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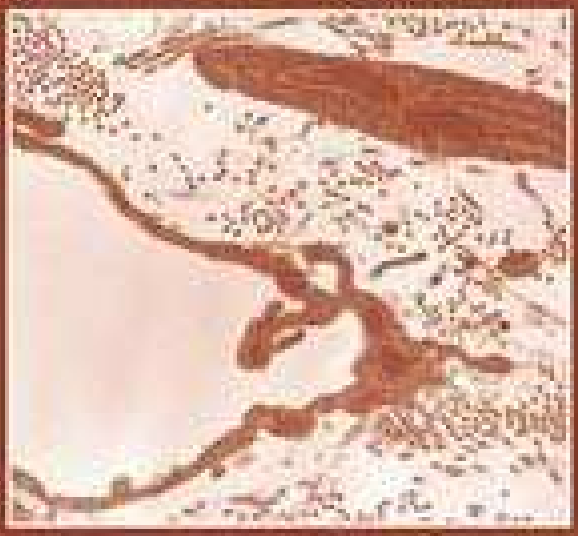
A SURVEY OF CELL BIOLOGY

Edited by
Kwang W. Jeon



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Adaptations for Nocturnal Vision in Insect Apposition Eyes

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Due to our own preference for bright light, we tend to forget that many insects are active in very dim light. Nocturnal insects possess in general superposition compound eyes. This eye design is truly optimized for dim light as photons can be gathered through large apertures comprised of hundreds of lenses. In apposition eyes, on the other hand, the aperture consists of a single lens resulting in a poor photon catch and unreliable vision in dim light. Apposition eyes are therefore typically found in day-active insects. Some nocturnal insects have nevertheless managed the transition to a strictly nocturnal lifestyle while retaining their highly unsuitable apposition eye design. Large lenses and wide photoreceptors enhance the sensitivity of nocturnal apposition eyes. However, as the gain of these optical adaptations is limited and not sufficient for vision in dim light, additional neural adaptations in the form of spatial and temporal summation are necessary.

KEY WORDS: Vision, Dim light, Nocturnal insects, Apposition eye, Landmark navigation, Optical and neural adaptations, Temporal and spatial summation, Theoretical modeling.

I. Introduction

Insects with apposition eyes are generally day-active since this eye design has significant limitations in dim light. Apposition eyes have small lenses and therefore a poor photon capture, resulting in unreliable visual signals at low light intensities. Nevertheless, some insects have managed the transition from a diurnal to a nocturnal lifestyle while retaining apposition eyes.

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As in the words of Autrum (1981), "the term *adaptation* denotes all those events that change the structure, form, function, or behaviour of organisms in such a way that they are better adjusted to their surroundings." Thus, the most intriguing question of this review is: what are the adaptations for nocturnal vision that have evolved in insect apposition eyes?

The structures of the major eye designs present in terrestrial insects will be introduced in Section II, illustrating why apposition eyes are highly unsuited for nocturnal vision. Section III will then describe dim-light foraging behavior in bees and other insects that use apposition eyes at night. Vision plays an important role in many of these insects, and this is demonstrated by the ability of nocturnal bees to use landmarks for their orientation. Section IV will point out the main limitations for vision in dim light, allowing us to understand the following paradox: insects are able to see at low light intensities despite using apposition eyes. Section V will finally attempt to solve this paradox. The evolution of unique optical adaptations in insect apposition eyes significantly enhances sensitivity for nocturnal vision. However, as the gain of these optical adaptations is limited, additional neural mechanisms are necessary. The well-established hypothesis of temporal and spatial summation is explained and supported by anatomical findings as well as a neural summation model.

So far, only one insect, the nocturnal bee *Megalopta genalis*, has been studied with respect to all aspects of vision and visual navigation at night and will thus be in focus throughout this review.

II. The Structure and Design of Compound Eyes

The compound eye's basic structure follows a general theme. The eye consists of repetitive visual units called ommatidia, each of which contains a dioptric apparatus that focuses light onto the layer of photoreceptors. The evolution of specific variations in either the optics of the eye or the neural wiring between the eye and the first optic ganglion (lamina) has led to the classification of three major compound eye types in insects: apposition, neural superposition, and refracting superposition compound eyes (Fig. 1).

The need for spatial resolution dictates the optical design of an eye, and the light intensity of the environment limits its application. As a general guideline, apposition and neural superposition eyes are designs typically found in day-active animals, while most nocturnal insects make use of the more sensitive superposition eye. Why this is the case, and how animals with less sensitive apposition eyes nevertheless manage to see in dim light, will be discussed in the following sections.

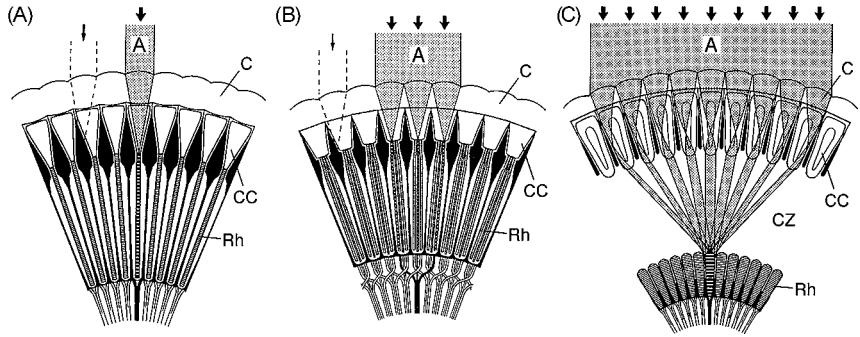

FIG. 1 Schematic longitudinal sections of the three major compound eye designs, (A) apposition, (B) neural superposition, and (C) refracting superposition eye, showing the axial ray paths of light absorbed by the photoreceptor (*shaded gray*) and off-axis light absorbed in the screening pigment (*dashed lines*). The aperture sizes (A-A) reflect the differences in sensitivity between the eye types. For further explanations see text. C, cornea; CC, crystalline cone; CZ, clear zone; Rh, rhabdom. (Modified from Nilsson, 1989.)

A. Apposition Eyes

Like in all compound eyes, the surface of the apposition eye consists of an array of tiny corneal lenses known as facets. Underneath each facet lies the crystalline cone, generally formed by four Semper cells. Together, the corneal lens and the crystalline cone build up the dioptric apparatus of the compound eye (Fig. 2). Each ommatidium usually contains 8–9 photoreceptors known as retinula cells, where the photon-absorbing, visual pigments are arranged within microvilli (rhabdomeres) of the light-sensitive rhabdom. These rhabdomeres can be either fused together as a rod-shaped rhabdom, or open (separated) throughout their length. In addition, several cell types containing screening pigments can be found: two primary pigment cells surround the crystalline cone, varying numbers of secondary pigment cells ensheath the entire ommatidium, and retinula cell pigments are present inside the retinula cells (Fig. 2). In general, a thick layer of pigments contained in the secondary pigment cells and the crystalline cone extensions covers the basement membrane to absorb stray light (Fig. 2).

A light-reflecting tapetal mirror proximal to the retina, formed by a well-developed tracheal system or reflective pigment granules, would be of great benefit for nocturnal insects. Via reflection of the light at the tapetum, photons get a second chance to be absorbed within the rhabdom, thus enhancing the eye's sensitivity. Tapeta are commonly found in the eyes of butterflies (Ribi, 1980) and dark-adapted crustacean apposition eyes (Debaisieux, 1944).

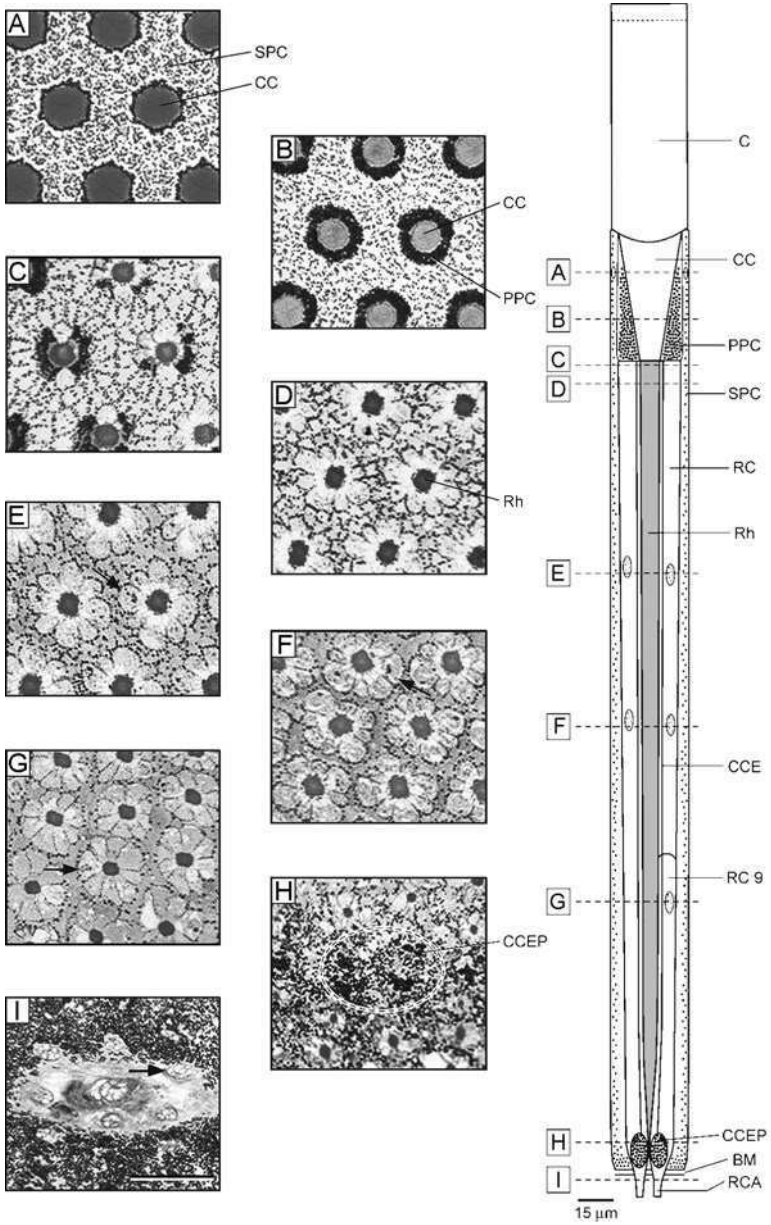

FIG. 2 Fine structure of the retina in *M. genalis* females. Longitudinal drawing of an ommatidium with photographs of transverse (A–I) sections at representative levels. The dioptric apparatus of the ommatidium consists of the prominent corneal facet (C) and the crystalline cone (CC). The crystalline cone is surrounded by 19 secondary pigment cells (SPC) (A) and two

However, tapeta have so far not been described in the apposition eyes of nocturnal insects.

The major characteristics of apposition eyes are the tight apposition of the crystalline cone and the rhabdom, as well as the pigment sheath of the secondary pigment cells. These pigments optically isolate the ommatidia from each other by absorbing light reaching the eye off-axis (Fig. 1A). Thus, only axial light from a single facet, representing the aperture of the apposition eye, is focused onto the respective rhabdom underneath. Due to this small aperture, the apposition eye design works best at bright light intensities, usually restricting the animal to a diurnal lifestyle. Low light intensities result in a poor photon catch and unreliable visual signals, as will be explained in Section IV.

B. Neural Superposition Eyes

The neural superposition eye of advanced flies (brachycerans) is similar to the apposition eye design, where each ommatidium receives light only through its own facet. However, instead of possessing a fused rhabdom, the rhabdomeres are open (separated) throughout their entire length and each of them receives light from a slightly different angle (Fig. 1B). This is also the case in most other flies (dipterans), hemipteran bugs, earwigs, and many beetles, but the unique characteristic found in brachycerans lies in the neural connections of the retinula cells to the first optic ganglion (lamina) of the brain. In conventional apposition eyes all retinula cell axons originating in one ommatidium project to a single neural unit (cartridge) in the lamina (Meinertzhagen, 1976; Ribi, 1974). In advanced flies, however, the retinula cell axons from those rhabdomeres in six adjacent ommatidia, which all have the same field of view, converge together onto the same cartridge (Braitenberg, 1967; Kirschfeld, 1967; Trujillo-Cenóz, 1965; Vigier, 1909). Thus, without sacrificing any spatial resolution, sensitivity can be increased 6-fold (Gemperlein and Smola, 1972; Strausfeld and Nässel, 1981).

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primary pigment cells (PPC) (B). Nine retinula cells (RC) build up the light-sensitive fused rhabdom (Rh) and their cell bodies are situated in three distinct layers (*arrows* in E–G). A ring of retinula cell pigments tightly surrounds the rhabdom in the light-adapted state (*arrow* in D). Four crystalline cone extensions (CCE) reach from the crystalline cone to the proximal retina and their pigments (CCEP) surround the most distal tip of the rhabdom (*dashed ellipse* in H). The retinula cell axons (RCA) pass the basement membrane (BM) as axon bundles forming pseudocartridges (*arrow* in I). Schematic pigments are not to scale. 1 μm transverse sections, toluidine blue stained, scale in I for (A–I). (Modified from Greiner *et al.*, 2004a.) (See also color insert.)

C. Superposition Eyes

In superposition eyes, unlike the eye types already described, a wide pigment-free "clear zone" spatially separates the optics and the light-absorbing rhabdom layer (Fig. 1C). The rhabdoms are usually wider and shorter compared to those of apposition eyes, and the optical isolation provided by screening pigments is absent (at least during dark adaptation). Through specialized optics, commonly via refractive index gradients in the crystalline cones (refracting superposition, Fig. 1C), parallel light rays from a large number of facets can be focused or "superimposed" across this clear zone onto a single rhabdom (Exner, 1891; Nilsson, 1989). Hence, each rhabdom receives light through wide superposition apertures comprising typically several hundred or even thousands of facets, which greatly improves photon catch and thus sensitivity. The gain in sensitivity is approximately equivalent to the number of superimposed facets, and due to this enhanced photon capture, superposition eyes are typically found in nocturnal insects.

D. Implications of Eye Design for Nocturnal Vision

The small apertures of apposition eyes clearly limit insects to vision at bright light intensities while superposition eyes are the most effective design for an insect active in dim light (Warrant, 2001, 2004). Thus, the driving question is how some insects have managed the transition to a nocturnal lifestyle while retaining their insensitive apposition eyes. Why have they not simply evolved a superposition eye? As convincingly explained by Nilsson (1989), a direct transition from an apposition to a superposition design is not as simple as one may think. Apposition eye optics generates multiple inverted images, whereas superposition optics creates a single upright image. A direct evolution from an apposition to a superposition eye would therefore need to convert this visual imaging from inverted to upright, which involves a passage through an unfocused system. As there exists no selective pressure for unfocused intermediate eye designs, superposition eyes cannot directly evolve from conventional apposition eyes. This has puzzled scientists for many years until the discovery of afocal apposition optics in butterflies presented an evolutionary pathway where every step results in improved vision (Nilsson *et al.*, 1984, 1988). In butterfly eyes a strong lens within the crystalline cone works in a similar manner to the telescope system of refractive superposition eyes. Hence, moths might have achieved their transition to a nocturnal lifestyle, and the evolution of superposition eyes, via these afocal apposition optics (Nilsson *et al.*, 1984, 1988). Although such an optical system may also exist in other apposition eyes, this has not yet been reported.

III. Nocturnal Life with Apposition Eyes

Dim light severely limits nocturnal insects with apposition eyes during foraging and visual navigation. A crepuscular or nocturnal lifestyle has nevertheless evolved repeatedly within bees and also other insects with apposition eyes, like wasps, ants, mosquitoes, and bugs. A major cost for enhanced sensitivity at low light intensities is decreased spatial resolution. In bees, this trade-off needs to be well balanced: finding a food source is known to be strongly guided by olfaction; however, upon return to their nest vision plays a large role in navigation. Reliable navigational cues (e.g., landmarks, sky polarization, canopy patterns, and path integration) are available even at low light intensities. The nocturnal bee *Megalopta genalis* is in fact the first insect known to orient using landmarks at very low light intensities (Section III.A.2). However, when spatial acuity becomes too low, such demanding tasks can no longer be achieved. In the apposition eyes of nocturnal mosquitoes, for instance, optical adaptations have evolved in favor of sensitivity (Section V.A). Having sacrificed almost their entire spatial resolution, nocturnal mosquitoes need to rely on simpler visual behaviors (e.g., phototaxis) and in addition use other specialized sensory systems (e.g., olfaction, mechanoreception) to guide them through the night.

A. Crepuscular and Nocturnal Bees

Worldwide there are more than 16,000 described bee species (Michener, 2000) and a large majority of them are only active during the day. Nevertheless, crepuscular or nocturnal species occur in at least four of the seven currently described bee families: the Apidae, Andrenidae, Colletidae, and Halictidae. Some of these bees are able to extend their foraging period into twilight or even throughout the night when the moon is present, and a few exceptional species have adopted a strictly nocturnal lifestyle.

1. Foraging in Dim Light

Most Apidae, like the European honeybee *Apis mellifera carnica*, forage only during the day despite their ability to retain achromatic vision down to moonlight intensities (Menzel, 1981; Rose and Menzel, 1981; Warrant *et al.*, 1996). This capability of the visual system may explain why some honeybee species, including the African race of the honeybee *A. mellifera adansonii* and the Asian giant honeybee *A. dorsata*, will actively forage into the night when at least a half-moon is present and temperatures are mild (Dyer, 1985; Fletcher, 1978). A similar situation can be found in the

carpenter bees (*Xylocopa,* Apidae), where dim-light foraging has been reported for some species in India, Thailand, and Mexico (Burgett and Sukumalanand, 2000; Janzen, 1964; Somanathan and Borges, 2001). A particularly interesting example is the occurrence of three *Xylocopa* species in the Western Ghats of India: *X. ruficornis* is strictly diurnal, *X. tenuiscapa* occasionally also forages in the evening, and *X. proximata* is strictly nocturnal (H. Somanathan and R. M. Borges, unpublished observations). Carpenter bees are large bees and their ability to forage despite low temperatures and strong winds, conditions in which not even moths are able to fly, makes them important pollinators for night–flowering plants (Somanathan and Borges, 2001). Similarly, the predawn flights of *Xenoglossa fulva* (Apidae) are of considerable importance for the pollination of its sole pollen host *Curcubita* sp. in Central and North America (Linsley *et al.*, 1955).

Dim-light foraging within the Adrenidae is only known from the crepuscular bee *Perdita bequaertiana* (Cockerell, 1923). This oligolectic species collects pollen from the evening primrose *Oenothera* sp., which opens its flowers just after sunset. Within the family of the Colletidae, predawn foraging is found in *Ptiloglossa* sp. and *Caupolicana* sp. (Linsley, 1962; Linsley and Cazier, 1970; Roberts, 1971), which close their burrows during the night. Within a population, the opening of the nests occurs simultaneously in the early morning, indicating that an internal circadian rhythm may play a role in the onset of their foraging (Linsley and Cazier, 1970).

Dusk and dawn foraging has also been reported for *Sphecodogastra galpinisiae*, a member of the large family Halictidae. *S. galpinisiae* starts to forage early on warm mornings and has a second period of evening flights lasting until late twilight (Bohart and Youssef, 1976).

This foraging period is even further extended in *S. texana*, which is crepuscular, but continues to forage for as long as the moon is present at night (Chandler, 1961; Kerfoot, 1967b). Foraging at low light intensities has been thoroughly studied in the halictid bees *M. genalis* (Fig. 3) and *M. equadoria* (Kelber *et al.*, 2006; Warrant *et al.*, 2004). Both species are native to parts of Central and South America with the main study site located on Barro Colorado Island, Panama (Leigh, 1999; Rau, 1933). Using their strong mandibles, they hollow out small wooden branches to construct nests and brood chambers, which they provision with nectar and pollen from a large variety of canopy and understorey flowers (Janzen, 1968; Roulston, 1997; Sakagami, 1964; Wcislo *et al.*, 2004). Although predominantly solitary, *M. genalis* and *M. equadoria* display facultatively social behavior (Arneson and Wcislo, 2003; Smith *et al.*, 2003).

Amazingly, these bees are able to forage and navigate at extremely low light intensities (Warrant *et al.*, 2004). Both species have been caught in light traps throughout the night (Roulston, 1997; Wolda and Roubik, 1986); however, recent behavioral studies show two distinct foraging periods during

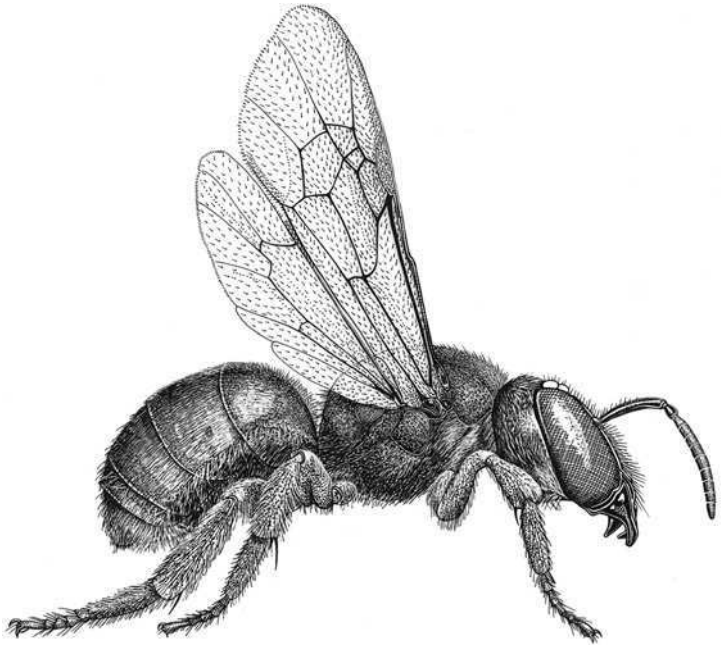

FIG. 3 Female halictid bee *Megalopta genalis* drawn after mounted material. (Reprinted from Greiner *et al.*, 2004a with kind permission of Springer Science and Business Media.)

early dawn and late dusk, when light levels underneath the forest canopy can reach less than starlight intensities (Kelber *et al.*, 2006; Warrant *et al.*, 2004). Although such extremely low light intensities should theoretically render their apposition eyes blind, these nocturnal bees are able to find their nest entrances with the aid of surrounding landmarks (Warrant *et al.*, 2004).

2. Landmark Navigation

Many insects are able to memorize visual landmarks around their nests and along the routes to their foraging sites (Collett and Collett, 2002). A variety of features (e.g., color, size, shape, and symmetry) can be used to learn and subsequently recognize visual landmarks. Two different classes of landmarks are likely to be available to insects: landmarks close to the goal that are used for determining its exact position, and larger and more distant landmarks for guiding the insect to the approximate position of the goal. Bees and wasps perform so-called orientation flights to acquire landmark information near to the nest site (Zeil, 1993) and around feeders (Collett and Lehrer, 1993; Lehrer, 1993). The shape of these flights resembles a series of increasing,

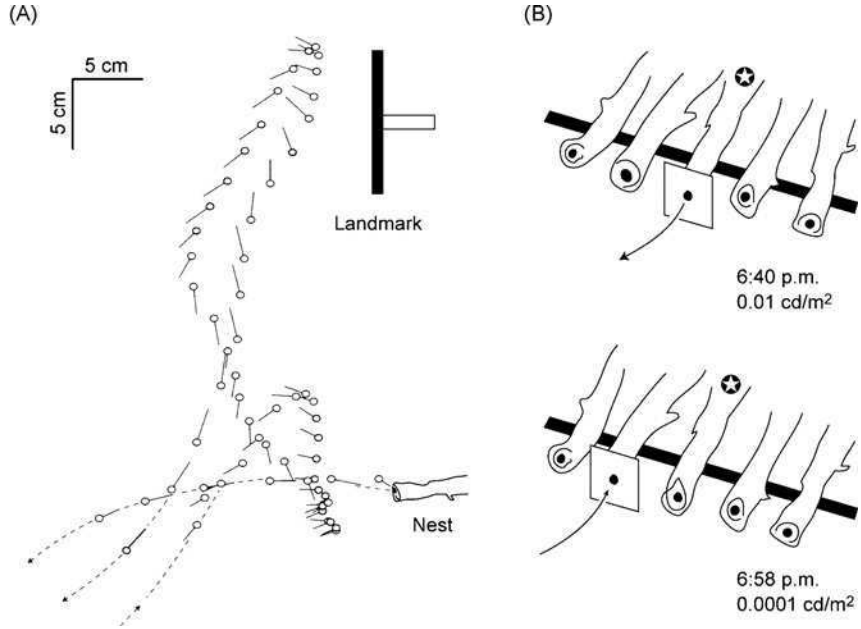

FIG. 4 (A) A reconstructed arc-shaped learning flight of the nocturnal bee *M. genalis* with the circle representing the head and the line the body of the bee at 40 ms intervals. (B) Landmark experiment, where the bees were given white square cards to learn, and which they use as reference upon return instead of other sensory cues from their original nest marked by the star. Time of day and light intensities are shown, where 0.0001 cd/m^2 is equivalent to an intensity of less than starlight. (Reprinted from Warrant *et al*., 2004 with kind permission from Elsevier.)

semicircular arcs that are kept at constant angular velocity while the insect is backing away from the goal (Zeil, 1993).

Such orientation flights around the nest entrance have recently been shown to occur even at very low light intensities (Warrant *et al.*, 2004). The nocturnal bee *M. genalis* performs arc-shaped learning flights that are very similar to those in day-active bees (Fig. 4A). Using these, *M. genalis* is able to navigate by means of natural as well as artificial landmarks near the nest site to locate the nest entrance (Fig. 4B) (Warrant *et al.*, 2004). Unlike some ground-nesting bees and wasps that perform learning flights each morning upon their first departure (Brünnert *et al.*, 1994; Zeil, 1993), *M. genalis* displays these flights occasionally both early in the morning and in the evening. To prove that it really is vision and not olfaction that guides these bees close to the nest, Warrant *et al*. (2004) performed a series of landmark experiments. By means of an artificial landmark mounted around the nest entrance, the bee was first allowed to learn the new structure during its

orientation flights. The bee was usually given several days to get used to the landmark. Then after a first flight on one evening, the landmark was moved to an unoccupied nest alongside and indeed the bee was "fooled" to fly into the now newly marked but wrong nest entrance (Fig. 4B). Once inside the wrong nest it discovers its mistake and leaves the nest quickly. However, instead of searching the surrounding area for its true nest, the bee was so convinced by the visual landmarks that it would fly back into the same nest and out again continuing with this behavior until the landmark was returned to the bee's own original nest.

An analysis of their collected pollen shows that these nocturnal bees are largely generalists and visit flowers both in the canopy as well as on understorey shrubs (Wcislo *et al.*, 2004) with foraging flights lasting up to 34 min (Kelber *et al.*, 2006). How can they navigate over the large spatial range of a long foraging trip? In addition to the described close-range landmark navigation, recent data suggest that polarization vision may also play a role in the long-distance foraging flights of *M. genalis* (Cronin *et al.*, 2006).

B. Other Nocturnal Insects with Apposition Eyes

Bees are not the only insects that use apposition eyes at low light intensities. Other hymenopterans (i.e., wasps and ants) are also active at night. In two genera of social wasps, *Apoica* and *Provespa*, nocturnal behavior has been documented (Hunt *et al.*, 1995; Matsuura, 1999). The nocturnal wasp *A. pallens*, for example, swarms during dusk and forages only at night (Howard *et al.*, 2002; Hunt *et al.*, 1995; Schremmer, 1972; Vesey-FitzGerald, 1938). Despite the importance of chemical cues in ant societies, specific optical adaptations to enhance sensitivity are present in the apposition eyes of night-active *Camponotus* species (Menzi, 1987). This suggests that at least in these nocturnal ants, vision plays a role in their orientation. Activity periods of mosquitoes can range from nocturnal blood-feeding species, like *Anopheles gambiae*, to the diurnal plant-feeder *Toxorhynchites brevipalpis*. *A. gambiae* is strictly nocturnal and shows behavioral optomotor responses down to light levels between moonlight and starlight (Clements, 1963; Gibson, 1995). As the apposition eyes of nocturnal mosquitoes trade spatial resolution for enhanced sensitivity, specializations of their olfactory system play a dominant role during host-finding (Takken and Knols, 1999). The nocturnal bug *Triatoma infestans* also possesses a highly sensitive visual system, which is mainly used to find refuges and to avoid predator exposure at night (Lazzari, 1992; Reisenman *et al.*, 1998).

C. Advantages of Being Nocturnal

What are the benefits of a nocturnal life for insects with apposition eyes? The current hypothesis is that the cover of night provides protection from diurnal predators and the reduced density of competitors secures early access to food sources. Bats, for example, discovered foraging on night-blooming flowers as a new niche and these animals act as important pollinators of nocturnal flora (Baker, 1961; Machado and Vogel, 2004; Marshall, 1983; Park, 1940).

Is this well-established hypothesis also true for insects? Wcislo *et al*. (2004) recently showed that these advantages are most likely responsible for the transition to nocturnal life in tropical bees and future work may further support the general validity of this hypothesis in other insects. Foraging at low light intensities is particularly common in tropical areas, an environment of severe food competition and a high risk of predation. As temperature is no limitation, even small species are able to exploit food sources early in the morning or late at night when most competitors are inactive. By hiding during the day, these bees are able to protect themselves, and also their brood, when most predators and parasites are active (Smith *et al.*, 2003). However, for bees the major limitation on their flight activity at night is light intensity (Kelber *et al.*, 2006).

IV. Limitations to Vision at Low Light Intensities

While spatial resolution determines eye designs in bright daylight, extracting information from unreliable signals is the main limitation for vision in dim light. Light intensity can be defined as the average number of photon arrivals per unit area and time, where photon arrival is a random event. Consequently, the fewer photons a photoreceptor absorbs per integration (or sampling) time, the larger the unreliability (noise) in the average number of photons sampled. Minimizing the noise and simultaneously maximizing the signal is the major task of an eye in order to function efficiently at low light intensities (Laughlin, 1990). Another limitation for eyes with small apertures may be diffraction. Parallel light passing through small apertures will suffer an angular spread, thus leading to a blurry image (Airy disc) and a loss in resolution. However, nocturnal apposition eyes generally have large lenses and wide rhabdoms where diffraction has little effect (Warrant and McIntyre, 1993).

A. Visual Noise

The major limitation for nocturnal vision in insects is consequently visual noise, the sources of which can be of three kinds within the photoreceptor:

photon shot noise, dark noise, and transducer noise (Warrant, 2004). As photon arrival follows Poisson statistics, a photoreceptor absorbing a number of N photons experiences an uncertainty (or photon shot noise) of $\sqrt{N}$ photons (Land, 1981; Warrant and McIntyre, 1993). Decreasing photon catch in dim light results in an increasing noise level that degrades the ability of two photoreceptors to discriminate contrast. As two visual channels need to detect sufficient photons in order to reduce this noise level below the actual difference in intensity, the eye has to enhance its sensitivity in order to reliably detect the contrast. However, improved sensitivity only comes at the cost of losses in spatial and/or temporal resolution.

Dark noise also originates within the photoreceptor and consists of spontaneous thermal responses in the absence of photons, which are indistinguishable from membrane potentials (quantum bumps) produced by photons (Barlow, 1956). These fluctuations are more frequent at higher temperatures and introduce uncertainty at low light intensities. Nevertheless, compared to the high rates measured in vertebrates (Aho *et al.*, 1988), dark noise is thought to have a low rate in invertebrates (Warrant, 1999).

Transducer noise is due to variations in the amplitude, latency, and duration of quantum bumps produced upon photon absorption within the photoreceptors (Lillywhite and Laughlin, 1979). This uncertainty adds to the unreliability of vision, although it is expected to be of lesser importance in dim light (Laughlin, 1990). Thus, as photon shot noise is the main source of uncertainty, nocturnal insects with apposition eyes have to maximize the photon catch or signal-to-noise ratio to enhance sensitivity, even if it comes at the cost of severe losses in resolution.

B. Resolution vs. Sensitivity

An eye has to fulfill two basic tasks: first and foremost for dim-light vision, it has to be sufficiently sensitive, collecting enough photons to reduce the effects of photon noise. Second, it needs to possess spatial resolving power in order to determine the direction of the incident light (Warrant and McIntyre, 1993). These two prerequisites often stand in a cost-benefit relation, meaning that if an eye needs to function at low light intensities it has to trade resolution for sensitivity (Warrant, 2004; Warrant and McIntyre, 1992). Despite the limitations for vision in dim light, various insects have managed the transition from a strictly diurnal to a nocturnal lifestyle. Hence, specific adaptations must have evolved in the apposition eyes of nocturnal insects to account for these shortfalls.

V. Adaptations for Vision in Dim Light

Activity at light intensities up to 8 log units dimmer than during the day has led to the evolution of various specific adaptations in the visual systems of insects with apposition eyes. In general, sensitivity can be enhanced via optically collecting more photons within the eye, or by neurally summing signals in space or time (Lythgoe, 1979).

A. Optical Adaptations

Independent of eye design, there are four major optical parameters that can affect light flux: (1) facet size, (2) changes of focal length, (3) pigment migrations within the retina, and (4) dimensional changes of the rhabdom (Nilsson, 1989). An ideal measure of quantifying optical sensitivity, which includes these factors, is to calculate the number of photons (N) a photoreceptor can capture per integration time (Δt) from a light intensity spectrum $I(\lambda)$ where λ is wavelength (Kelber *et al.*, 2002; Warrant and Nilsson, 1998; Warrant *et al.*, 2004; Warrant, 1999):

$$N = 1.13\left(\frac{\pi}{4}\right)\Delta\rho^2 D^2 \kappa\tau\Delta t \int (1 - e^{-kR(\lambda)l})I(\lambda)d\lambda \qquad (1)$$

N depends on a number of optical and physiological parameters, namely on the size of the aperture (D), the length of the photoreceptor (l), the receptive field of the photoreceptor ($\Delta\rho$), the length of time a sample of photons is counted in the photoreceptor (integration time Δt), and on the absorption rate within the photoreceptor, which is based on the quantum efficiency of transduction (κ), the transmission of the optics (τ), the absorption coefficient of the rhabdom (k), and the spectral sensitivity of the photoreceptor $R(\lambda)$.

Thus, in apposition eyes specific optical and structural adaptations have evolved to enhance optical sensitivity, including changes in the facet diameter and the receptive field of the photoreceptors. This section describes these optical adaptations (i.e., facet size, focal length, pigment migrations, and rhabdom diameter) and discusses their effect on sensitivity.

1. Facet Size and Corneal Surface Structure

Nocturnal bees and wasps typically possess prominent eyes and huge ocelli with an eye area 1.8 times larger than in their diurnal relatives (Greiner, 2006; Greiner *et al.*, 2004a; Jander and Jander, 2002; Kerfoot, 1967a). In the case of bees, these large eyes show a regional increase of facet size (Fig. 5C), which

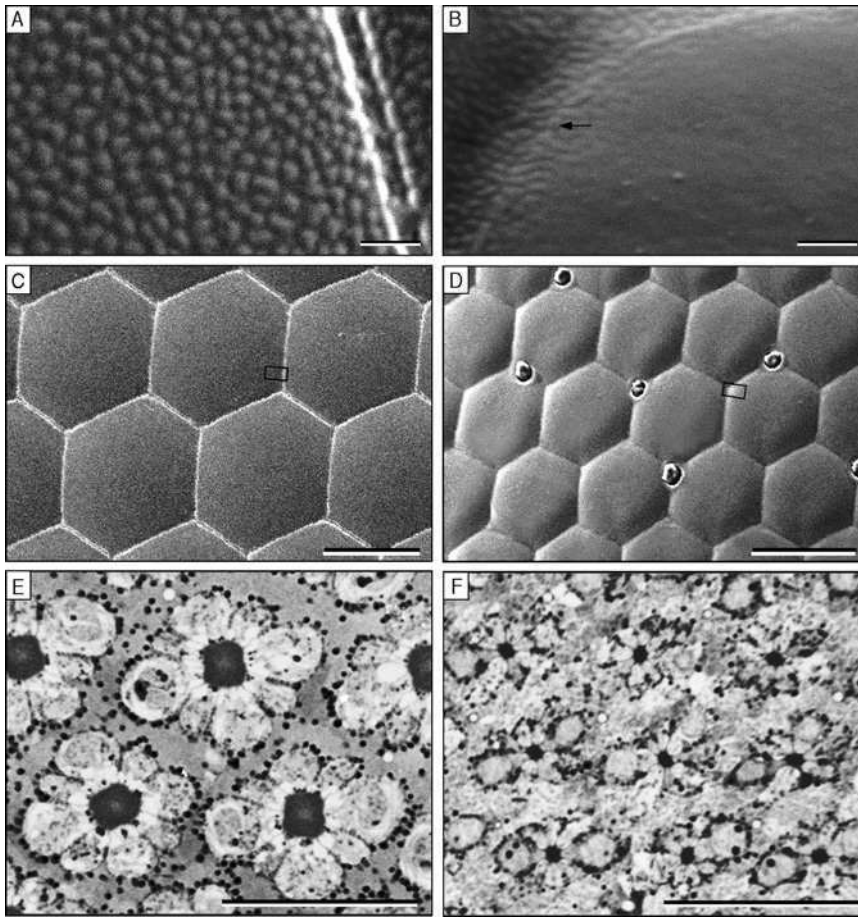

FIG. 5 Comparisons of corneal surface (A, B), facet size (C, D), and rhabdom diameter (E, F) in the nocturnal bee *M. genalis* (A, C, E) and the worker honeybee *A. mellifera* (B, D, F). The corneal outer surface features a distinct corneal nipple-array in *M. genalis* (A), while in the worker honeybee nipples are restricted to the facet borders (*arrow* in B). The corneal facets of the frontomedial eye region are approximately twice as wide in *M. genalis* (C) than in the worker honeybee (D). The *black squares* mark the regions shown in (A, B). The diameter of the rhabdom in *M. genalis* (E) is 4–5 times wider than in the honeybee (F). (A–D) Scanning electron micrographs. (E, F) 1 μm cross sections, toluidine blue stained. Scale (A, B) 1 μm, (C – F) 25 μm. (Reprinted from Greiner *et al.*, 2004a with kind permission of Springer Science and Business Media.) (See also color insert.)

together with the wide photoreceptor rhabdom diameter (Fig. 5E) directly affects the optical sensitivity of the eye (Greiner *et al.*, 2004a). In contrast, the larger eyes of the nocturnal wasp *A. pallens* contain 2000 more facets when

compared to the eye of a diurnal relative, as facet size distribution is similar (Greiner, 2006). Optical measurements demonstrate that greater sensitivity in nocturnal wasps is instead achieved only by the wide photoreceptor rhabdom diameter and the large visual acceptance angle (Greiner, 2006).

In mosquitoes, clear differences in eye structure are also related to light intensity (Land *et al.*, 1999). Nocturnal species have significantly fewer facets of generally larger diameter compared to diurnal species (Land *et al.*, 1999), indicating that nocturnal mosquitoes trade sensitivity for spatial resolution. Despite the obvious benefit of enlarging facets for nocturnal vision, nocturnal ants possess both smaller eyes and fewer facets of equal diameter compared to their diurnal relatives (Menzi, 1987). A pupil mechanism and extremely wide rhabdoms (see below) nevertheless support the importance of vision for these ants.

A detailed investigation of the corneal microsurface reveals an array of nipple structures in some nocturnal species, e.g., the nocturnal bee *M. genalis* (Fig. 5A, Greiner *et al.*, 2004a), which is thought to further enhance sensitivity (Miller, 1979; Stavenga *et al.*, 2006). The anti-reflectance effect of this corneal nipple array facilitates the transition of light into the eye by reducing reflection. In nocturnal bees, the effect is calculated to be 4 % (Greiner *et al.*, 2004a) and even such a small increase of sensitivity is likely to be important for a photon-starved eye. Despite these benefits for dim-light vision, corneal nipples are not present on the lens surface of the nocturnal wasp *A. pallens* (Greiner, 2006).

2. Dioptric Apparatus and Focal Length

The dioptric apparatus of the compound eye, consisting of the corneal lens and the crystalline cone, focuses incoming light onto the distal rhabdom tip. Generally, the lens, either via refractive index gradients within the lens cylinder, or its convex curvature, forms the image. The crystalline cone represents a mere spacer and has no direct optical function (Nilsson, 1989). As most of the refractive power comes from the outer corneal surface, it is surprising that the corneal facets of nocturnal bees and wasps feature flattened outer and strongly convex inner curvatures instead (Fig. 6A,B) (Greiner, 2006; Greiner *et al.*, 2004a). As this inner curvature is not present in their diurnal relatives (Fig. 6C,D), it may represent an optical adaptation for nocturnal vision. However, despite this inner corneal curvature, theoretical calculations using the thick lens formula (Land *et al.*, 1999) predict a graded refractive index within the lens of the nocturnal bee *M. genalis*, in order to focus light onto the distal rhabdom (Greiner *et al.*, 2004a).

In nocturnal mosquitoes, in contrast, focusing is achieved via an almost hemispherical curvature of the facet lens (Fig. 7A), which transmits a 65° cone of light onto the rhabdom (Land *et al.*, 1997, 1999). As the outermost

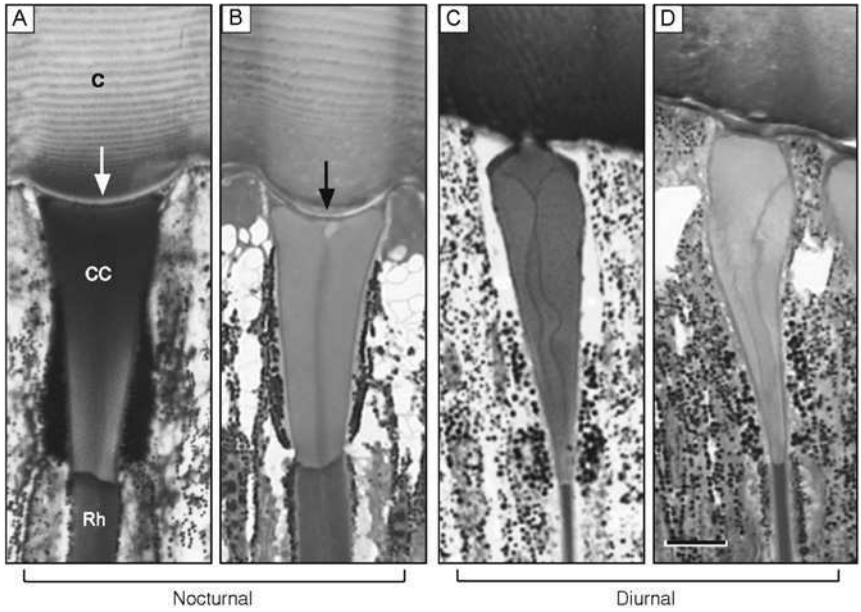

FIG. 6 The dioptric apparatus in the apposition eyes of (A) *M. genalis*, (B) *Apoica pallens*, (C) *Apis mellifera,* and (D) *Polistes occidentalis*, showing the proximal cornea (C), crystalline cone (CC), and the distal tip of the rhabdom (Rh). Note the strongly convex inner corneal curvature, its tight apposition to the CC, and the extremely wide rhabdom diameter in the nocturnal bee (A) and wasp (B), which are not present in their diurnal relatives (C, D). Scale for (A–D) in (D) 10 μm. (Modified from Greiner, 2006.) (See also color insert.)

rays of such lenses are highly overfocused and cause severe spherical aberration, spatial resolution is sacrificed in order to enhance sensitivity. Despite this trade-off, the system minimizes spherical aberration by allowing the position of the distal rhabdom to coincide with the smallest blur circle of the refracted light (Land *et al.*, 1999).

As for cameras, a large aperture (or facet diameter D in apposition eyes) and a relatively short focal length (f) lead to a system of high sensitivity defined by a low F-number:

$$F = f/D \tag{2}$$

Low F-numbers are adaptations commonly found in nocturnal animals, as large apertures and short focal lengths allow light to reach the rhabdom over a wide range of incident angles (Warrant and McIntyre, 1991). Diurnal insects with apposition eyes usually have eyes of high F-number, typically above 2.1 (Warrant and McIntyre, 1993). Eyes of low F-number, while sensitive, tend to have poorer spatial resolution. Due to the small difference

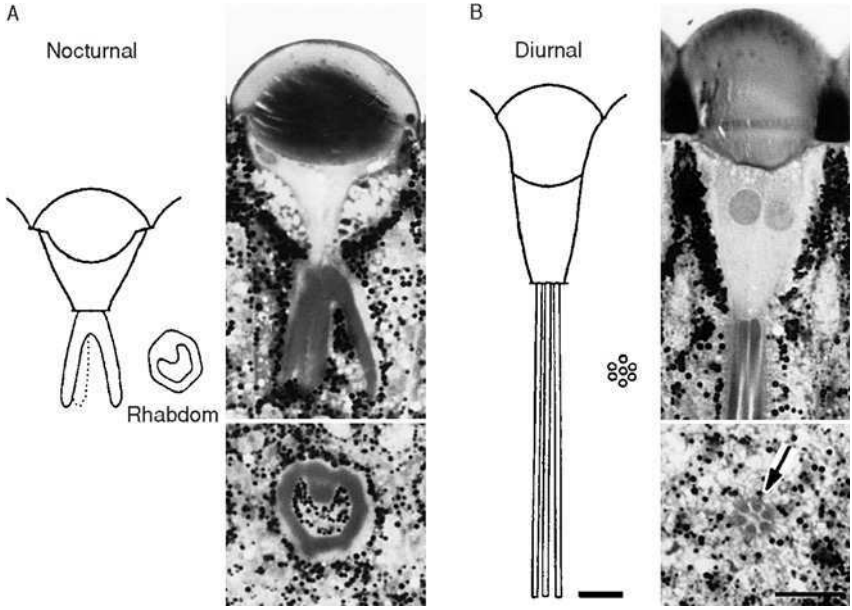

FIG. 7 The ommatidial structure of (A) nocturnal and (B) diurnal mosquitoes, shown as a schematic drawing and as histological sections. (A) The rhabdom of nocturnal mosquitoes has a unique hollow cone shape with a blunt apex and traps all the light reaching the eye through the large, almost hemispherical lenses. (B) In contrast, diurnal mosquitoes have narrow, open rhabdomeres and much smaller apertures. Scale for (A, B) in (B) 10 μm; *arrow* in (B) points at the open rhabdomeres in the eyes of the diurnal mosquito. (Modified from Land *et al.*, 1999.)

in refractive index between the rhabdom and its surroundings, only rays with a maximum incident angle of about 10° can be trapped within a cylindrical rhabdom (Warrant and McIntyre, 1993). The minimum *F*-number for light to be totally reflected within the rhabdom is consequently 2.8 (Warrant and McIntyre, 1993). Most diurnal apposition eyes, and even many nocturnal insects, have *F*-numbers close to this value, ensuring that most of the light remains trapped within their rhabdoms (Greiner, 2006; Greiner *et al.*, 2004a).

For those eyes with *F*-values less than 2.8, several solutions exist to avoid the severe spreading of unused stray light (Warrant and McIntyre, 1991). A shield of light-absorbing pigments would remove scattered light, but as this comes only at the cost of sensitivity it is not an ideal option for dim-light vision. In eyes where photoreceptors have large visual fields, noncylindrical rhabdoms can trap light from an incident angle much greater than 10°, thus enhancing sensitivity, as demonstrated in nocturnal mosquitoes (Land *et al.*, 1997). The most complete solution is to surround the rhabdom with a tapetal sheath (reflective pigment granules or tracheoles); however, this has not yet been shown to exist in the apposition eyes of nocturnal insects.

Even though the trade-off between sensitivity and resolution represents a distinct limitation of eye design, most insect eyes also need to function over a wide range of light intensities. This is mediated by migrations of screening pigments.

3. Pigment Migrations within the Retina

Movements of screening pigments during light adaptation (LA) and dark adaptation (DA) constitute the most important pupillary mechanism found in compound eyes. Without optical mechanisms that adapt the eye to different light intensities, photoreceptors are unable to operate over a range of more than about 3 log units (Walcott, 1975). Animals active in a broader intensity range therefore need mechanisms to adjust the sensitivity of their eyes accordingly.

Just as humans use the iris as a pupil, screening pigments serve the same function in apposition eyes. Radial pigment movements, either in the retinula cells or in the primary pigment cells, are typical for apposition eyes, while longitudinal migrations are mostly found in superposition eyes (Warrant and McIntyre, 1996). The onset of pigment migration is often controlled directly by light intensity, but endogenous circadian rhythms can also influence migration (Menzi, 1987; Reisenman *et al.*, 2002).

a. Radial Pigment Migration In most apposition eyes, radial pigment migration is mediated by retinula or primary pigment cells (Autrum, 1981; Walcott, 1975). Within the retinula cells, pigments tightly surround the rhabdom in the LA state and migrate away from the rhabdom during the DA state (Ribi, 1978). Close to the rhabdom, these pigments absorb the light propagating outside the microvilli, which sharpens spatial acuity but decreases sensitivity (Land and Osorio, 1990). Many day-active insects use the benefits of this mechanism (Stavenga, 1979), but interestingly it is also present in some night-active insects, like cockroaches (Butler and Horridge, 1973) and nocturnal bees (Greiner *et al.*, 2004a).

By effectively reducing the intensity during bright light, radial migrations of retinula cell pigments have an analogous effect to dynamic pupils located distally to the rhabdom (Autrum, 1981). Such pupils often cause strong retinomotor movements affecting not only the primary pigment cells, but also the shape of the crystalline cone and the position of the distal rhabdom (Eckert, 1968; Lüdtke, 1953). During LA, when the primary pigment cells form a closed pupil, a long narrow crystalline cone tract appears and the rhabdom distance toward the lens increases. During DA, in turn, this adaptive mechanism shortens the focal length and enhances the eye's sensitivity by widening the visual field of the photoreceptor. Little is known, however, regarding the mechanisms driving these retinomotor movements,

as microtubuli can be found in the visual cells as well as in the primary pigment cells and the crystalline cone (Autrum, 1981).

Primary pigment pupils are typically found in apposition eyes with open rhabdoms that lack neural superposition (Ioannides and Horridge, 1975; Lüdtke, 1953; Nilsson and Ro, 1994; Reisenman *et al.*, 2002; Walcott, 1971; Williams, 1980). The pupil mechanism mediates high resolution/low sensitivity via the central rhabdomeres when the pupil is narrow (during LA) and low resolution/high sensitivity when the pupil is wide open and exposes the outer rhabdomeres to light (during DA) (Nilsson, 1989). The significant gain in sensitivity, when the eye is dark-adapted, is of great benefit for crepuscular and nocturnal species. Primary pigment pupils also occur in some eyes with fused rhabdoms, where the pupil instead regulates the visual angle of the entire rhabdom during LA and DA (Land *et al.*, 1999; Menzi, 1987).

b. Longitudinal Pigment Migration Longitudinal pigment migrations are generally present only in superposition eyes, where secondary pigments may migrate into the clear zone to optically isolate the ommatidia in the light-adapted state (Autrum, 1981; Nilsson, 1989). However, in the apposition eye of the nocturnal bug *Triatoma infestans* and the common European earwig *Forficula auricularia*, movement of retinula cell pigments along the longitudinal axis of the rhabdomeres adds to the effect of the primary pigment pupil during LA (Nilsson and Ro, 1994; Reisenman *et al.*, 2002). Longitudinal retractions of secondary and retinula cell pigments during DA could greatly improve the sensitivity of an apposition eye, but without specific superposition optics this would result in severely blurred images as light crosses between the ommatidia.

Not all insects use the dynamic changes of pigment migrations. Instead, some have large cisternae (or palisades) surrounding the rhabdom in the DA state (Butler and Horridge, 1973; Horridge and Barnard, 1965), which could function as spacers between the rhabdom and the light-absorbing pigment granules (Snyder and Horridge, 1972). As the optical density of these palisades is lower than the cytoplasm, they are also thought to improve the light-guiding properties of the rhabdom by retaining the light energy within the rhabdom (Horridge and Barnard, 1965; Ioannides and Horridge, 1975).

4. Changes of Rhabdom Size and Shape

Rhabdom size plays a crucial role in the apposition eyes of nocturnal insects as enlarging its effective area directly enhances sensitivity. Most insects that are active at low light intensities have wide rhabdoms where the diameter either remains constant or changes according to a circadian rhythm. Comparative studies between diurnal and nocturnal hymenopterans show a

constant rhabdom diameter of 8 μm in the nocturnal apposition eyes of the halictid bee *M. genalis* (Figs. 5E and 6A) (Greiner *et al.*, 2004a), the wasp *A. pallens* (Fig. 6B) (Greiner, 2006), and the ant *Camponotus ligniperda* (Menzi, 1987).

A circadian rhythm, where rhabdom diameter widens significantly during DA, is present in the apposition eyes of locusts and mantids (Horridge *et al.*, 1981; Rossel, 1979; Williams, 1982). As locusts perform migration flights at low light intensities (Chapman, 1980), this circadian change in rhabdom diameter widens the receptive field of the photoreceptors to enhance sensitivity at night and subsequent narrowing optimizes spatial resolution during the day (Tunstall and Horridge, 1967).

The nearly hemispherical lenses of crepuscular and nocturnal mosquitoes focus a 65° wide cone of light onto the rhabdom during DA. To capture this wide cone, a unique rhabdomeric shape, consisting of a wide fused apex and proximal hollow cone, has evolved in these species (Fig. 7A) (Land *et al.*, 1997, 1999; Mazokhin-Porshnyakov and Kazyakina, 1978). Only with such a conical rhabdom is all the light effectively trapped within the rhabdom (Land *et al.*, 1997). As diurnal species have open rhabdomeres with a narrow cylindrical shape (Fig. 7B), conical rhabdoms represent a purely nocturnal adaptation (Land *et al.*, 1999).

Another extreme case is the apposition eye of the deep-sea crustacean *Cirolana borealis*, where the rhabdom width and length are equal and remarkably large (90 × 90 μm) (Nilsson and Nilsson, 1981). However, the overall cost of widening the visual field to such an extent is the almost complete loss of spatial resolution.

5. Resolution, Visual Field, and Optical Sensitivity

The anatomical resolution of an apposition eye is determined by the ommatidial packing density, which is given by the interommatidial angle ($\Delta\phi$). In conventional apposition eyes, $\Delta\phi$ is typically narrow, and together with a small acceptance angle ($\Delta\rho$, half-width of the photoreceptor's angular sensitivity curve) this leads to good spatial resolution. Surprisingly, in the nocturnal bees *M. genalis*, the minimal $\Delta\phi$ of 1.4° in the frontal eye region is extremely narrow for an insect that needs to maximize sensitivity (Fig. 8A) (Warrant *et al.*, 2004).

However, as insects active in dim light have in general larger facets, we can examine the trade-off between sensitivity and resolution using the eye parameter (p), which relates $\Delta\phi$ to the facet diameter D (Snyder, 1979):

$$p = D\Delta\phi \tag{3}$$

Diurnal insects active in bright light intensities typically optimize resolution by having smaller eye parameters. In *M. genalis*, the values for p reach a

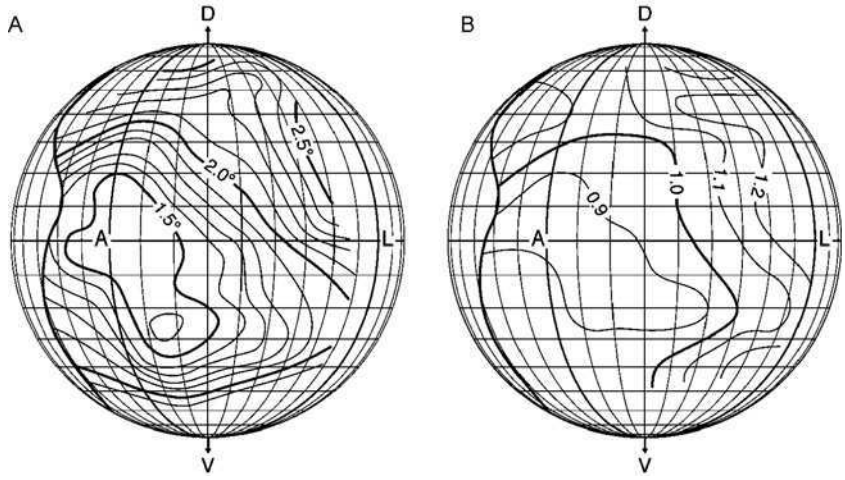

FIG. 8 (A) Interommatidial angles $\Delta\phi$ and (B) the eye parameter p of *Megalopta*'s left eye. Data are plotted onto a sphere representing the three-dimensional space around the bee. Latitude and longitude are shown in intervals of 10°. D, dorsal; V, ventral; A, anterior; L, lateral. (Reprinted from Warrant *et al.*, 2004 with kind permission from Elsevier.)

minimum of 0.9 μm/rad in the frontal eye region (Fig. 8B) (Warrant *et al.*, 2004). This is high compared to most diurnal insects but still lower than generally predicted for nocturnal insects, which is greater than 2 μm/rad (Snyder, 1979). Paradoxically, in nocturnal bees the anatomical resolution of the eye seems more suited for activity in bright light, which brings us to the question of whether the visual fields of the photoreceptors also reflect this.

Despite its detrimental effects on resolution, a useful and common mechanism to enhance sensitivity in dim light is to increase the angle in space from which the photoreceptor receives light (Land, 1981; Snyder, 1977). Essentially all the optical mechanisms discussed earlier in this section affect the size of the photoreceptor's visual field: large apertures, short focal lengths, wide rhabdom diameters, and large dark-adapted pupils all effectively widen $\Delta\rho$ (Snyder, 1979). Typically, day-active insects like bees, butterflies, and dragonflies have a narrow $\Delta\rho$ even during DA. However, insects active in dim light are able to effectively use these optical mechanisms to widen their dark-adapted $\Delta\rho$. The wide rhabdom diameter in the nocturnal bee *M. genalis*, for example, leads to a $\Delta\rho$ of around 6° (Fig. 9A) (Warrant *et al.*, 2004), which is more than twice as wide as in the worker honeybee ($\Delta\rho_{DA} = 2.6°$, Fig. 9B) (Laughlin and Horridge, 1971). These values compare to a dark–adapted $\Delta\rho$ of almost 6° in locusts (Tunstall and Horridge, 1967), and about 7° in cockroaches (Butler and Horridge, 1973), both also caused by wider rhabdom diameters at night. Pupil mechanisms usually have a much stronger

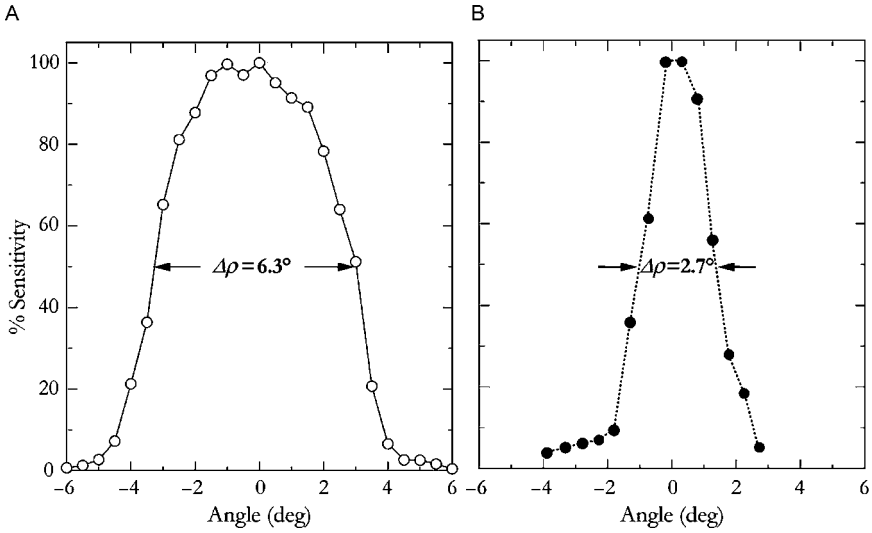

FIG. 9 The angular sensitivity function of dark-adapted photoreceptors in (A) the nocturnal bee *M. genalis* and (B) the worker honeybee *A. mellifera*. The half-width $\Delta\rho$ of *M. genalis* is more than twice as wide as in *A. mellifera*. (A, Reprinted from Warrant *et al.*, 2004 with kind permission from Elsevier; B, redrawn from Laughlin and Horridge, 1971.)

effect on the dark-adapted $\Delta\rho$: in the backswimmer *Notonecta glauca* and the cranefly *Tipula pruinosa*, $\Delta\rho$ increases almost 3- and 4-fold during dark adaptation, respectively (Nilsson and Ro, 1994).

At bright light intensities, a visual overlap $\Delta\rho/\Delta\phi$ of less than 2 is optimal for resolution (Snyder, 1979). For example, in the worker honeybee, $\Delta\rho$ matches the anatomical resolution of the eye ($\Delta\phi = 1.9°$) (van Hateren *et al.*, 1990), leading to a visual overlap of only 1.4. However, in dim-light conditions a larger $\Delta\rho$ improves the signal-to-noise ratio of the photoreceptors for low spatial frequencies. This is when a large visual overlap is beneficial, because without enhanced sensitivity, photon noise will disable the detection of all spatial frequencies (Land, 1981; Nilsson and Ro, 1994; Snyder, 1979). Nocturnal mosquitoes have a visual overlap of almost 5 during DA (Land *et al.*, 1999), while in the tenebrionid beetle *Zophobas morio*, the earwig *Forficula auricularia*, the backswimmer *N. glauca*, and the cranefly *T. pruinosa* ratios of about 6 are present (Nilsson and Ro, 1994). In *M. genalis*, with its large $\Delta\rho$ and small $\Delta\phi$, the visual overlap is 4, clearly showing that these nocturnal bees have prioritized sensitivity over spatial acuity (Warrant *et al.*, 2004). Moreover, in eyes where $\Delta\rho$ is larger than $2{\cdot}\Delta\phi$, additional neural pooling of signals from neighboring ommatidia to an angle of $\Delta\rho$ will result in an increase of sensitivity without further loss in resolution.

For eyes where only anatomical and optical parameters are available, an alternative measure of sensitivity [compared to Eq. (1)] is the optical sensitivity (S). This value defines the amount of light energy that is absorbed by a photoreceptor when it views an extended source of white light (Land, 1981; Kirschfeld, 1974; Warrant and Nilsson, 1998):

$$S = (\pi/4)^2 D^2 (d/f)^2 [kl/(2.3 + kl)] \quad (4)$$

Similar to Eq. (1), the parameters in Eq. (4) include facet diameter and the receptive field, where the term $[(\pi/4)D^2]$ is the facet area, $[(\pi/4)(d/f)^2]$ is the solid angular visual field of the rhabdom, l the rhabdom length, and k the absorption coefficient of the photoreceptors. Wider facets, larger visual fields, and longer, as well as more absorptive, photoreceptors all increase sensitivity.

In eyes with F-numbers less than 2, Eq. (4) has limitations (Stavenga, 2003). However, in those nocturnal apposition eyes with F-numbers larger than 2, it can be applied with safety. Recent work on nocturnal bees and wasps has shown that despite differences in facet size, S is very similar: 2.7 μm^2 sr in *Megalopta* and 3.0 μm^2 sr in *Apoica*. Compared to S-values of 0.1 μm^2 sr in their diurnal relatives, this represents a 25- to 30-fold enhancement of sensitivity (Greiner, 2006; Greiner *et al.*, 2004a). Superposition eyes are in contrast much more efficient in gathering light as shown by an optical sensitivity of 69 μm^2 sr in the nocturnal elephant hawmoth *Deilephila elpenor* and 59 μm^2 sr in the nocturnal dung beetle *Onitis aygulus* (Warrant, 2004).

In *M. genalis*, the physiological parameters are known and the number of photons N a single photoreceptor absorbs within its integration time (Δt), and at a nocturnal light intensity (I), was calculated to be 0.15 photons (Warrant *et al.*, 2004). As the photoreceptors of *M. genalis* absorb 28 times more photons per integration time than those of *A. mellifera* at the same light intensity, the results calculated with either Eq. (1) or Eq. (4) are highly comparable.

A 30-fold improvement in photon capture is remarkable for an apposition eye; however, this improvement alone is not sufficient to account for a light intensity difference of up to 8 log units. As the photoreceptors of *M. genalis* would need to absorb about 100 times more photons per integration time to detect the contrast at its nest entrance (Warrant *et al.*, 2004), additional mechanisms need to be involved.

B. Neural Adaptations

Due to their small facet apertures, optical improvements in sensitivity are particularly limited in apposition eyes. An increase in the response gain of the photoreceptors with decreasing light intensity can further enhance sensitivity

but does not improve photon capture itself (Laughlin, 1981). Instead, the ultimate solution to optimize sensitivity at low light intensities is to process the incoming visual signal using a strategy of neural summation in space and time (Laughlin, 1981; Lythgoe, 1979; Pirenne, 1967; Snyder, 1979; Warrant, 1999; Warrant *et al.*, 1996).

1. Temporal Summation

As is well known to photographers, a longer camera exposure time leads to a brighter image. This improvement in sensitivity, however, comes at a cost—fast moving objects appear "smeared" or could even completely disappear from the photograph. In an analogous manner, visual systems can also improve image reliability at night by slowing vision down. By lengthening the eye's visual integration time at night, the signal-to-noise ratio of lower temporal frequencies is improved at the expense of noisier and less reliable higher temporal frequencies. This temporal summation results in a slower but more reliable visual world. The duration of the visual integration time can be altered via the transduction cascade in the photoreceptor membrane, or by higher circuits that neurally integrate temporal information (Warrant, 2004). Extremely long photoreceptor integration times of about 1.5 s in nocturnal toads (Aho *et al.*, 1993; Donner, 1989), and 160 ms in a deep-sea crustacean (Moeller and Case, 1995), indicate the presence of temporal summation in the photoreceptors. For sit-and-wait predators and slowly moving animals, temporal summation is certainly a good strategy; however, the challenge to perceive flow-field information during nocturnal flight may instead be better met via neural photon summation in space.

2. Spatial Summation

Another strategy photographers use to improve image quality at night is to choose films of coarser grain (higher ASA value), which makes the image brighter at the expense of larger pixels. In apposition eyes, such spatial summation can be achieved by neurally summing the output of neighboring visual channels, or ommatidia, which would dramatically increase photon capture and thus image brightness. Hence, with an increasing extent of neural pooling, the brighter but also the blurrier the image becomes (Fig. 10). This is due to the fact that photons are integrated over wider visual fields, which is similar to a widening of the angular sensitivity function, and therefore has the same effect: reduced spatial acuity. Only when neural summation is matched to the extent of the visual overlap present in the eye, can sensitivity be maximized without further degrading resolution (Section V.C, Fig. 18).

Laterally spreading neurons that sum photons from a large number of ommatidia may provide the neural basis of spatial summation. Such

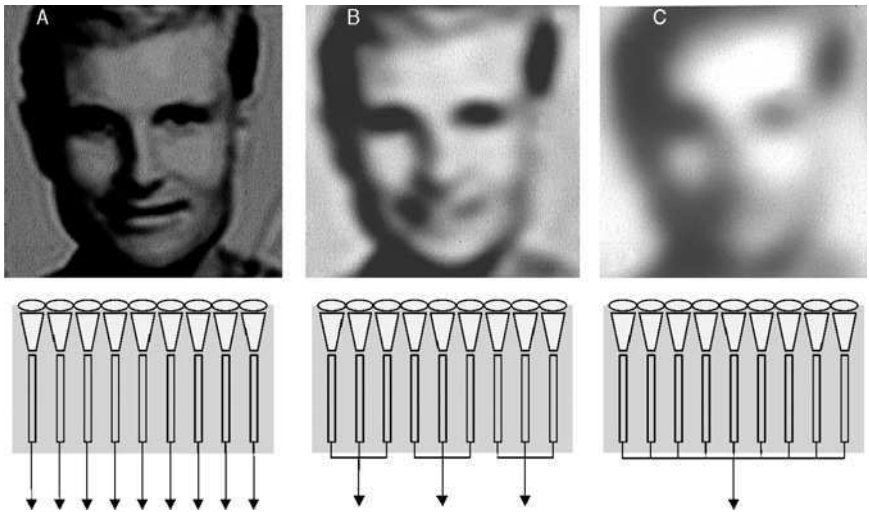

FIG. 10 Theory of spatial summation. (A) Without summation each visual channel within an array of ommatidia remains isolated from all others, creating a sharp but dark image. (B, C) Increasing the amount of spatial summation (pooling of ommatidia) enhances the brightness but degrades the resolution of the image.

neurons, which in insects are likely to be found within the first optic ganglion (or lamina), could create a wide receptive field or "neural pupil." Prime neural candidates, due to their regular arrangement and extensive lateral spreading in nocturnal insects (Greiner *et al.*, 2004b), are the retinula cell axons of the photoreceptors (Fig. 11A), and the first-order interneurons, also known as L-fibers or lamina monopolar cells (Fig. 11B).

3. Organization of the Lamina and Spatial Summation

Remarkably, the neural circuitry of the lamina (i.e., cell identity and position) has remained highly conserved over more than 200 million years (Shaw and Moore, 1989). Instead of the evolution of novel neurons, changes in the branching pattern and synaptic connectivity of existing neurons appear to mediate the high adaptability of the visual system. The optic lobes of insects contain an array of repeated neural units known as cartridges (Meinertzhagen, 1991; Strausfeld, 1976). In apposition eyes, all the retinula cells from one ommatidium directly project, via an axon bundle, to one specific lamina cartridge (Horridge and Meinertzhagen, 1970; Ribi, 1974). Thus, there are as many cartridges in the lamina as there are ommatidia in the eye and neighboring ommatidia project to neighboring laminar cartridges. This retinotopic representation of the visual environment is maintained

across the three optic neuropils (lamina, medulla, and lobula) via an outer chiasm between the lamina and medulla and an inner chiasm between the medulla and the lobula (Fig. 12) (Meinertzhagen, 1976; Strausfeld, 1976).

There are two types of retinula cell axons: short visual fibers, which terminate within the lamina, and long visual fibers, which pass through the lamina and end in the medulla (Fig. 12B). A set of first-order interneurons (L-fibers) joins each cartridge in the distal lamina and a dense network of

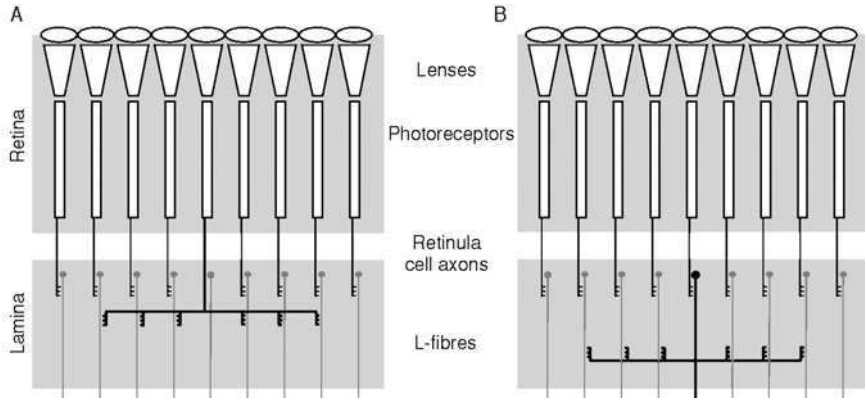

FIG. 11 Possible neural basis of spatial summation in the lamina. Visual information is pooled from several ommatidia via (A) lateral branching of retinula cell axons from the photoreceptors or (B) wide dendritic fields of L-fibers. (Modified from Warrant *et al*., 2004.)

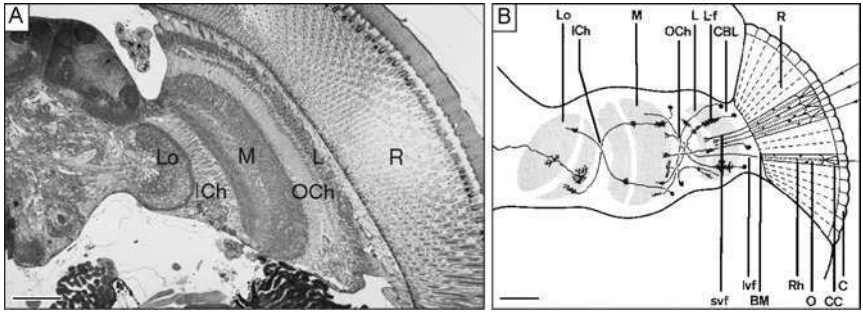

FIG. 12 The hymenopteran visual system features the retina (R) and three optic ganglia: the lamina (L), the medulla (M), and the lobula (Lo). Retinula cell axons project as short visual fibers (svf) to the lamina and as long visual fibers (lvf) to the medulla. In the lamina, a set of first-order interneurons, or L-fibers (L-f), joins the receptor cell axons forming neural units known as cartridges. ICh, inner chiasm; OCh, outer chiasm. D, dorsal; V, ventral; A, anterior; p, posterior. Scales in A, B 200 μm. (B, Modified from Ribi, 1987b.) (See also color insert.)

tangential glial cells tightly surrounds the cartridge bundles. In addition, the lamina contains various accessory cells, including amacrine cells, tangential fibers, and centrifugal cells (Strausfeld, 1976). However, due to our limited understanding of their functions, these cells are discussed no further.

a. Lateral Branching of Retinula Cell Axons In apposition eyes, the exact projection of the retinula cell axons from neighboring ommatidia to the lamina cartridges directly beneath serves the purpose to conserve the image within the optic lobes. However, in eyes where a significant visual overlap is present, pooling of signals from neighboring ommatidia, which matches this overlap, will enhance sensitivity without further decreasing resolution (Snyder, 1979). Such large visual overlaps exist in many dark-adapted apposition eyes of the open-rhabdom type, such as aquatic hemipterans and primitive dipterans (Nilsson and Ro, 1994). As predicted, lateral branching can be found in the short visual fibers of these insects, and interestingly, many dendritic collaterals diverge in an asymmetrical manner into several cartridges (Fig. 13).

Lateral branching over at least 3–5 rows of neighboring cartridges is present in all of the species studied: the backswimmer *N. glauca*, the water strider *Gerris lacustris*, the water boatman *Corixa punctata*, the waterbug *Benatus griseus*, the phantom midge *Chaoborus crystallinus,* and the cranefly *T. rufina* (Meinertzhagen, 1976; Melzer *et al.*, 1997; Strausfeld, 1976; Wolburg-Buchholz, 1979). From these, the backswimmer (Fig. 13C) and the cranefly possess the longest collaterals (Melzer *et al.*, 1997; Wolburg-Buchholz, 1979). The pupil mechanisms of both species, and the spectral sensitivity of *Notonecta*'s outer rhabdomeres, seem to be optimized for vision at low light intensities (Ro and Nilsson, 1995).

The asymmetrical lateral branching pattern of the primitive dipterans has been intensely discussed regarding the evolution of neural superposition (Melzer *et al.*, 1997; Nilsson and Ro, 1994). The hypothesis is that asymmetrical pooling originally evolved to enhance sensitivity (explaining its presence in nocturnal insects), and subsequently led to the neural superposition eyes of advanced flies, by narrowing the pooling fields in favor of resolution. All of these insects show strong dynamic pupils, which narrow to the approximate width of the central rhabdomere during light adaptation (Nilsson and Ro, 1994). These central rhabdomeres remain within their own cartridge and terminate as long visual fibers in the medulla and may therefore mediate high spatial resolution. Consequently, the proposed neural pooling would occur only in the dark-adapted eye, when the pupil is open and the outer six rhabdomeres (R1–6) are exposed to light. In animals that are active at twilight and experience large variations in light intensity, several parallel spatial summation channels may be an additional benefit. In *Notonecta*, for

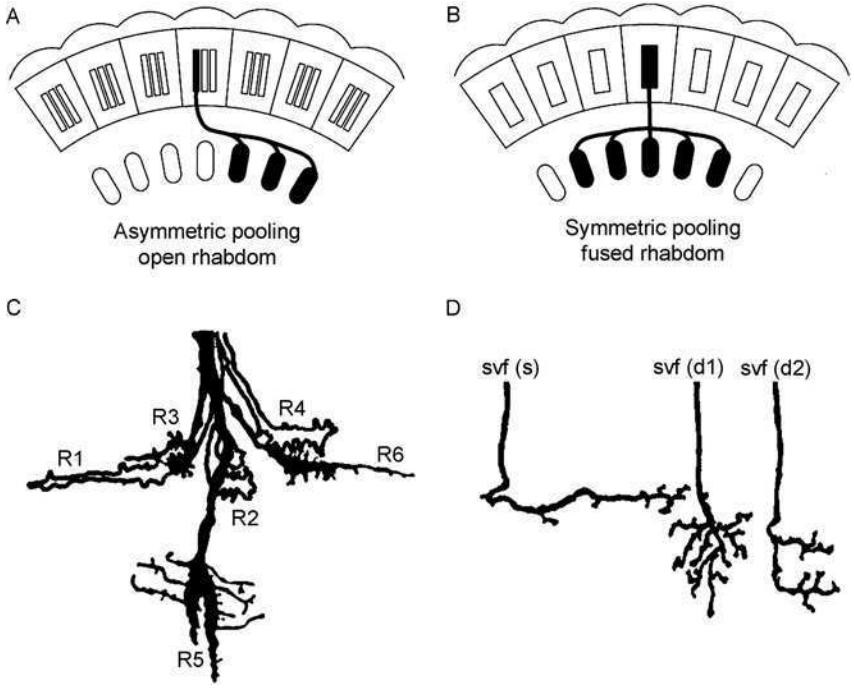

FIG. 13 Lateral branching of short visual fibers (svf) may be involved in either (A) asymmetric or (B) symmetric pooling to enhance sensitivity. In the lamina of (C) the backswimmer *Notonecta glauca* and (D) the cockroach *Periplaneta americana*, asymmetric branching can be found in retinula cell axons R1 and R6 of the backswimmer, as well as svf (s) and svf (d2) of the cockroach, while symmetric branching is present in R5 of the backswimmer and svf (d1) in the cockroach. (A, B, Redrawn from Nilsson and Ro, 1944; C, redrawn from Wolburg-Buchholz, 1979; D, redrawn from Ribi, 1977.)

example, this might be mediated by a different extent of lateral spreading in the six short visual fibers (Fig. 13C).

In most apposition eyes with fused rhabdoms, the retinula cell axons from one ommatidium project to a single cartridge and their dendritic branches remain within that same cartridge (Meinertzhagen, 1976; Meinertzhagen *et al.*, 1980; Ribi, 1975, 1987a). As apposition eyes are typically present in diurnal insects, neural summation to enhance sensitivity should not be necessary. Accordingly, one might expect lateral branching in nocturnal insects. Indeed, in the lamina of the cockroach, all short visual fiber types show wide asymmetric and symmetric lateral branching (Fig. 13D) (Ribi, 1977). The dendritic fields of these retinula cell axons (as well as those of their L-fibers) could thus enhance sensitivity through neural pooling. The reason

for the presence of asymmetric projections in fused rhabdoms is, however, unclear, as all photoreceptors receive light from the same visual angle in space. In nocturnal bees, lateral branching was found in one of the three short visual fiber types (Greiner *et al.*, 2004b, 2005). This is the first time that lateral branching of retinula cell axons has been demonstrated in hymenopterans. Thus, in order to relate this lateral spreading to nocturnal vision, more comparative studies of other nocturnal and diurnal hymenopterans are needed. In nocturnal mosquitoes, lateral branching into adjacent cartridges may also exist (Land and Horwood, 2005), and due to large visual overlap (Land *et al.*, 1997), spatial summation of adjacent cartridges would be beneficial.

Neural pooling in fused rhadoms does not allow separation of spatial channels, like those proposed for open rhabdom eyes. Thus, to retain good spatial resolution during the day and use spatial summation at night, a dynamic control in the neurons involved in spatial summation would be of great benefit (Warrant and McIntyre, 1993). Circadian changes of synapses in the fly lamina indicate that a neural basis for such dynamics may in fact exist (Pyza and Meinertzhagen, 1993).

Surprisingly, the lamina of the diurnal skipper butterfly *Parnara guttata* features lateral branching of both short and long visual fibers (Shimohigashi and Tominaga, 1999). Skipper butterflies, despite living in bright light, are highly unusual in that the secondary pigment granules of their superposition eyes always remain in a position typical of dark-adapted nocturnal moth eyes (Horridge *et al.*, 1972). However, as light intensity represents no limitation to their eyes, these collaterals may be involved in visual tasks other than enhancing sensitivity.

b. Lateral Branching of L-Fibers Lateral spreading of L-fibers is commonly found in insects and shows an interesting correlation, where the amount of spreading is greater in the lamina of crepuscular and nocturnal insects than in diurnal species (Greiner *et al.*, 2004b). This hypothesis—that a nocturnal lifestyle should affect the amount of lateral branching in L-fibers—was recently investigated in nocturnal and diurnal bees (Fig. 14) (Greiner *et al.*, 2004b, 2005). In bees, the structure of the lamina as well as the number and identity of the four different L-fibers is highly conserved. The lamina contains three morphologically distinct layers, A, B, and C, and the four L-fiber types L1–L4 are categorized by their branching pattern within these three layers. L-fibers with branches in all three layers are categorized as the L1-type, L-fibers with processes in layers A and B are of the L2-type, L-fibers arborizing in layer B (in the honeybee) or A and C (in the nocturnal bee) are of the L3-type, and L-fibers branching exclusively in layer C are of the L4-type (Fig. 14) (Greiner *et al.*, 2004b; Ribi, 1976).

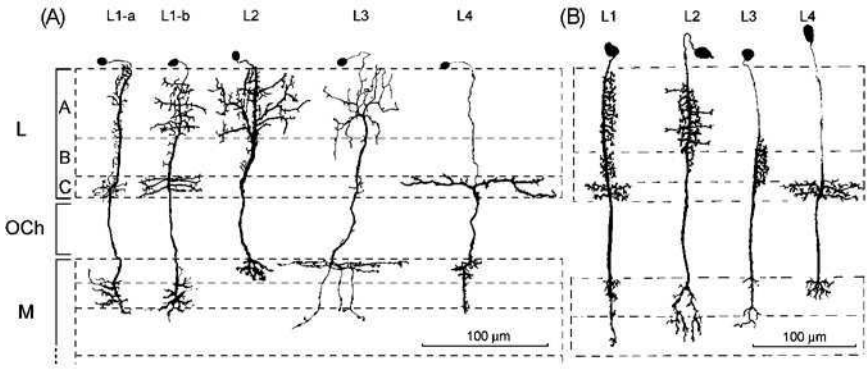

FIG. 14 The L-fiber types L1–L4 of (A) the nocturnal bee *M. genalis* and (B) the worker honeybee *A. mellifera* shown in frontal orientation. All L-fiber types feature extensive lateral branching in the nocturnal bee, which is significantly wider than in the honeybee. Only the distal part of the medulla containing the L-fiber terminals is shown. Note the wide lateral branching of L3 in the medulla. L, lamina; OCh, outer chiasm; M, medulla; A, B, C, layers of the lamina. (a, Modified from Greiner *et al.*, 2004b; b, modified from Ribi, 1975.)

Extensive lateral branching is present in the lamina of nocturnal bees (Fig. 14A), and this branching is significantly wider than in diurnal bees (Fig. 14B) (Greiner *et al.*, 2004b). The largest dendritic fields of the L-fibers, L2 and L3, extend to 12 and 13 cartridges, respectively (Section V.C, Fig. 19). L4 targets a remarkable 18 cartridges (Fig. 15), and this is considerably larger than the largest dendritic fields of L-fibers found in the honeybee (L2, which visits 9 cartridges: Ribi, 1981).

Behavioral data show that the honeybee *A. mellifera* is also able to see at moonlight intensities, even though the optical structure of its eyes should render it blind by mid-dusk (Warrant *et al.*, 1996). Theoretical modeling predicts that neural summation should occur in the honeybee to enhance its vision at low light intensities (Warrant *et al.*, 1996). The lateral branching of L2 and L4 may well serve this purpose and might also explain why the African race of the honeybee *A. mellifera adansonii* and the Asian giant honeybee *A. dorsata* are able to continue to forage on nights when at least a half-full moon is present in the sky (Dyer, 1985; Fletcher, 1978). Subsequent widening of the dendritic fields in L1, L2, and L4, together with the modifications of the branching pattern in L3, may all have evolved in bees as a response to the benefits of a nocturnal lifestyle. Whether other nocturnal hymenopterans have followed a similar evolutionary path, or whether they have evolved an alternative solution to enhance vision at night, is a fascinating open question that needs further attention.

How do these findings in bees compare to the L-fiber branching present in other insects? Is there a correlation with their preferred light intensity? In

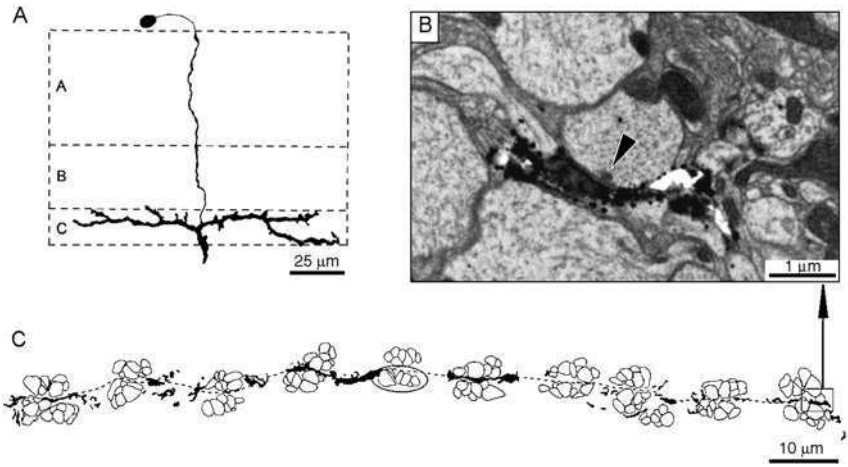

FIG. 15 (A) The dendritic field of the L-fiber L4 showing wide lateral branching exclusive to lamina layer C and oriented in an extreme dorsoventral orientation. (B) Example of an EM section where the membrane of L4 is silver-stained (Golgi) and therefore appears black. *Arrowhead* marks a synapse in an adjacent axonal stem of another L-fiber. (C) L4's dendritic field of 18 cartridges in a reconstructed serial cross-sectioned layer C. The L4's own cartridge is circled in the center. Dendritic branching pattern was reconstructed from alternating Golgi-electron microscopy (EM) ultrathin sections (drawn in black) and light microscopy semi-thin sections (*dotted lines*). A, B, C, lamina layers A, B, C. (Modified from Greiner *et al.*, 2005.)

most strictly diurnal species, like the cabbage butterfly *Pieris* (Strausfeld and Blest, 1970), the dragonfly *Sympetrum* (Meinertzhagen and Armett-Kibel, 1982), and the housefly *Musca* (Boschek, 1971; Strausfeld, 1971), the dendrites of all L-fibers are confined to their own parental cartridge. However, there are interesting exceptions, like the orchard butterfly *Papillio*, which shows a branching pattern similar to that of the diurnal honeybee (Ribi, 1981, 1987a). Again, these branches may be involved in other visual tasks (e.g., color vision), as diurnal insects with apposition eyes have no need for spatial summation.

An interesting correlation with intensity and L-fiber branching can be found within the previously discussed aquatic hemipterans, the backswimmer *N. glauca*, the water boatman *C. punctata,* and the waterstrider *G. lacustris* (Wolburg-Buchholz, 1979). In all three species, four L-fiber types L1–L4 are present. In *Notonecta* three of them contact neighboring cartridges, with L3 having the largest dendritic field with contact over eight cartridges. In the water boatman, only L3 spreads laterally, in this case over five cartridges, and in the water strider all four L-fibers remain in their own cartridge. Their behavior matches the hypothesis: *Notonecta* visually navigates during crepuscular periods (Schwind, 1983), *Corixa* spends time on

the pond floor (Chinery, 1973), where a sensitive visual system might be beneficial, and the water strider hunts on the water surface in bright light.

The cockroach *Periplaneta americana* is the only insect with apposition eyes where all L-fibers are wide-field neurons with large dendritic fields (Ribi, 1977). As *Periplaneta* is purely nocturnal, these laterally branching neurons may effectively sum the visual input reaching the lamina from several ommatidia in order to enhance sensitivity.

Similar wide-field neurons are present in the lamina of nocturnal insects with superposition eyes, like the male firefly *Phausis splendidula* (Ohly, 1975) and nocturnal hawkmoths (Strausfeld and Blest, 1970). Interestingly, when comparing nocturnal and diurnal hawkmoths, nocturnal species have significantly wider lateral branching L-fibers (A. Balkenius, unpublished observations). This correlation, similar to the one found in bees, strongly indicates that neural summation may be a general strategy to enhance sensitivity in insects.

As pointed out by Ribi (1977), differences in the lamina organization found across insect groups may depend on their visual requirements. A more differentiated lamina is characterized by a multilayered appearance and a defined location of the neurons within the lamina (Kral, 1987). Cockroaches have a less organized lamina, where a cartridge may contain anything from 6 to 20 fibers (Ribi, 1977). The greater visual demands of day-flying insects might have led to the evolution of highly organized laminas, such as those of neural superposition eyes, whose exact wiring arose from the unorganized asymmetric cell branching found in primitive dipterans. In bees, however, nocturnal activity has most likely evolved from an originally diurnal lifestyle, and the extent of lateral L-fiber branching simply widened in response to the low light intensities.

How does the receptive field size of photoreceptors compare to that of L-fibers? In dragonflies, dark-adapted L-fibers receive their major input from retinula cell axons and therefore have the same field of view (Laughlin, 1973). As no lateral connections into neighboring cartridges exist in the lamina of dragonflies (Meinertzhagen and Armett-Kibel, 1982), it makes sense that the receptive field is not increased. Such recordings have not yet been done in the L-fibers of nocturnal insects and an interesting question is whether the receptive fields of wide-field L-fibers are actually larger than the receptive fields of the photoreceptors. In addition, one might expect the receptive field size to be controlled by a circadian rhythm and/or changes in light intensity. Future investigations, especially with respect to these possible dynamic regulations during day and night, both physiologically and anatomically, will also advance our knowledge of neuronal plasticity.

Spectral sensitivity recordings of the L-fibers in the honeybee lamina revealed a highly complex response pattern (de Souza *et al.*, 1992). Whether responses in the nocturnal bee *M. genalis* are equally complex remains to be

seen as no attempts have so far been made to physiologically characterize the functions and receptive fields of the four L-fiber types. The possible roles of the different L-fibers in the lamina of *M. genalis* can therefore only be discussed on the basis of their anatomy and by direct comparison to the thoroughly studied synaptic connectivity in the lamina of the worker honeybee (Greiner *et al.*, 2005; Ribi, 1981, 1987b). For example, in *M. genalis*, L4 shows the largest dendritic field, extending over 18 cartridges in an extreme dorsoventral direction (Fig. 15). As L4 is restricted to layer C, it does not receive direct input from short visual fibres, but probably does so via L1 instead. In addition, L2 and L3 have large dendritic fields of 12 and 13 cartridges and are therefore potential candidates for spatial summation (Section V.C, Fig. 19).

Only a combination of single cell recordings (receptive fields) and intracellular staining can reveal whether wide-field L-fibers actually mediate spatial summation in the lamina. Nevertheless, theoretical modeling predicts a great deal about the optimal amount of spatial and temporal summation that is needed in order to allow nocturnal vision in bees (Theobald *et al.*, 2006). Whether these theoretical predictions match the anatomical data is the topic of the final section.

C. A Model of Neural Summation

As discussed earlier, spatial and temporal summation enhances sensitivity at the cost of spatial and temporal resolution. Using the analytical model developed by Warrant (1999), one can now test this trade-off theoretically and determine whether neural summation can improve vision in dim light or whether the sacrifices in resolution actually outweigh the sensitivity gain and make vision worse. By calculating the number of photons N sampled by a visual channel [Eq.(4), explained in Section III.A] and the total visual noise (photon shot noise $\sqrt{N}$ plus the effect of dark noise σ^2_D), at a particular light intensity I and image velocity v, the model predicts the maximum detectable spatial frequency ν_{max} that an animal can see with and without optimal spatial and temporal summation. A criterion used in the model is that ν_{max} occurs when signal and noise become equal: all spatial frequencies below ν_{max} can be reliably distinguished, whereas those higher than ν_{max} are lost in the noise (Fig. 16).

The Gaussian angular sensitivity function describes visual performance in the eye. Similarly, the summation model defines a Gaussian output channel receptive field (with a half-width $\Delta\rho_T$) that predicts visual performance after spatiotemporal summation (Fig. 17). This summation function is defined by the convolution of three Gaussians: the receptive field of each input channel

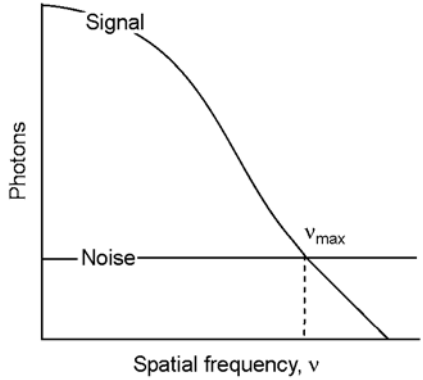

FIG. 16 Signal decreases with increasing spatial frequency ν (cycles deg^{-1}), while noise remains constant. At the maximum detectable spatial frequency ν_{max}, signal and noise become equal and are indistinguishable from each other. (Redrawn from Warrant, 1999.)

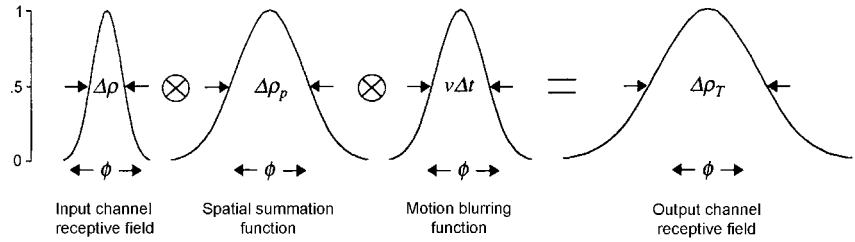

FIG. 17 In the spatiotemporal summation model, the output channel receptive field $\Delta\rho_T$ depends on the input channel receptive field $\Delta\rho$, the extent of spatial summation (spatial summation function $\Delta\rho_P$), and the extent of temporal summation (motion blurring function $\nu\Delta t$). The circles with crosses signify convolution, the ϕ-axis defines angles in degrees, and the vertical amplitudes of the function relates to sensitivity. (Reprinted from Warrant, 1999 with kind permission from Elsevier.)

of half-width $\Delta\rho$, the spatial summation function of half-width $\Delta\rho_P$, and a motion blurring function $\nu\Delta t$ (temporal summation) (Warrant, 1999).

The spatial summation function (of half-width $\Delta\rho_P$) also describes the strength of coupling within the field of channels. This Gaussian assumes that nearest-neighbor channels contribute most to the summed signal, which gradually decreases with increasing distance. In dim light, this function is generally much wider than the angular sensitivity function, or input receptive field (half-width $\Delta\rho$), of the photoreceptor. Thus, a much greater visual overlap is present between the channels. This leads to an increased photon capture in each visual channel, but only at the cost of spatial resolution

(Fig. 18A). Despite a drastic decrease in resolution, spatial summation nevertheless proves to be beneficial in dim light due to the enhanced signal-to-noise ratio at low spatial frequencies (Warrant, 1999).

Irrespective of whether the visual system increases sensitivity by optically widening the receptive field of the input channels ($\Delta\rho$) or neurally widening the receptive field of the output channels ($\Delta\rho_P$), resolution will always be limited by the larger of the two. Consequently, to optimize both resolution and sensitivity, the size of $\Delta\rho$ and $\Delta\rho_P$ should be matched. In eyes where the input receptive field $\Delta\rho$ is much larger than the anatomical resolution of the eye, spatial summation from the number of ommatidia representing $\Delta\rho$ will enhance sensitivity without further decreasing resolution (Fig. 18B) (Nilsson and Ro, 1994). In the case of dynamic or circadian changes in $\Delta\rho$ (e.g., pupil mechanisms or changes in rhabdom diameter), $\Delta\rho_P$ could also be under dynamic control (possibly via synaptic changes within neurons).

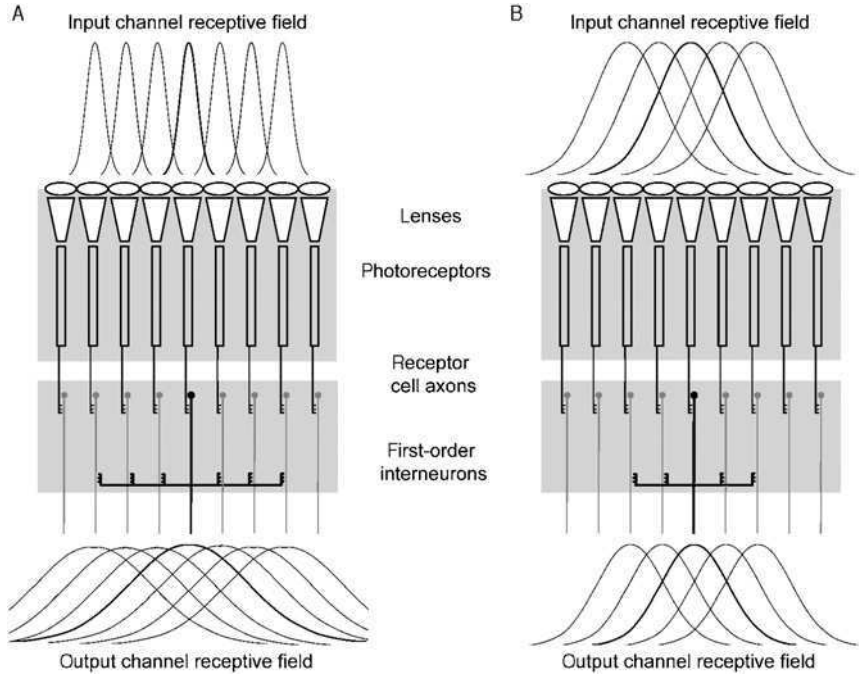

FIG. 18 Theory of spatial summation mediated by the receptive field of a wide-field first-order interneuron, which couples the input channels from the photoreceptors. (A) In a conventional apposition eye this leads to a large visual overlap and poor spatial resolution. (B) In some nocturnal apposition eyes large visual overlap is already present in the retina. In these eyes, a laterally branching neuron with a matched summation field would enhance sensitivity without further losses in resolution.

Accordingly, the model assumes intensity-related dynamics, where pooling is turned off at bright light intensities and gradually "switched on" as the intensity level decreases. Subsequent Fourier transformation of the output channel receptive field yields the modulation transfer function (MTF), which is used to calculate the signal. MTFs show the potential range of spatial frequencies seen by the eye, where wider receptive fields lead to a narrower range of frequencies. In practice, inherent visual noise reduces this range significantly (for more details on the model see Warrant, 1999).

As apposition eyes have small apertures and isolated visual channels, the model predicts that spatial and temporal summation will be of great benefit for this eye design in dim light. Indeed, in locusts and bees, the model predicts that neural summation can dramatically improve visual performance as measured by the maximum detectable spatial frequency ν_{max} (Theobald *et al.*, 2006; Warrant, 1999; Warrant *et al.*, 1996). Using spatiotemporal summation, locusts can extend their vision from mid-dusk into starlight, a remarkable difference of 5 log units (Warrant, 1999). In the honeybee, the optical sensitivity of their apposition eyes alone should render them blind by mid-dusk. However, behavioral data show that visual performance in dim light is much greater than expected from the optics, and the model predicts that this performance can be achieved by optimal spatiotemporal summation (Warrant *et al.*, 1996). In the interesting case of the nocturnal bee *M. genalis*, the limited optical sensitivity of its eyes does not allow reliable vision at the low light intensities in which it is active. Thus, neural summation is crucial for the visual performance of nocturnal bees (Fig. 19A) (Theobald *et al.*, 2006).

The model also predicts the optimum balance between spatial and temporal summation for different light intensities and image velocities. For an animal experiencing low image velocities, visual performance is better enhanced via temporal summation, whereas at high image velocities temporal resolution needs to be retained. In the latter situation, enhanced visual performance in dim light is best achieved by spatial summation, as seen in insects capable of fast flight even at low light intensities, like locusts and nocturnal bees (Theobald *et al.*, 2006; Warrant, 1999; Warrant *et al.*, 1996). At constant image velocity and in dim light, spatial summation contributes most to the enhancement of visual performance.

How does the spatial summation function relate to the branching pattern of wide-field L-fibers? Lateral branching is present in one of the six L-fibers of the locust lamina (Nowel and Shelton, 1981); however, it is unknown whether the dendritic field of its branching pattern matches this amount of spatial summation. In nocturnal bees, however, the L-fiber's dendritic fields are known (Greiner *et al.*, 2005), and after transforming the receptive field of the summation function into numbers of cartridges, the results from the theoretical model can be directly compared with the anatomical data

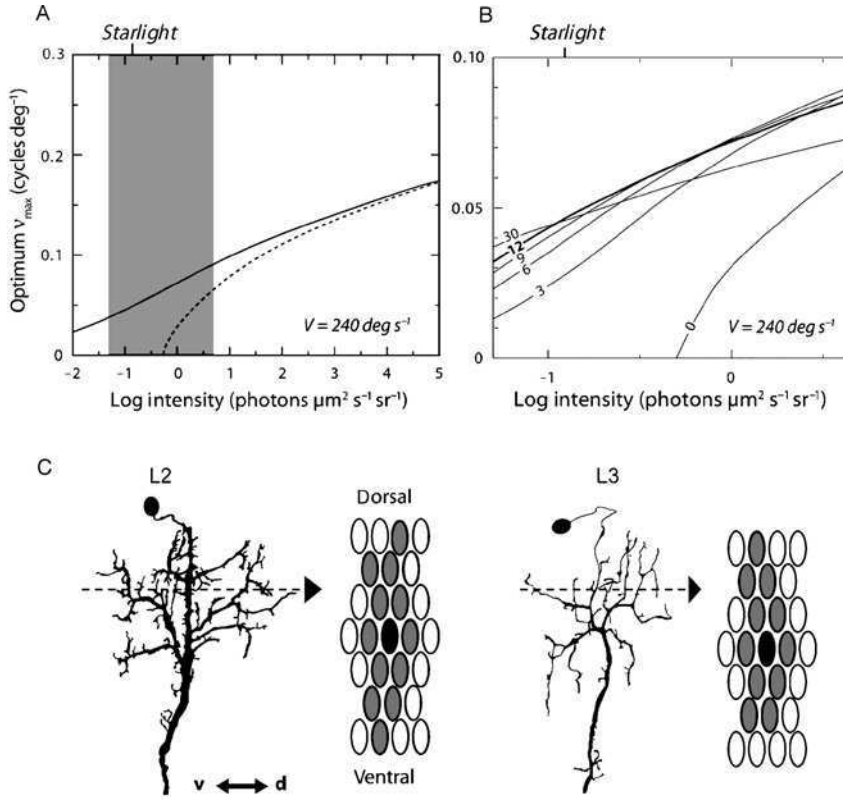

FIG. 19 Spatial and temporal summation modeled for *Megalopta genalis* at different light intensities for a constant image velocity of 240 deg s^{-1}. *Gray areas* mark the light intensity window at which *M. genalis* is normally active. With optimal summation (*solid line*) the maximum detectable spatial frequency ν_{max} is significantly higher over the entire light intensity window than without summation (*dashed line*). (B) Optimum summation in *M. genalis* expressed as various extents of spatial summation: curves for no summation (0), and for 3, 6, 9, 12, and 30 summing cartridges. ν_{max} was measured at a constant velocity (240 deg s^{-1}) and over the range of light intensities experienced by *M. genalis* (−1.3 to 0.7 log units). The *heavy black line* marks the summation of 12 cartridges, which overall proves optimal with respect to the entire range of light intensities. (C) Equivalent dendritic field sizes can be found in L-fibers L2 and L3 of *M. genalis* reconstructed from frontal sections. The schematic drawings represent the cross-sectioned layer marked by the *dashed arrows*. The ovals illustrate the schematic arrangement of the L-fiber's own cartridge (*black*) and its dendritic field (*gray*). v, ventral; d, dorsal. (A, B, Modified from Theobald *et al.*, 2006; C, modified from Greiner *et al.*, 2005.)

(Theobald *et al.*, 2006). Over the entire intensity range of *Megalopta*'s activity period, the model predicts a summation of about 12 cartridges to be optimal (Fig. 19B). Even though summation from 30 cartridges would be better at the lowest intensities, it reduces visual performance at brighter

intensities. The data generated by the model match the anatomical results remarkably well, where the dendritic fields of L2 and L3 visit 13 and 12 cartridges, respectively (Fig. 19C), and L4 as many as 18 (Fig. 15, Greiner *et al.*, 2005). Furthermore, a simulation of the spatial resolution confirms that the optical resolution of the bee's eye is already blurred at the level of the retina, so that spatial summation would hardly worsen the image (Theobald *et al.*, 2006).

Thus, neural summation is extremely useful for insects active in dim light and is able to convert an apposition eye into a reasonably well functioning nocturnal eye. With optimal spatiotemporal summation, vision can be extended into significantly dimmer light intensities, and one can expect that this clearly beneficial strategy is widely used by nocturnal insects with apposition eyes.

VI. Concluding Remarks

Despite our own preference for bright light, several groups of insects seek the cover of night in search of food and to reduce the risk of predation and competition. The fact that some insects were able to achieve this transition to a nocturnal lifestyle while retaining their apposition eyes is remarkable and intriguing, as this eye design is highly unsuitable for vision at low light intensities. How is it possible that small tropical bees can navigate in an entangled rainforest using landmarks at light intensities where it is not possible to see your own hand in front of your face? This review presents numerous optical and neural adaptations that nocturnal insects have evolved to significantly enhance the sensitivity of their apposition eyes for dim-light vision. One remarkable example is certainly the apposition eye of nocturnal mosquitoes, where unique structural changes of the photoreceptors match the wide cone of light reaching the eye through almost hemispherical lenses. Additional neural adaptations are crucial in other insects, like nocturnal bees, and a hypothesis of spatiotemporal summation for further enhancing sensitivity is proposed. Although a large amount is still unknown, recent anatomical evidence of laterally spreading neurons in the first optic ganglion, and convincing neural modeling, strongly suggest that spatial summation is present in the eyes of these nocturnal bees. Further knowledge of the neural networks within the first optic ganglion of nocturnal insects, and the mechanisms of neural plasticity modulating their early visual processing, will reveal the principles responsible that regulate vision at low light. Such discoveries may be instrumental in the development of new technologies, including night-vision devices.

Acknowledgments

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Kinase and Phosphatase: The Cog and Spring of the Circadian Clock

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Reversible phosphorylation is an important regulatory mechanism for many biological processes in eukaryotic organisms. The phosphorylation state of a protein is controlled dynamically by both protein kinases and phosphatases. Phosphorylation of circadian clock proteins is an essential posttranscriptional mechanism in the regulation of circadian clocks, and several protein kinases and phosphatases have been shown to regulate key clock components in eukaryotic systems, including *Arabidopsis*, *Neurospora*, *Drosophila*, and mice. In this review, recent progress in the characterization of protein kinases and phosphatases involved in circadian rhythms is summarized. The protein kinase CK2 has been proposed as an evolutionary link between the divergent circadian systems of plants, animals, and fungi. The roles of CK2 in this process are discussed here in detail.

KEY WORDS: *Arabidopsis*, Casein kinase II (CK2), Circadian rhythms, LHY/CCA1, Photoperiod, Protein kinase, Protein phosphatase, Protein phosphorylation.

I. Introduction

The circadian clock optimizes the relationship, or phase angle, between biological activities and dawn and dusk, thereby allowing specific biological activities to occur at precise times of day, or phases (McClung *et al.*, 2002). The hallmarks of the circadian clock are that it persists without environmental time cues and maintains a period of about 24 h. The circadian clock

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controls numerous physiological and molecular processes in organisms ranging from bacteria and fungi to humans. For example, the circadian rhythms of sleep, melatonin secretion, body core temperature, and the cell cycle are generated by clocks within us. Autosomal semidominant mutations in rodents with fast or slow circadian clocks (i.e., short or long endogenous period lengths; *tau*) are associated with phase-advanced or delayed sleep–wake rhythms, respectively (Lowrey *et al.*, 2000). For clock mutants in plants, the situation is similar. Two closely related genes, *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), encode Myb proteins that are highly associated with clock functions in *Arabidopsis*. The *lhy cca1* mutation, which results in a pronounced short period, is associated with phase-advanced rhythms of clock controlled gene (CCG) expression during light/dark cycles (Mizoguchi *et al.*, 2002).

These models predict the existence of familial human circadian rhythm variants. In a behavioral trait known as familial advanced sleep phase syndrome (FASPS), sleep onset occurs at approximately 1930 h, when most people are actively socializing. Sleep duration is normal but is terminated by spontaneous awakening at approximately 0430 h, just when conventional sleepers are at their sleepiest time of the 24-h cycle. The FASPS gene was mapped near the telomere of chromosome 2q by Toh *et al.* (2001). A candidate gene (*hPer2*), a human homolog of the *period* gene in *Drosophila*, maps to the same locus. Affected individuals have a serine to glycine mutation within the casein kinase I (CKI)-binding region of the clock component hPER2, which causes hypophosphorylation by CKI *in vitro*. A variant in human sleep behavior can thus be attributed to a missense mutation in hPER2 that alters the circadian period. Identification of a phosphorylation-site mutant of hPER2 in a family with an inherited circadian rhythm abnormality strongly suggests that PER2 is a physiologically relevant substrate of CKI. Furthermore, a missense mutation (T44A) in the human *CKIdelta* gene has been shown to result in FASPS (Xu *et al.*, 2005). This mutant kinase has decreased enzymatic activity *in vitro*. Transgenic mice carrying the same mutation have a shorter circadian period, a phenotype mimicking human FASPS. These results clearly indicate that protein phosphorylation plays important roles in circadian rhythm regulation in humans.

The stability of self-sustained oscillation and the length of the circadian period are key features of circadian rhythms. These properties are likely to be realized by interactions between multiple feedback processes (Roenneberg and Merrow, 2002; Somers, 1999) and multiple posttranslational controls of the core feedback process, including subcellular translocation, protein–protein interactions, and phosphorylation of some circadian clock proteins (Dunlap, 1999; Young and Kay, 2001). An understanding of the role of protein phosphorylation status has become essential to understanding the mechanisms of the circadian clock. This review summarizes the recent

progress made in elucidating the roles of protein kinases and phosphatases in the biological clock (Table I).

II. Protein Phosphorylation and Clock Functions

A. Circadian Rhythms

Circadian clocks oscillate with an approximately 24-h period, are ubiquitous, and presumably confer a selective advantage by anticipating the transitions between day and night. The circadian system was proposed to be composed of three components: an input pathway (usually described as photoreceptors) that entrains the clock, the central oscillator (clock), and output pathways to generate the overt rhythms (Fig. 1A; Dunlap, 1999). These three components

TABLE I
Glossary of Chronobiology Terms[a]

Amplitude	Difference between maximum (or minimum) value and mean value in a sinusoidal oscillation
Circadian	Literally, about a day (24 h)
Clock	Generally refers to the entire circadian system, although it is sometimes used to mean the oscillator
Clock gene	A rhythmically expressed negative element of a transcription/translation feedback loop; this term is also used to refer to a gene that encodes any oscillator component
Free running rhythms	Self-sustained oscillations under constant conditions
Entrainment	The setting of the oscillator to match environmental cycles of light and dark, or of temperature
Input pathways	The sequence of events via which information from the environment, such as changes in light and temperature, is transduced to the oscillator
Oscillator	The cell-autonomous timekeeper responsible for generating self-sustained rhythmicity; also called the pacemaker
Output	The pathways linking the oscillator with the various biological processes it controls
Period	Time after which a definite phase of the oscillation reoccurs
Phase	The relationship of some point in a rhythm to a marker, such as another rhythm; for instance, the relationship of the peak expression of a gene to daybreak during a day/night cycle
Phase shift	A single displacement of an oscillation along the time axis
Rhythm	The regular oscillations of a process

[a]Modified from Barak *et al.* (2000).

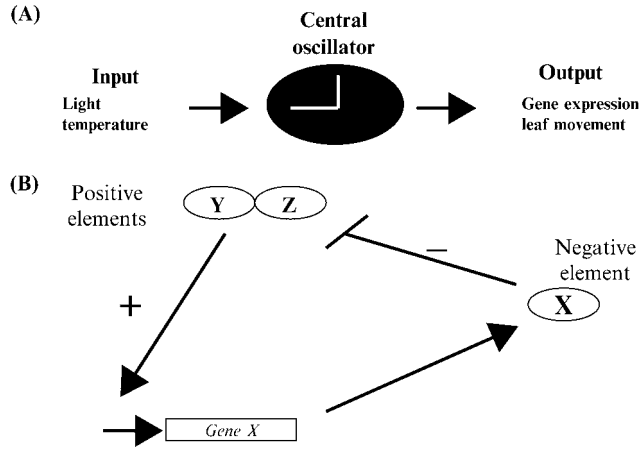

FIG. 1 Three components of the circadian system and a negative feedback model. (A) Conceptual scheme showing simple linear information flow from input pathways through the central oscillator to output pathways. (B) General scheme showing the core feedback loop central to circadian oscillators. In this model, paired positive elements Y and Z form heterodimers that act as transcriptional activators to induce clock gene (X) expression. The protein products (negative element) of the clock gene X, in turn, block the action of the positive elements, thus indirectly repressing their own expression.

are believed to be arranged in a linear array, with information flowing unidirectionally from the input through the oscillator to the output. At the core of the circadian clock, the central oscillator consists of a negative autoregulatory feedback loop (Fig. 1B). This negative feedback model was based on the transcription and translation of genes that play important roles in the oscillator (often referred to as clock components). However, it has become increasingly clear that a simple linear array of the circadian system is inadequate, and a more complex model has emerged. In particular, the importance of posttranslational modifications (e.g., phosphorylation) of clock components has been highlighted.

B. Clock Functions in Animals, Fungi, and Bacteria

Although the simple transcription/translation feedback loops described above allow the construction of satisfactory models, these models are not sufficient to describe circadian rhythmicity. Multiple levels of posttranscriptional controls are built into these systems, presumably to delay the cycles so that they take a full 24 h, maintain robust amplitude of cycling from the transcription of clock components all the way to physiological outputs, and

buffer the clock mechanism against abrupt changes. In addition, post-transcriptional control provides mechanisms by which the clock can be reset by environmental inputs. These mechanisms include the splicing and stability of RNA, the control of protein translation, the nuclear transport of protein, and the stability and degradation of protein. Recently, protein phosphorylation has been shown to be important in the posttranscriptional regulation of the clock, at least in the latter two mechanisms (Schoning and Straiger, 2005).

Protein kinases, such as CKI, casein kinase II (CKII or CK2), glycogen synthase kinase 3β (GSK3β), mitogen-activated protein kinase (MAPK), and tyrosine kinase (PTK), are involved in the control of circadian rhythms in animals and fungi. Two protein phosphatases, protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1), dephosphorylate clock components in *Drosophila* and *Neurospora*. Regulation of the clock components PERIOD (PER) in *Drosophila* and FREQUENCY (FRQ) in *Neurospora*, by reversible phosphorylation, has been well characterized. Protein phosphorylation is essential to cell cycle control, which is closely associated with circadian rhythms. Moreover, a protein kinase, WEE1, has been shown to participate in this process. In this section, protein phosphorylation and clock function in animals, fungi, and bacteria are summarized.

1. Key Players in the *Drosophila* Clock

Circadian locomotor rhythms in *Drosophila* are regulated by a clock located in the central brain (Staewwsky *et al.*, 2002). The clock itself consists of at least two autoregulatory feedback loops generated by several core clock molecules (Glossop *et al.*, 1999). In the major loop, the transcriptional activators CLOCK (dCLK) and CYCLE (CYC) bind a CACGTG E-box in the promoters of *per* and *timeless* (*tim*) and activate transcription (Glossop *et al.*, 1999). PER and TIM proteins inhibit their own transcription by binding to CLK and CYC. The timely degradation of PER and TIM relieves this repression and maintains a 24-h rhythm in the RNA and protein levels of *per* and *tim*.

The phosphorylation status of PER and TIM affects protein stability and is thought to confer oscillations at the protein level. PER is phosphorylated and destabilized by DOUBLETIME (DBT), a CKIϵ homolog (Bao *et al.*, 2001; Kloss *et al.*, 1998; Price *et al.*, 1998). In addition, PER is phosphorylated by CK2 (Akten *et al.*, 2003; Lin *et al.*, 2002). PER cycling may also depend on its interaction partner, TIM, which stabilizes PER by inhibiting DBT-mediated phosphorylation (Kloss *et al.*, 1998). TIM is phosphorylated by GSK3β (or SHAGGY; Martinek *et al.*, 2001), and hyperphosphorylated TIM is targeted for degradation (Grima *et al.*, 2002).

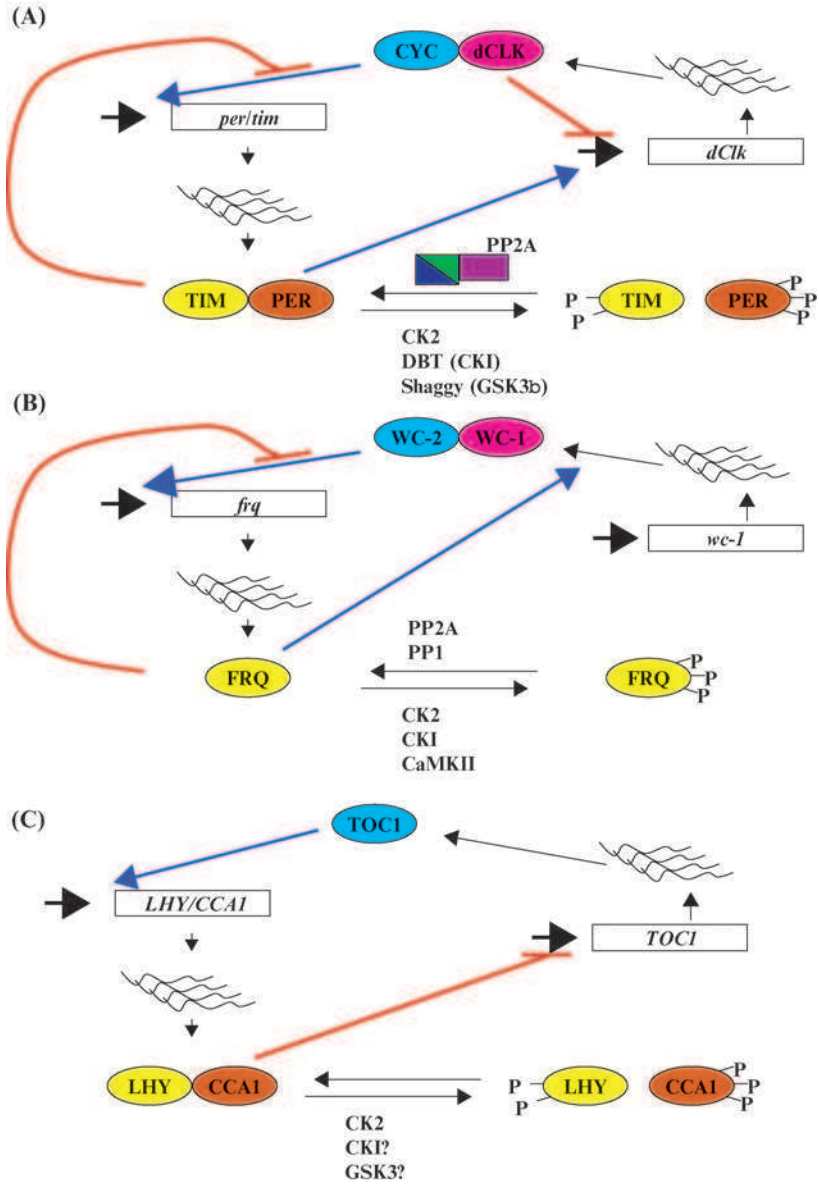

FIG. 2 Circadian clocks in *Drosophila*, *Neurospora*, and *Arabidopsis*. (A) In *Drosophila*, a heterodimer of two bHLH-PAS domain-containing transcription factors, dCLK and CYC, binds to the E-box in the *per* and *tim* promoters, activating their transcription. DBT (CKI) phosphorylates cytoplasmic TIM-free PER protein (-P) and triggers its degradation. As TIM progressively accumulates, it binds to PER, prevents DBT activity, and stabilizes PER. SHAGGY (GSK3) phosphorylates TIM, and regulates nuclear entry of the PER/TIM/DBT

2. Roles of CK2 in the *Drosophila* Clock

CK2 is composed of two α (catalytic) and two β (regulatory) subunits. The CK2α of *Drosophila* is expressed predominantly in the cytoplasm of key circadian pacemaker neurons. CK2α mutant flies (*timekeeper*) show a lengthened circadian period, decreased CK2 activity, and delayed nuclear entry of PER (Lin *et al*., 2002; Table II, Fig. 2A). These effects are probably direct, as CK2α specifically phosphorylates PER *in vitro*. CK2 is highly conserved in eukaryotes (Fig. 3A and B), and CK2 has been proposed as an evolutionary link between the divergent circadian systems of animals, plants, and fungi (Lin *et al*., 2002).

The loss-of-function mutation *andante* causes abnormally long circadian periods in *Drosophila* (Akten *et al*., 2003; Table II). The mutation occurs in the gene encoding the CK2β subunit and is predicted to result in perturbation of CK2β subunit dimerization. In addition, *andante* is associated with reduced β-subunit levels, indicative of a defect in α:β association and production of the tetrameric α2:β2 holoenzyme. Consistent with a direct action on the clock mechanism, CK2β is localized within clock neurons and the clock proteins PER and TIM accumulate to abnormally high levels in the *andante* mutant. Furthermore, the nuclear translocation of PER and TIM is delayed in *andante*, and this defect accounts for the long-period phenotype of the mutant. These results suggest a key function for CK2-dependent phosphorylation in the molecular oscillator.

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complex. TIM is later released from the nuclear complex, allowing repression of dCLK/CYC function. In the absence of TIM, DBT promotes the phosphorylation and degradation of nuclear PER, thereby derepressing dCLK/CYC function and starting a new wave of transcription from the E-box; dCLK constitutes another feedback loop by repressing its own transcription. PER promotes *dClk* transcription, although the transcription factor(s) that binds to the *dClk* promoter is poorly understood. PP2A is involved in dephosphorylation of PER proteins. (B) The WC-1–WC-2 heterodimer activates expression of the clock gene FRQ. The FRQ protein has two roles. In one loop, it acts as a negative regulator of the WC-1–WC-2 heterodimer. In a second loop, FRQ acts as a positive regulator of WC-1 protein synthesis from existing WC-1 mRNA. (C) *LHY/CCA1* gene expression is activated by light and directly, or indirectly, by the TOC1 protein. LHY/CCA1 proteins bind to the *TOC1* promoter and inhibit *TOC1* expression, causing the levels of TOC1 proteins to decrease. CK2 phosphorylates LHY/CCA1 proteins and is proposed to affect DNA-binding activity of the proteins. Since *TOC1* is required for *LHY/CCA1* expression, a decrease in the TOC1 protein level causes a decrease in their expression. The decrease in these *TOC1* gene repressors allows *TOC1* and other evening-expressed genes, such as *ELF4* and *GI*, to increase. As the TOC1 levels and those of other evening-expressed genes rise during the night, *LHY/CCA1* expression begins to increase and the cycle starts again. It is still not known whether the TOC1 protein interacts directly with the *LHY* or *CCA1* promoter.

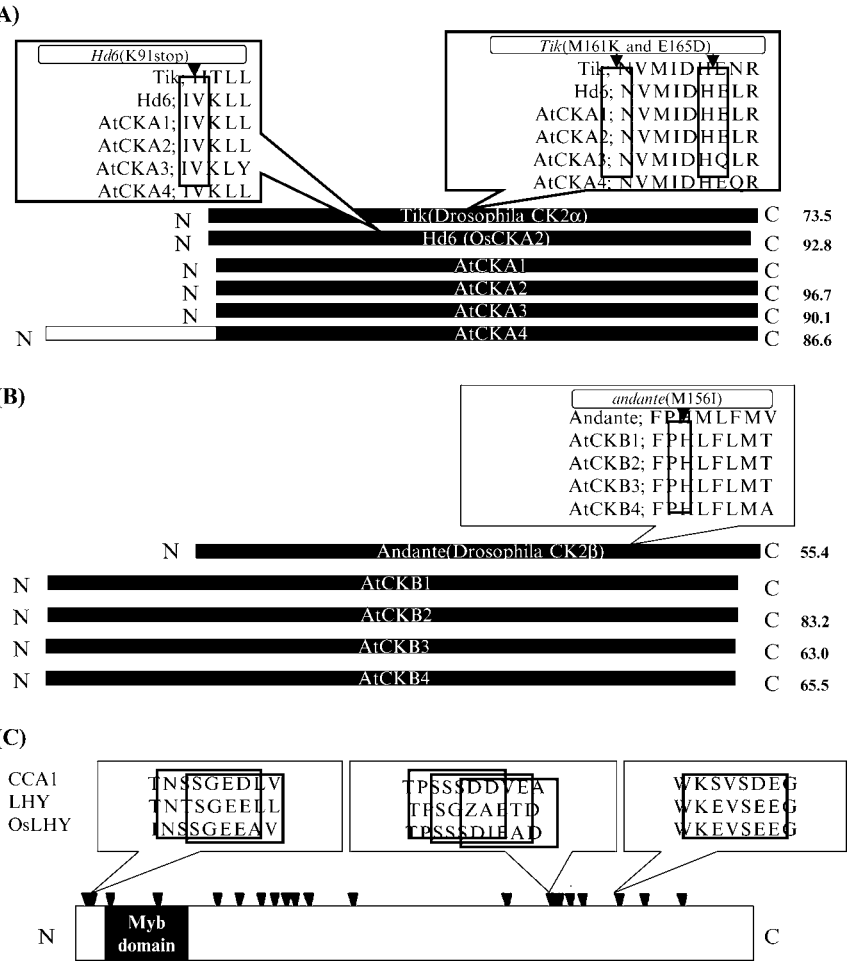

FIG. 3 Comparisons of CK2 α-subunit genes and the phosphorylation consensus sequence of CCA1, LHY, and OsLHY recognized by CK2. *Arabidopsis* has four CK2 α-subunit genes that are similar to the *Timekeeper* (*Tik*) gene in *Drosophila* (Akten *et al*., 2003). Amino acid identity (%) between *Arabidopsis* CKA1 and each protein is shown on the right. M161 and E165 are changed to K and D, respectively, in the *tik* mutant of *Drosophila*, and sequences around these residues are highly conserved among CK2 α-subunit genes in *Drosophila* and *Arabidopsis*. *Hd6* is a quantitative trait locus involved in the photoperiod sensitivity of rice, a short-day (SD) plant (Takahashi *et al*., 2001). *Hd6* (*OsCKA2*) encodes a CK2 α, and the Nipponbare allele of CK2 α contains a premature stop codon at K91, causing early flowering. (B) *Arabidopsis* has four CK2 β-subunit genes that are similar to the *Andante* gene in *Drosophila* (Atken *et al*., 2003). Amino acid identity (%) between *Arabidopsis* CKB1 and each protein is shown on the right. M156 is changed to I in the *andante* mutant of *Drosophila*, and sequences around this residue are highly conserved among CK2 β-subunit genes in *Drosophila* and *Arabidopsis*. (C) CK2 phosphorylation sites of the CCA1 protein are conserved in *Arabidopsis* LHY and a rice

3. Roles of PP2A in the *Drosophila* Clock

PP2A is an abundant heterotrimeric enzyme composed of a highly conserved catalytic subunit (C), a variable regulatory subunit (B), and a structural subunit (A). The A subunit forms a structural scaffold on which the B and C subunits assemble (Groves *et al.*, 1999). Diverse functions of PP2A are mediated largely by the regulatory B subunit, which directs the phosphatase to distinct substrates and intracellular locations (Janssens and Goris, 2001; Virshup, 2000).

TABLE II
The CK2 Mutants[a]

	CK2 mutants	Free-running rhythms	Other phenotypes	References
Arabidopsis	*CKB3-ox*	Short period in LL	Early flowering	Sugano *et al.* (1999)
Drosophila	*timekeeper* (CK2α)	Long period in DD	Delayed nuclear entry of Per	Lin *et al.* (2002)
	andante (CK2β)	Long period in DD	Increased levels and delayed nuclear translocation of Per/Tim protein	Akten *et al.* (2003)
Neurospora	cka^{RIP}	Arrhythmic in DD	Hypophosphorylated and increased levels of FRQ protein Severe defects in growth and development	Yang *et al.* (2002)
	$ckb1^{RIP}$	Arrhythmic (after long period and low amplitude) in DD	Hypophosphorylated and increased levels of FRQ protein No severe defects in growth and development	Yang *et al.* (2003)

[a]RIP, repeat induced point mutation; LL and DD, continuous light and dark.

homolog of LHY/CCA1 (OsLHY: XP_480189). A schematic structure of CCA1 is shown. N and C indicate the N- and C-terminus, respectively. A black box and triangles indicate a Myb DNA-binding domain and putative CK2 phosphorylation sites (S/T XX D/E) in CCA1, respectively (Daniel *et al.*, 2004). *Arabidopsis* CK2 phosphorylates Ser-5, Ser-6, and one or more of four additional serine residues (Ser-431, Ser-432, Ser-433, and Ser-484) of the 21 putative phosphorylation sites. The six Ser residues of CCA1 are shown in bold, and the putative CK2 phosphorylation sites conserved among CCA1, LHY, and OsLHY are boxed.

PP2A has recently been shown to play an important role in the cyclic expression of the PER protein (Sathyanarayanan *et al.*, 2004; Figs. 2A and 4). PP2A regulatory subunits TWINS (TWS) and WIDERBORST (WDB) target PER and stabilize it in Schneider (S2) cells. In adult fly heads, *tws* expression cycles robustly under control of the circadian clock. Hypomorphic *tws* mutants show delayed accumulation of PER, while overexpression of *tws* in clock neurons produces shorter, weaker rhythms. Reduction of PP2A activity reduces PER expression in central clock neurons and results in long periods and arrhythmia. In addition, overexpression of the PP2A catalytic subunit causes a loss of behavioral rhythms and constitutive nuclear expression of PER. PP2A also affects PER phosphorylation *in vitro* and *in vivo*. These results suggest that the posttranslational mechanisms that drive cycling of PER require the rhythmic expression of PP2A.

4. The Cell Cycle and Circadian Rhythms

In mammals, circadian rhythms affect the timing of cell divisions. Day–night variations in both the mitotic index and DNA synthesis occur in many tissues (e.g., oral mucosa, tongue keratinocytes, intestinal epithelium, skin, and bone marrow), and some of them persist even in constant darkness. However, how the circadian clock controls the timing of cell divisions has not been elucidated. In the regenerating liver of mice, the circadian clock controls the expression of cell cycle-related genes that in turn modulate the expression of active cyclin B1-Cdc2 kinase, an essential regulator of mitosis (Matsuo *et al.*, 2003). Among these genes, expression of the protein kinase encoded by *wee1* is directly regulated by the molecular components of the circadian clock. In contrast, the circadian clock oscillates independently of the cell cycle in single

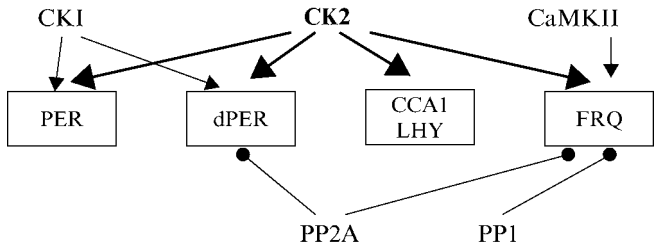

FIG. 4 CK2 is commonly used to control eukaryotic clock proteins. CK2 phosphorylates PER in animals, LHY/CCA1 in *Arabidopsis*, and FRQ in *Neurospora*. Two other protein kinases, CKI and CaMKII, are also involved in the control of PER in animals and FRQ in *Neurospora*, respectively. The phosphorylation states of clock proteins are controlled dynamically by protein kinases and phosphatases. *Drosophila* PER is dephosphorylated by PP2A, while FRQ is regulated by PP2A and PP1.

cells, suggesting that the intracellular circadian clock may control the cell-division cycle directly and unidirectionally in proliferating cells.

5. Roles of Protein Kinases and Phosphatases in the *Neurospora* Clock

In *Neurospora*, FRQ, WHITE COLLAR-1 (WC-1), and WHITE COLLAR-2 (WC2) are three major components of the *frq–wc*-based circadian oscillator (Loros and Dunlap 2001; Fig. 2B). Several protein kinases (CK2, CKI, and CaMKII) phosphorylate FRQ and promote its degradation (Yang *et al.*, 2001, 2002, 2003; Figs. 2B and 4). Mutations of the FRQ phosphorylation sites lead to the stabilization of FRQ and long-period rhythms of the clock. In strains in which genes encoding the CK2 catalytic subunit or one of its regulatory subunits are disrupted, the FRQ protein level is high and more stable, and the clock function is either completely abolished or severely affected (Yang *et al.*, 2002, 2003; Table II). Also, CK2-mediated FRQ phosphorylation regulates the FRQ–WC interaction. In contrast, the disruption of a gene encoding a PP1 catalytic subunit causes instability of the FRQ protein as well as its advanced phase and short period (Yang *et al.*, 2004). The stability of FRQ is not altered in a mutant with loss-of-function of a regulatory subunit of PP2A, but levels of FRQ protein and mRNA are low, resulting in a low-amplitude and long-period oscillation of the clock. Furthermore, PP1 and PP2A can dephosphorylate FRQ *in vitro*. These data strongly suggest that the two phosphatases differentially regulate the FRQ protein.

6. Protein Phosphorylation in the Control of Protein Stability in *Neurospora*

FRQ is likely ubiquitylated *in vivo*, and its proper degradation requires FWD1, an F-box/WD-40 repeat-containing protein (He *et al.*, 2003). In *fwd1*-disrupted strains, FRQ degradation is severely impaired, resulting in the accumulation of hyperphosphorylated forms of the protein. Circadian rhythms of gene expression and circadian conidiation rhythms are abolished in these *fwd1* mutants. FRQ and FWD1 interact physically *in vivo*, suggesting that FWD1 is the substrate-recruiting subunit of an SCF-type ubiquitin ligase responsible for FRQ ubiquitylation and degradation. Together with the recent finding that SLIMB (the *Drosophila* homolog of FWD1) is involved in the degradation of the PERIOD protein in flies (Grima *et al.*, 2002), the results indicate that FWD1 regulates the degradation of FRQ in *Neurospora* and is an evolutionarily conserved component of the eukaryotic circadian clock.

7. KaiC Phosphorylation in Cyanobacteria

Three clock genes, *kaiA*, *kaiB*, and *kaiC*, have been identified in cyanobacteria as essential timekeeping components. Some features of circadian clocks in eukaryotic organisms are conserved in the clocks of prokaryotic cyanobacteria. For example, a transcription/translation-based autoregulatory (negative feedback) loop of *kaiBC* expression has been proposed to drive circadian rhythms (Ishiura *et al.*, 1998). In this model, KaiA and KaiC are presented as positive and negative regulators of *kaiBC* expression, respectively (Ishiura *et al.*, 1998). KaiA-mediated activation of *kaiBC* expression is KaiC–dependent, indicating that KaiC also functions in a positive feedback process in the molecular oscillatory mechanism (Iwasaki *et al.*, 2002).

One of the similarities between eukaryotic clock proteins and cyanobacterial KaiC is that KaiC is phosphorylated *in vivo*. The circadian period in cyanobacteria is determined by the phosphorylation status and the degradation rate of KaiC (Imai *et al.*, 2004; Iwasaki *et al.*, 2002; Kitayama *et al.*, 2003; Xu *et al.*, 2003). Cyanobacteria do not have CK2, which plays important roles in eukaryotic clocks. KaiC undergoes autophosphorylation (Nishiwaki *et al.*, 2000), and KaiA and KaiB modulate the status of KaiC phosphorylation. KaiA inhibits KaiC dephosphorylation, and KaiB antagonizes this action of KaiA (Xu *et al.*, 2003).

KaiC is proposed to form the transcription–translation feedback loop to generate circadian rhythms (Iwasaki and Kondo, 2004). However, it has been demonstrated that temperature-compensated robust circadian cycling of KaiC phosphorylation is sustained under continuous dark conditions even without KaiC mRNA accumulation (Tomita *et al.*, 2005). This rhythm persisted in the presence of a transcription or translation inhibitor, and kinetic profiles in the ratio of KaiC autophosphorylation–dephosphorylation were also temperature compensated *in vitro*.

Recently, Kondo's group reconstituted the self-sustainable oscillation of KaiC phosphorylation *in vitro* by incubating KaiC with KaiA, KaiB, and adenosine triphosphate (Nakajima *et al.*, 2005). The *in vitro* oscillation period was stable despite temperature change (temperature compensation), and the circadian periods observed *in vivo* in KaiC mutant strains were consistent with those measured *in vitro*. These results indicate that at least the cyanobacterial clock can keep time independent of the transcription–translation feedback loop believed to be common in eukaryotic clocks. Homologous genes of *kaiA*, *kaiB*, and *kaiC* have not been found in other eukaryotic model organisms. Thus, whether this kind of autophosphorylation–autodephosphorylation of clock component(s) is common—perhaps in order to keep time without the negative feedback loop based on

transcription–translation—will require further biochemical analyses of clock components in each model system.

C. Clock-Associated Processes in Plants

Although several genes implicated in circadian clock function have recently been isolated in *Arabidopsis*, there is as yet no clear model in which to place them. This is mainly due to the lack of biochemical studies on clock-associated proteins in plants. However, the predicted functions of the protein products of these genes, based on characterization of clock mutants (loss- and gain-of-function), are suggestive and several models of plant circadian clock have been proposed (Alabadí *et al*., 2001; Mizoguchi *et al*., 2002, 2005). Recent transgenomic comparisons clearly demonstrate that *Arabidopsis* also has homologs of the protein kinases and phosphatases of animal and fungal clocks. Among these, CK2, which is involved in circadian rhythms and flowering in *Arabidopsis*, is one of the best characterized (Sugano *et al*., 1999). *Hd6* is a quantitative trait locus involved in the photoperiod sensitivity of rice, a short-day (SD) plant (Takahashi *et al*., 2001). Hd6 encodes a CK2α, and the Nipponbare allele of CK2α contains a premature stop codon. Genetic complementation analysis revealed that the Kasalath allele of CK2α increases days-to-heading (flowering time). Therefore, CK2 plays an important role in the photoperiodic control of flowering both in a long-day (LD) plant, *Arabidopsis*, and an SD plant, rice. In this section, we discuss the current status of our understanding of how CK2 affects the circadian rhythms. In addition, we summarize recent research on the molecular mechanisms by which the plant clock controls flowering.

1. Trans-genomic Comparisons

Whole-genome sequencing of *Arabidopsis* has been completed (*Arabidopsis* Genome Initiative, 2000), and systematic transgenomic comparison of protein kinases in *Arabidopsis* and *Saccharomyces cerevisiae* has been performed (Wang *et al*., 2003). This comparison and previous work (Mizoguchi *et al*., 1993) have demonstrated that *Arabidopsis* has 4 catalytic (α) and 4 regulatory (β) genes for CK2; 17 genes for CKI; 10 genes for GSK3β/SHAGGY; and 19 genes for MAPK. Also, in *Arabidopsis* there are at least 7 genes encoding PP1 catalytic subunits and 6 genes encoding PP2A catalytic subunits (Kerk *et al*., 2002). As discussed above, only CK2 has been shown to be closely associated with a circadian clock in *Arabidopsis*. A systematic analysis of the effects of knockout mutants of these genes on circadian rhythms would be of interest to test whether a similar set of phosphorylation units is commonly used in eukaryotes.

2. Roles of LHY, CCA1, and GI in the Control of Circadian Rhythms in *Arabidopsis*

In plants, the circadian clock controls many processes, such as leaf movement, stomatal opening, and the expression of several genes. Photoperiodic control of flowering time is also closely associated with the circadian clock in plants (Mizoguchi and Coupland, 2000; Mizoguchi *et al.*, 2002, 2005). In *Arabidopsis*, at least four genes, *LHY*, *CCA1*, *TOC1* (*TIMING OF CAB EXPRESSION 1*), and *GI* (*GIGANTEA*), have been suggested to be components of or closely associated with the oscillator, based on analyses of loss-of-function and over-expression in *LHY* (Mizoguchi *et al.*, 2002, 2005; Schaffer *et al.*, 1998), *CCA1* (Green and Tobin, 1999; Wang and Tobin, 1998), *TOC1* (Mas *et al.*, 2003; Matsushika *et al.*, 2002; Millar *et al.*, 1995; Somers *et al.*, 1998), and *GI* (Fowler *et al.*, 1999; Mizoguchi *et al.*, 2002, 2005; Park *et al.*, 1999).

LHY and *CCA1* are closely related and encode Myb DNA-binding proteins. *LHY* and *CCA1* mRNA levels are controlled by a circadian clock and peak at dawn. *TOC1* encodes an atypical response regulator protein, similar to those found in bacterial two-component signal transduction systems (Strayer *et al.*, 2000). *TOC1* was identified independently by Mizuno's group and named ARABIDOPSIS PSEUDO-RESPONSE REGULATOR 1 (APRR1), because the TOC1/APRR1 protein lacks some critical amino acid residues required for normal response-regulator function (Matsushika *et al.*, 2000). *TOC1* mRNA levels also cycle, peaking at the end of the subjective day.

Negative feedback loop models in *Arabidopsis* have been proposed (Alabadí *et al.*, 2001, 2002; Mizoguchi *et al.*, 2002). LHY/CCA1 has been suggested to repress *TOC1* expression, and TOC1 promotes *LHY*/*CCA1* expression (Alabadí *et al.*, 2001). The promotion of *LHY*/*CCA1* expression by GI suggests that GI might also play a role in this type of feedback mechanism (Mizoguchi *et al.*, 2002, 2005). GI does not show substantial homology to any other proteins. Apart from its general function in the plant clock, it is important in mediating between the clock and flowering (a transition from a vegetative phase to a reproductive phase; Mizoguchi *et al.*, 2005). GI is proposed to be a transcription factor, based on its nuclear localization and strong influence on the expression of floral activators CONSTANS (*CO*) and FLOWERING LOCUS T (*FT*). Other proteins, such as EARLY FLOWERING 4 (*ELF4*; Doyle *et al.*, 2002), TIME FOR COFFEE (*TIC*; Hall *et al.*, 2003), the ZEITLUPE (*ZTL*) family members (*ZTL*, *FKF1*, and *LKP2*; Kiyosue and Wada, 2000; Mizoguchi and Coupland, 2000; Nelson *et al.*, 2000; Schultz *et al.*, 2001; Somers *et al.*, 2000), and the TOC1/APRR1 family members (*APRR3*, *5*, *7*, and *9*; Makino *et al.*, 2000; Matsushika *et al.*, 2000; Mizuno and Nakamichi, 2005) have been shown to be closely associated with clock function in *Arabidopsis*.

3. Functional Interaction between LHY/CCA1 and CK2

A possible involvement of CK2 in circadian rhythms was first demonstrated in *Arabidopsis* (Sugano *et al*., 1998). A series of papers by Tobin's group showed that CK2 appears to closely associate with CCA1 and LHY and thus have a vital function in the circadian rhythms of *Arabidopsis* (Daniel *et al*., 2004; Sugano *et al*., 1998, 1999). CCA1 was first identified as a factor involved in the phytochrome regulation of *Lhcb1*3* (*CAB*) expression (Wang *et al.*, 1997) and then shown to participate in the function of the circadian oscillator in *Arabidopsis* (Wang and Tobin, 1998). CCA1 interacts with the β-subunits of CK2 (CKB3 and CKB1), both in a yeast two-hybrid system and in an *in vitro* interaction assay (Sugano *et al*., 1998). The catalytic subunits of CK2 (CKA1 and CKA2) also interact with CCA1 *in vitro*. CK2 β-subunits stimulate binding of CCA1 to the CCA1 binding site on the *Lhcb1*3* promoter, and CK2 phosphorylates CCA1 *in vitro*. Furthermore, *Arabidopsis* plant extracts contain a CK2-like activity that affects the formation of a DNA–protein complex containing CCA1, suggesting that CK2 can modulate CCA1 activity both by direct interaction and by phosphorylation of CCA1, and that CK2 plays a role in the function of CCA1 *in vivo*.

Overexpression of CKB3 (CKB3-ox) causes shorter periods of rhythmic expression of *CCA1* and *LHY* (Sugano *et al*., 1999; Table II). CK2 interacts with and phosphorylates not only CCA1 but also LHY *in vitro*. Additionally, the *CKB3-ox* transgene shortens the periods of four other CCGs with different phase angles, demonstrating that many clock outputs are affected. *CKB3-ox* also reduces phytochrome induction of an *Lhcb* gene. The photoperiodic flowering response, which is influenced by circadian rhythms, is diminished in *CKB3-ox* lines, with the plants flowering earlier in both LD and SD photoperiods. These phenotypes of the *CKB3-ox* are quite similar to that of the loss-of-function mutant of either *cca1* or *lhy* (Green and Tobin, 1999; Mizoguchi *et al*., 2002). The data support the idea that CK2 is involved in regulation of the circadian clock in *Arabidopsis*.

To ascertain whether the effects of CK2 on the clock were due to its phosphorylation of clock components, we determined the sites on CCA1 that are phosphorylated by CK2 (Daniel *et al*., 2004). Rice has a CCA1/LHY homolog, and the CK2 phosphorylation consensus sequence reported by Daniel *et al*. (2004) is highly conserved (Fig. 3C). A mutant form of CCA1 (mCCA1) that cannot be phosphorylated by CK2 was constitutively overexpressed in *Arabidopsis* (*mCCA1-ox*), and the effects of *mCCA1-ox* on circadian rhythms and flowering time were examined (Daniel *et al*., 2004). Unlike *CCA1-ox*, *mCCA1-ox* does not abolish the circadian rhythms of either the central oscillator (expression of *CCA1* and *LHY*) or the output genes (*CCR2* and *CAB*). In addition, *mCCA1-ox* does not delay flowering

and reduce viability to the extent that *CCA1-ox* does (Green *et al.*, 2002). Results obtained by Tobin's group demonstrated that CCA1 phosphorylation by CK2 is important for normal functioning of the central oscillator.

CKB3-ox causes shortening of period and acceleration of flowering, especially under SD conditions, as discussed above (Table II). However, it has not been demonstrated whether loss-of-function of β-subunits of CK2 in plants affects circadian rhythms. Also, the roles of CK2 α-subunits on circadian rhythms in plants are not fully understood. One possible experiment is to investigate phenotypes of knockout mutants of genes encoding CK2α. *Arabidopsis* has four genes encoding the catalytic subunits (α) of CK2, whereas other typical model organisms have only two genes for CK2α. One of the *Arabidopsis* CK2α genes (AtCKA4: At2g23070) has been predicted to encode a protein that locates in chloroplasts (Loschelder *et al.*, 2004; Ogrzewalla *et al.*, 2002). The four genes are highly related and may have redundant functions in *Arabidopsis*. Thus, constructing multiple mutants of the four *ck2α* and the four *ck2β* genes would be useful to understand the roles played by protein phosphorylation in the clock function of *Arabidopsis*.

4. Posttranscriptional Control of the PEPC Kinase Level by a Circadian Clock

Phosphorylation of phosphoenolpyruvate carboxylase (PEPC) is crucial to the control of plant metabolism (Nimmo, 2000). PEPC kinase is activated by a process involving protein synthesis in response to a range of signals in different plant tissues (Hartwell *et al.*, 1999). The PEPC kinase genes were cloned from the Crassulacean acid metabolism plant *Kalanchoë fedtschenkoi* and the C_3 plant *Arabidopsis* (Hartwell *et al.*, 1999). While PEPC kinase is a member of the Ca^{2+}/calmodulin-regulated group of protein kinases, it lacks the autoinhibitory region and EF hands of plant Ca^{2+}-dependent protein kinases, explaining its Ca^{2+} independence. Furthermore, the sequence of PEPC kinase is novel in that it comprises only a protein kinase catalytic domain with no regulatory regions; thus, it appears to be the smallest known protein kinase. In *K. fedtschenkoi*, the abundance of PEPC kinase transcripts increases during leaf development. The transcript level in mature leaves is very low during the photoperiod, reaches a peak in the middle of the dark period, and correlates with kinase activity. The protein exhibits a circadian oscillation under constant conditions. Protein kinases are typically regulated by second messengers, phosphorylation, or protein–protein interactions. PEPC kinase is an exception to this general rule, being controlled only at the level of expression. In *K. fedtschenkoi*, its expression is controlled both developmentally and by a circadian oscillator.

5. Protein Phosphorylation by the Plant Photoreceptors CRY and PHY

In plants, environmental light information, such as quality, intensity, and duration, in red (ca. 660 nm) and far-red (ca. 730 nm) wavelengths is perceived by phytochromes, and that in blue (ca. 400 nm) wavelengths by cryptochromes. Phytochromes (PHY) and cryptochromes (CRY) are major photoreceptors in plants and have important functions in the input pathway to the clock (Fig. 1A). Phytochromes interconvert between red light-absorbing Pr and biologically functional far-red light-absorbing Pfr forms. PHYA itself has protein kinase activity and interacts not only with a protein kinase (Ahmad *et al.*, 1998; Fankhauser *et al.*, 1999; Yeh and Lagarias, 1998) but also with protein phosphatases (Ryu *et al.*, 2005). Phytochromes are phosphorylated at specific serine residues. A type 5 protein phosphatase (PAPP5) has recently been shown to specifically dephosphorylate biologically active Pfr phytochromes and enhance phytochrome-mediated photoresponses (Ryu *et al.*, 2005).

An uncharacterized kinase phosphorylates CRY2 (Shalitin *et al.*, 2002), and phosphorylation is associated with CRY2 function and regulation. In *Arabidopsis*, the cryptochrome mediates light regulation of seedling development and photoperiodic flowering and undergoes blue light-dependent phosphorylation. These results suggest that in the absence of light, CRY2 remains unphosphorylated, inactive, and stable, whereas the absorption of blue light induces CRY2 phosphorylation, triggering photomorphogenic responses, and eventually, degradation of the photoreceptor (Shalitin *et al.*, 2002).

III. Other Approaches to Understanding the Roles of Protein Phosphorylation in the Plant Circadian Clock

A. Yeast Two-Hybrid Assay

TOC1 is believed to act close to or in the central clock in *Arabidopsis*, and mutation of *TOC1* causes a short-period phenotype (Somers *et al.*, 1998). Circadian-regulated homologs of *TOC1/APRR1* (*APRR3*, *5*, *7*, and *9*) are also known (Matsushika *et al.*, 2000). Transcripts of these *APRR*s peak at different times throughout the day, suggesting that they are important for the proper phasing of clock outputs through the day. Loss-of-function of *aprr3* shortens the period length of leaf movements (Michael *et al.*, 2003), while overexpression of *APRR3* causes a longer period (and/or

delayed phase) of rhythms in certain circadian-controlled genes under continuous white light (Murakami *et al.*, 2004). Loss-of-function and over-expression of other *APRR*s also affect circadian rhythms as well as some of the output processes, such as hypocotyl elongation and flowering (Mizuno and Nakamichi, 2005).

In yeasts, APRR3 interacts with an *Arabidopsis* protein kinase (WNK1), which has high similarity to a mammalian protein kinase, WNK (with no lysine [K] at a key catalytic residue; Murakami-Kojima *et al.*, 2002). Hypertension is a major public health problem of largely unknown cause. Mutations of human WNK1 and WNK4 genes cause pseudohypoaldosteronism type II, an inheritable condition featuring hypertension, increased renal salt reabsorption, and impaired K^+ and H^+ excretion (Wilson *et al.*, 2001).

WNK1 phosphorylates APRR3 *in vitro*, and both *APRR3* and *WNK1* are controlled by the circadian clock in *Arabidopsis*. Furthermore, the rhythmic expression patterns of the two genes are quite similar to each other. *Arabidopsis* has at least eight members of the WNK1 family of protein kinases (WNK1–8; Nakamichi *et al.*, 2002). Expression of the *WNK2*, *WNK4*, and *WNK6* genes is also under the control of circadian rhythms. It has not been demonstrated whether loss- or gain-of-function of the *WNK* genes affects circadian rhythms in *Arabidopsis*.

B. Pharmacological Approaches

Protein phosphatases have been implicated in the regulation of circadian rhythmicity in the marine dinoflagellate *Gonyaulax polyedra* based on the effects of three inhibitors specific for protein phosphatases 1 and 2A (okadaic acid, calyculin A, and cantharidin) (Comolli *et al.*, 1996). Chronic exposure to okadaic acid resulted in significant period lengthening, as measured by the bioluminescent glow rhythm, whereas cantharidin and calyculin A caused large phase delays but had no persistent effect on period. Short pulses of the phosphatase inhibitors resulted in phase delays that were greatest near subjective dawn. Unlike 6-dimethylaminopurine, a protein kinase inhibitor, okadaic acid, calyculin A, and cantharidin did not block light-induced phase shifts. The three inhibitors also increased radiolabeled phosphate incorporation into *Gonyaulax* proteins *in vivo* and blocked protein phosphatase 1 and 2A activities in *Gonyaulax* extracts. These results suggest that protein phosphorylation–dephosphorylation is necessary for proper functioning of the circadian system.

The protein kinase inhibitor staurosporine was found to cause a dramatic increase in the free-running period (FRP) of circadian rhythms in *Gonyaulax*, and its effect was similar when added at different phases of the circadian cycle

(Comolli *et al*., 1999). Long-term exposure to staurosporine lengthened the FRP by as much as 7 h without greatly affecting the amplitude or waveform of the bioluminescence rhythm. The effect on the length of the FRP occurred only above a threshold concentration, and it lasted for a limited number of cycles, depending on the dose of the drug. FRP lengthening was not evident until 23 to 26 h after staurosporine addition, even though the drug entered *Gonyaulax* cells in 1 h or less. When tested in combination with bright light pulses, staurosporine was found to enhance both light-induced phase advances and delays, indicating that the drug acts on circadian phototransduction. At concentrations that alter the FRP and the response to light pulses, staurosporine appears to act on a small number of protein kinases, attenuating the activity of two individual protein kinases without affecting overall phosphate incorporation into proteins *in vitro*. The effects of inhibitors of protein kinase and phosphatase in higher plants are not well characterized.

C. Microarray- and Gene Chip-Based Approaches

Clock-controlled genes in *Drosophila* were identified by high-density oligonucleotide arrays (McDonald and Rosbash, 2001). The 10 genes with the highest amplitude of cycling expression include *per*, *tim*, *clk*, and *vri*. Loss-of-function of these genes affects circadian rhythms in *Drosophila*, suggesting that genes with clear circadian rhythms are necessary for clock function. Extensive microarray analyses of *Arabidopsis* circadian rhythms were conducted by two independent groups (Harmer *et al*., 2000; Schaffer *et al*., 2001). Both identified genes for protein kinases and phosphatases that are under the control of a circadian clock (Table III); however, none of these has been reported as clock genes in *Arabidopsis* so far.

LHY and CCA1 are proposed to be regulated by CK2, as described above (Section IV.C). Usually, this kind of process can be antagonistically regulated by protein phosphatase(s). FRQ is controlled by both protein kinases (CK2 and CaMKII) and protein phosphatases (PP1 and PP2A) in *Neurospora*. Similar regulation of PER by CK2 and PP2A has been shown in *Drosophila*, and genes for PP2A and PP1 are highly conserved in *Arabidopsis*. Two genes for the catalytic subunits of PP1 and PP2A are under the control of circadian rhythms, based on results of microarray experiments (Harmer *et al*., 2000; Table III). It has not been demonstrated whether PP2A and PP1 have important roles in circadian rhythms in *Arabidopsis*. It would thus be of interest to determine whether loss-of-function and overexpression of the genes listed in Table III affect circadian rhythms in *Arabidopsis*.

TABLE III
Protein Kinase and Phosphatase Genes Controlled by a Clock in *Arabidopsis*

Accession no.	MIPS code	Annotation	Reference
108J22T7	At1g68830	Putative protein kinase	Schaffer *et al.* (2001)
122H23T7	At5g66890	Putative receptor-like protein kinase	Schaffer *et al.* (2001)
202D4T7	At1g14370	APK2a	Schaffer *et al.* (2001)
F6D5T7	At1g18030	Protein phosphatase 2C	Schaffer *et al.* (2001)
CAB82751	At5g01810	ATPK10	Harmer *et al.* (2000)
BAA77716	At3g23000	ATSRPK1, SNF1 related protein kinase	Harmer *et al.* (2000)
D42061	At3g08720	ATPK19, putative ribosomal-protein S6 kinase	Harmer *et al.* (2000)
CAA55395	At4g14340	Casein kinase I	Harmer *et al.* (2000)
P30366	At2g29400	PP1 catalytic subunit	Harmer *et al.* (2000)
AF030864	At3g45780	NPH1, phot1	Harmer *et al.* (2000)
Q07099	At1g59830	PP2A catalytic subunit	Harmer *et al.* (2000)
CAB79516	At4g26610	Putative protein kinase (PVPK1 type)	Harmer *et al.* (2000)
AAB80785	At2g17700	Putative protein kinase	Harmer *et al.* (2000)
AAC33221	At2g28930	Putative protein kinase	Harmer *et al.* (2000)
AAF33112	At2g30510	RPT2, phot2	Harmer *et al.* (2000)
CAB37508	At4g38520	Putative protein phosphatase 2C	Harmer *et al.* (2000)
AAB65472	At1g11130	LRR receptor-like protein kinase strubbelig (SUB)	Harmer *et al.* (2000)
AAF21151	At1g72770	Protein phosphatase 2C (AtP2C-HA)	Harmer *et al.* (2000)
	At3g04910	WNK1	Murakami-Kojima *et al.* (2002)
	At3g22420	WNK2	Nakamichi *et al.* (2002)
	At5g58350	WNK4	Nakamichi *et al.* (2002)
	At3g18750	WNK6	Nakamichi *et al.* (2002)

IV. Concluding Remarks

Protein phosphorylation plays several important roles in controlling various aspects of clock function. Control of protein stability of clock components by phosphorylation is common in model organisms such as cyanobacteria, *Neurospora*, *Drosophila*, mice, and *Arabidopsis*. In plants, the circadian

clock has a strong impact on the control of flowering time, which, along with the length of the plant's life cycle, is of great agricultural interest.

Human behavior is also affected by a circadian clock. Some behaviora disorders, such as FASPS, are caused by mutations of the *PER2* and *CKI* genes. Researchers have started to search for small molecules that affect the phosphorylation state of PER2 as a first step toward developing drugs that either speed up or slow down the clock (Chicurel, 2001). Some small molecules may increase the kinase activity of mutated forms of CKI, and when administered therapeutically, could help not only patients with behavioral disorders but also jet-lagged travelers and night-shift workers.

Whole genome sequencing has been completed in several model organisms, including *Arabidopsis*, and knockout mutants produced by insertional mutagenesis (e.g., T-DNA tagging methods) are available for more than 90% of the *Arabidopsis* genes (Alonso *et al.*, 2003; Pennisi, 2004). Genetic approaches have led to the identification of mutants that have altered sensitivity to plant hormones, lights, and abiotic and biotic stresses. Sequencing of the *Arabidopsis* genome, however, revealed that approximately 65% of the genes have homologs or related genes, suggesting that they have redundant functions in *Arabidopsis* (*Arabidopsis* Genome Initiative, 2000). This situation makes it difficult to identify monogenic mutations that cause major altered responses of the plant.

Pharmacological approaches provide powerful tools not only for biochemical analysis but also for the identification of mutants. It is quite reasonable to assume that in response to a certain drug, the sum of activity of the target enzyme decreases. Consequently, it should be possible to identify monogenic mutations whose phenotypes are visible only when enzymatic activity is extremely low. Pharmacologically based screening of T-DNA null collections will make it possible to identify novel, mutated genes affecting the response to a variety of environmental conditions. Since it is clear that many steps in circadian clock mechanisms are affected by protein phosphorylation and dephosphorylation, the development of novel methods to identify such mutants will result in a wide range of medical and agricultural applications.

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A Model for Lymphatic Regeneration in Tissue Repair of the Intestinal Muscle Coat

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The gastrointestinal lymphatic system, which comprises a network of thin-walled vessels, is essential for the regulation of tissue fluid volume, immune function, and transport of fatty nutrients. The identification of specific lymphatic endothelial markers has facilitated analyses of lymphatic organization and lymphangiogenesis during individual development and tissue repair. The intestinal muscle coat producing motor activity develops a dense maze-like lymphatic network by vascular sprouting consisting of thin lymphatic endothelial projections and splitting of the vessels. The lymphatic regeneration in the tissue repair of the intestinal muscle coat is essentially attributable to sprouting from preexisting lymphatics, and it progresses vigorously with vascular maturation. The regrowing lymphatic endothelial cells exhibit structural changes indicating a high migratory potential and a close association with regenerating stromal cells. The upregulation of VEGF-C, a specific lymphangiogenic molecule, in a subpopulation of the stromal cells probably contributes to lymphatic regeneration by activating its receptor, VEGFR-3, on the regrowing lymphatic endothelial cells.

KEY WORDS: Lymphatics, Lymphatic endothelial markers, Organization, Development, Regeneration, Intestinal muscle coat.

I. Introduction

The lymphatic system is composed of a generalized vascular network, which starts with initial lymphatics in most organs. The lymphatic vessels play a critical role in the control of tissue fluid homeostasis by draining protein-rich

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lymph from the extracellular space. The lymphatics also transport immune cells and nutrients, such as the rich emulsion of fat, from the tissues both to the lymph nodes and to the bloodstream (Allen, 1967). While the blood vascular network is a closed circular system, the lymphatic network is a one-way lymph-draining system from the interstitial space of most organs to the blood vascular system for recirculation by connecting with large veins such as the subclavicular vein. Both of these vascular systems share many anatomical features, but each of them has its own structural and functional features.

Since the gastrointestinal tract physiologically demonstrates great absorptive and motor activities, it is equipped with a well-developed lymphatic network. The broad structure and distribution of lymphatic vessels in the gut have been studied by a variety of methods including the injection of dyes or silver nitrate into the parenchyma or blood vessel (Mori, 1969; Satomura *et al.*, 1978; Vajda and Tömböl, 1964; Zweifach, 1973). Transmission and scanning electron microscopy (TEM and SEM) demonstrated the fine structure and three-dimensional organization of the gastrointestinal lymphatics of several mammals (Ohtani, 1987, 1989, 1992; Ohtani and Murakami, 1992; Ohtani and Ohtsuka, 1985; Sugito *et al.*, 1996; Ushiki, 1990; Yamanaka *et al.*, 1995). However, it often proves difficult to differentiate the lymphatic from the blood vasculature, when the histological morphology of the two systems is the only basis on which a distinction is made. Longstanding technical difficulties in identifying lymph vessels within tissues has hindered studies of the lymph vascular system, in comparison to studies of the blood vascular system.

Recently, a number of interesting molecules have been identified that show different expression profiles in the blood and lymphatic vasculature (Hirakawa *et al.*, 2003; Petrova *et al.*, 2002). The discovery of lymphatic endothelial cell markers has facilitated detailed analyses of the nature and structural organization of the lymphatic vessels and their growth (lymphangiogenesis). These advances in lymphatic research have demonstrated that the lymphatic vessels play an important role in the pathogenesis and progression of a variety of disorders including wound healing. Concomitant lymphatic restoration to ideal tissue repair is requisite for the gut, which frequently suffers from tissue injury due to various diseases and surgical operations, to recover its integrated function. This article delineates the regeneration of the lymphatic vessels in tissue repair of the intestinal muscle coat, along with their organization and development, and includes a survey of lymphatic endothelial markers.

II. Basic Structure of Lymphatic Vessels

Lymphatic vessels are generally sorted into two segments: one is composed only of attenuated endothelium (initial lymphatics; lymphatic capillaries),

while the other is endowed with periendothelial cells such as smooth muscle cells (collecting lymphatics). The lymphatic capillaries are lined with a single cell layer of overlapping endothelial cells and lack a continuous basement membrane and periendothelial cells (Fig. 1a). The structural profiles afford a high permeability to the lymphatics. The endothelial cells, which are fixed in the connective tissue space by anchoring filaments, display an incomplete connection between the adjacent cells. With this structural device, interstitial pressure provides them with open and closed lymphatic endothelial microvalves along the wall of the capillaries (Schmid-Schönbein, 1990). The endothelial cells of the initial lymphatics have thus been thought to play an important role in the regulation of lymph formation and/or flow (Hogan and Unthank, 1986; Leak, 1976; Leak and Burke, 1966, 1968). The capillaries exhibit no spontaneous contractile activity.

In contrast, the collecting lymphatic vessels, including the thoracic duct, develop a smooth muscle media, thus showing periodic contraction, and adventitia (Fig. 1B). A number of studies have disclosed the innervation of

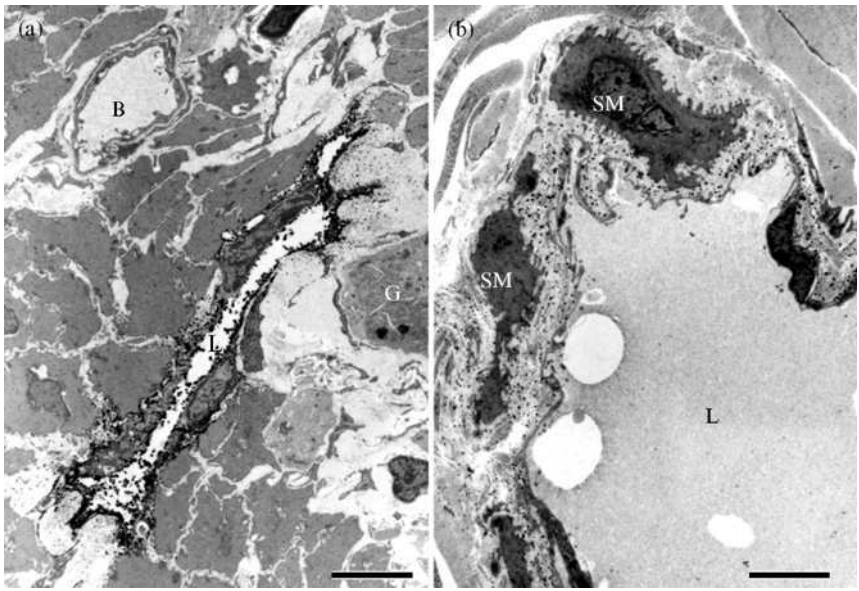

FIG. 1 Transmission electron images of a lymphatic capillary (a) in the jejunal muscle coat and of a collecting lymphatic (b) in the mesentery from monkey tissue specimens with 5′-Nase staining. The reaction products for 5′-Nase activity are densely distributed on the lymphatic endothelial cells. (a) The lymphatic capillary (L) is composed of attenuated endothelial cells arranging in a single layer among the intestinal muscles, but no periendothelial cells belong to the vessel. B, blood vessel; G, ganglion. (b) The large collecting lymphatic (L) is endowed with its proper periendothelial smooth muscle cells (SM). Bars: 7 μm (a), 5 μm (b).

the collecting lymphatics, and their contractile activity is considered to be under nervous control (Alessandrini *et al.*, 1981; Allen and McHale, 1986; Allen *et al.*, 1988; Guarna *et al.*, 1991; McHale *et al.*, 1980; Ohhashi *et al.*, 1982; Sacchi *et al.*, 1994; Todd and Bernard, 1973; Vajda, 1966). Lymph is produced from the tissue fluid and cellular elements at the site of the initial lymphatics, which drained into the large vessels, and then returns to the venous circulation via the collecting lymphatics. Valve structures, which appear to prevent a convective reflow, are arranged in places en route from the lymphatic capillaries in some organs including the gut to the collecting vessels. In the gastrointestinal tract, almost all lymphatics are of the initial type, and the muscular collecting lymphatics arise only outside of the organ *per se*.

III. Lymphatic Endothelial Cell Markers

The discovery of unique lymphatic endothelial cell markers has enabled us to examine the lymphatic system and gain new insights into the mechanism that controls the function and growth of the lymphatic vessels.

A. 5′-Nucleotidase (5′-Nase)

5′-Nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.2.3.5; 5′-Nase) is a sialoglycoprotein bound to the plasma membrane via a glycosylphosphatidylinositol anchor (Low and Finean, 1978; Taguchi and Ikezawa, 1978; Taguchi *et al.*, 1994). The enzyme is biochemically exploited as a marker enzyme for the cell surface membrane (ectoenzyme) (Baron *et al.*, 1986; Evans, 1980) and shows a wide distribution in animal tissues and between cell types, including hepatocytes, lymphocytes, and epithelial cells of renal tubules (Hardonk, 1968; Kreutzberg *et al.*, 1978; Robinson and Karnovsky, 1983; Usitalo and Karnovsky, 1977). The extracellular hydrolysis of nucleotides into membrane-permeable nucleosides and phosphate, in particular the production of adenosine from 5′-adenosine monophosphate, has been assumed to play a major role in this enzyme (Wada *et al.*, 1984), but its physiological role is still not completely understood.

In the mammalian circulation system, we observed the 5′-Nase activity located on the cell membrane of the lymphatic endothelial cells to be remarkably higher than that in the blood vascular endothelium (Kato, 1990) (Fig. 1). The lymphatic vessels possess an extremely intense 5′-Nase activity in the tissues of humans, monkeys and rodents, but rabbits and canines show less enzyme activity in the lymphatics. We applied these facts to elucidate the

lymphatics in the tissues, and developed an enzyme-histochemical method with 5′-Nase staining to visualize the lymphatics in various tissue preparations of several mammals (Kato *et al*., 1991; Shimoda *et al*., 1997). Immunohistochemistry using a specific antibody against ecto-5′-Nase (CD73) is also useful for demonstrating the lymphatics (Fig. 2a), and the combination of 5′-Nase and blood vascular markers such as CD34 and von Willebrand factor can be used to distinguish lymphatic vessels from blood vessels (Shimoda *et al*., 2003).

Cells of various types including mesenchymal cells display 5′-Nase activity during embryogenesis, but the lymphatic vessels postnatally tend to show a more abundant amount of 5′-Nase than the other tissue elements, except for hepatocytes, lymphocytes, and some absorptive epithelia. Hence, the level of expression and distribution of 5′-Nase in tissue elements presumably depend on the developmental stage of the tissues.

Since 5′-Nase is predominantly distributed on the luminal surface of the lymphatic endothelium, this enzyme possibly serves to prepare the lymph. However, its precise function on the lymphatic vessels remains obscure.

B. Vascular Endothelial Growth Factor Receptor (VEGFR)-3

The current progress on the molecular control of the lymphatic system commenced with the work of Alitalo and colleagues, who identified vascular endothelial growth factor (VEGF)-C as a specific lymphangiogenic factor (Joukov *et al*., 1996), and its tyrosine kinase receptor VEGFR-3, also known as flt4, expressed on lymphatic endothelial cells (Kaipainen *et al*., 1995) (Fig. 2b). The fully processed and activated form of VEGF-C binds to and activates VEGFR-3 at low concentrations and VEGFR-2, which is also known as Flk1 when present in the blood (Terman *et al*., 1991) and the lymph vascular endothelium (Hirakawa *et al*., 2003; Makinen *et al*., 2001), at high concentrations (Joukov *et al*., 1996, 1997). VEGF-C almost exclusively induces lymphangiogenesis through VEGFR-3 in adult murine tissues (Jeltsch *et al*., 1997; Veikkola *et al*., 2001) and chicken chorionic allantoic membrane (Oh *et al*., 1997), but also stimulates angiogenesis via VEGFR-2 in the ischemic limb of rabbits (Witzenbichler *et al*., 1998) and in the mouse cornea (Cao *et al*., 1998).

VEGF-C is expressed by a multitude of cell types, including mesenchymal cells around the embryonic veins, activated macrophages, skeletal muscle cells, smooth muscle cells surrounding large arteries, and blood vascular endothelial cells during embryogenesis (Eichmann *et al*., 1998; Hirakawa *et al*., 2003; Joukov *et al*., 1996; Karkkainen *et al*., 2004; Kukk *et al*., 1996). During embryonic lymphangiogenesis, VEGF-C is the paracrine

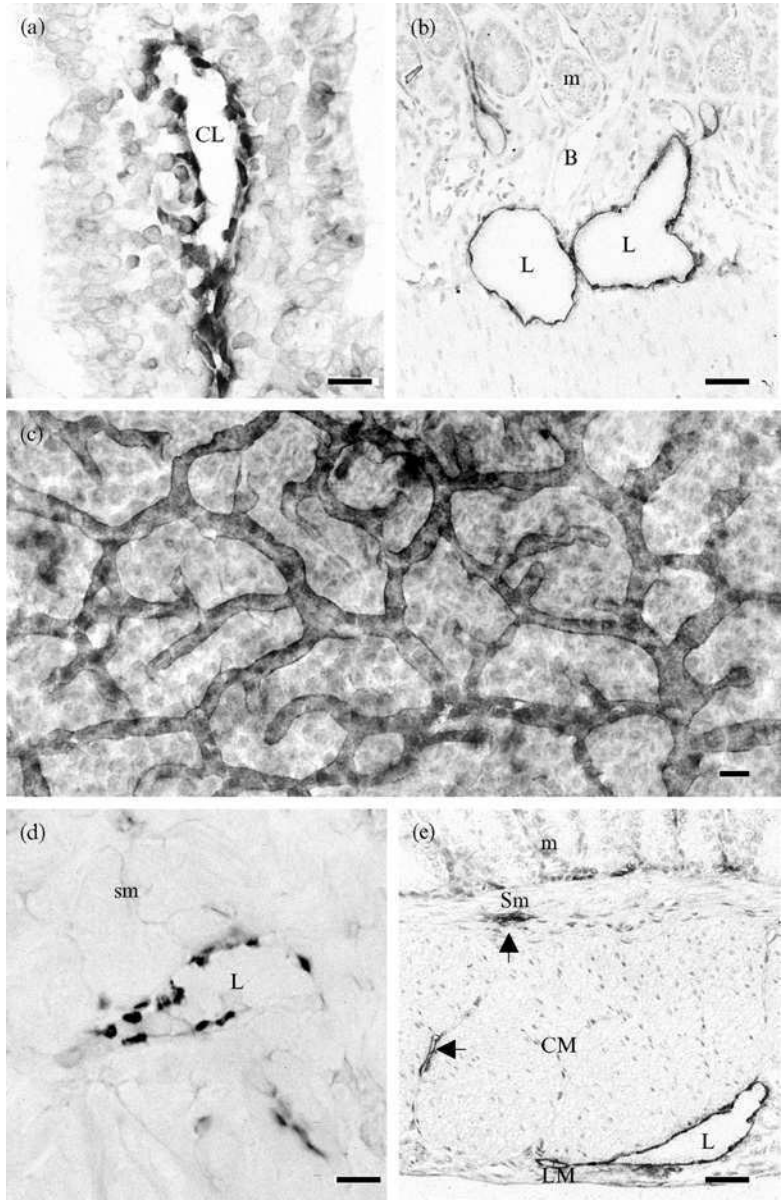

FIG. 2 Light micrographs of immunostaining for CD73 (a), VEGFR-3 (b), LYVE-1 (c), Prox1 (d), and podoplanin (e) on tissue sections (a, b, d, e) and a whole-mount preparation (c) from the rat (a, d) or murine (b, c, e) small intestine. (a) A central lacteal (CL) in jejunal villus shows an intense immunoreaction to CD73. (b) VEGFR-3 is immunolocalized in lymphatic vessels (L), but not in blood vessel (B). m, mucosa. (c) A dense network of LYVE-1-immunopositive lymphatic vessels is seen throughout the intestinal submucosal layer. (d) The lymphatic endothelial cells show Prox-1 immunoreactivity in their nuclei. Sm, submucosa. (e) Lymphatic

factor requisite for regular lymphatic development, but blood vascular formation does not necessarily demand VEGF-C (Karkkainen *et al*., 2004).

Another member of the VEGF family of glycoproteins, VEGF-D, also known as c-fos-induced growth factor (FIGF) also binds to and activates VEGFR-3 and VEGFR-2 (Achen *et al*., 1998), while displaying similar angiogenic and lymphangiogenic properties to VEGF-C in a variety of animal models (Marconcini *et al*., 1999; Stacker *et al*., 2001; Veikkola *et al*., 2001). The activation of VEGFR-3 by VEGF-C and/or VEGF-D promotes the proliferation, migration, and survival of cultured human lymphatic endothelial cells (Makinen *et al*., 2001).

VEGFR-3 is a member of the fms-like tyrosine kinase family and is structurally related to the two receptors for VEGF-A, VEGFR-1/Flt1 and VEGFR-2/ flk1 (Kaipainen *et al*., 1993, 1995). VEGFR-3 serves as a signaling receptor for VEGF-C and VEGF-D, which are lymphatic endothelial growth factors, but it does not respond to VEGF-A. During embryogenesis, this receptor is initially expressed in angioblasts of murine head mesenchyme, dorsal aorta, cardinal vein, and allantois (Kaipainen *et al*., 1995). The expression of VEGFR-3 is then distributed both in developing venous and presumptive lymphatic endothelia, and it later becomes restricted to the lymphatic endothelium (Kaipainen *et al*., 1995).

While VEGFR-3 is predominantly expressed in lymphatic endothelial cells in normal adult tissues, the expression is upregulated in some blood capillaries in some pathological conditions including tumor neovascularization (Valtola *et al*., 1999) and wound granulation (Paavonen *et al*., 2000; Witmer *et al*., 2001). VEGFR-3 is involved in the development of both the blood vascular and lymphatic system (Dumont *et al*., 1998; Kukk *et al*., 1996; Persaud *et al*., 2004), and the downregulation of VEGFR-3 expression causes severe defects in blood vessel development and abnormal cardiovasculature including cardiomegaly (Dumont *et al*., 1998). VEGFR-3-mediated signaling represents the main determinants of the lymphatic endothelium.

VEGF-C signaling and/or VEGF-D signaling through VEGFR-3 are not only regulators of lymphatic vascular growth, but also promote tumor lymphangiogenesis and lymph node metastasis (He *et al*., 2002; Karpanen *et al*., 2001; Mandriota *et al*., 2001; Shimizu *et al*., 2004; Skobe *et al*., 2001; Stacker *et al*., 2001). Many human tumors express VEGF-C and/or VEGF-D, which have been suggested to correlate with tumor metastasis via the lymphatic system (Stacker *et al*., 2002). Tumor lymphangiogenesis thus promises to be a useful factor for evaluating the metastatic spread.

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vessels (arrows, L) in each layer are immunoreactive for podoplanin. CM, circular muscle; LM, longitudinal muscle; m, mucosa; Sm, submucosa. Bars: 20 μm (a), 50 μm (b), 200 μm (c), 20 μm (d), 40 μm (e).

VEGFR-2, another receptor for VEGF-A, VEGF-C, and VEGF-D, is also expressed by blood vascular and lymphatic endothelial cells (Hirakawa *et al.*, 2003, 2005; Hong *et al.*, 2004b; Kriehuber *et al.*, 2001; Veikkola *et al.*, 2001). While the essential role of VEGFR-2 in angiogenesis has been well established, the lymphangiogenic potential of VEGF-A through VEGFR-2 has been indicated in such cases as isolated lymphatic endothelial cells (Hirakawa *et al.*, 2003), overexpression of VEGF-A in the skin of immunodeficient mice (Nagy *et al.*, 2002), cutaneous wound healing (Hong *et al.*, 2004b), cutaneous delayed-type hypersensitivity reactions in VEGF-A transgenic mice (Kunstfeld *et al.*, 2004), and tumor-associated lymphangiogenesis (Hirakawa *et al.*, 2005).

C. LYVE-1

LYVE-1 is a homologue of the CD44 glycoprotein and an endocytic factor for hyaluronan identified on lymphatic endothelium (Banerji *et al.*, 1999; Prevo *et al.*, 2001) (Fig. 2c). Hyaluronan, a mediator of cell migration, is transported through lymphatic vessels to lymph nodes and the liver to be degraded.

LYVE-1 is also expressed in discrete populations of activated tissue macrophages and sinusoidal endothelium of the liver and spleen, where high-molecular-weight hyaluronan is picked up and degraded (Grant *et al.*, 2002; Mouta-Carreira *et al.*, 2001). However, the blood vascular endothelium in normal and pathological tissues bearing granulation (Hong *et al.*, 2004b), inflammation (Baluk *et al.*, 2005; Cursiefen *et al.*, 2004; Kunstfeld *et al.*, 2004; Xu *et al.*, 2003), and tumors (He *et al.*, 2004; Hirakawa *et al.*, 2005; Mandriota *et al.*, 2001; Shimizu *et al.*, 2004; Skobe *et al.*, 2001) does not express LYVE-1, with the exception of the sinusoidal vessels. In contrast, the lymphatic vessels also display LYVE-1 expression in pathological conditions (Baluk *et al.*, 2005; Cursiefen *et al.*, 2004; He *et al.*, 2002, 2004; Hirakawa *et al.*, 2005; Hong *et al.*, 2004b; Kunstfeld *et al.*, 2004; Mandriota *et al.*, 2001; Shimizu *et al.*, 2004; Skobe *et al.*, 2001; Xu *et al.*, 2003). LYVE-1, therefore, serves as one of the reliable lymphatic-specific markers (Jackson *et al.*, 2001).

D. Prox1

The homeobox gene *Prox1* was originally identified by homology to the *Drosophila* gene *prospero* (Oliver *et al.*, 1993). The protein Prox1 has been confirmed in human, mouse, chicken, rat, frog, and zebrafish, and displays highly conserved amino acid sequences across the species (Oliver *et al.*, 1993; Schaefer *et al.*, 1999; Tomarev *et al.*, 1996). While Prox1 is distributed in a variety of cell types in the developing liver, nervous system, pancreas, and

heart to establish many different cell lineages (Oliver *et al*., 1993), the expression is exclusively detected in lymphatic endothelial cells among the vascular system both in embryonic (Wigle and Oliver, 1999) and adult tissues (Wigle *et al*., 2002) (Fig. 2d).

During murine embryogenesis, endothelial cells of the cardinal vein exhibit lymphatic competence due to their expression of VEGFR-3 and LYVE-1, and a subpopulation of the cells located at one side of the vein expresses Prox1 to migrate from the site in a polarized manner (lymphatic vias; Wigle and Oliver, 1999; Wigle *et al*., 2002). The sprouting lymphatic endothelial precursor cells achieve a lymphatic phenotype by expressing additional molecules characteristic of lymphatics and by losing blood vascular-specific molecules such as CD34 and laminin to form rudimentary lymph sacs (lymphatic specification; Wigle *et al*., 2002). In contrast, the Prox1-negative venous endothelial cells downregulate the expression of VEGFR-3 and LYVE-1 to attain a blood vessel phenotype.

In Prox1 null mice, budding of the lymphatic endothelial precursor cells appears unaffected, but the lymphatic specification of the cells is arrested (Wigle *et al*., 2002). Prox1, thus, is regarded as a master regulator of the lymphatic fate of embryonic venous endothelial cells with lymphatic competence by reprogramming their transcriptome (Wigle *et al*., 2002). Hence, Prox1 is regarded as an excellent lymphatic marker because this molecule controls lymphatic lineage (Hong *et al*., 2004a).

Recent evidence concerning development of the lymph sacs via Prox1 supports Sabin's "centrifugal" concept (Sabin, 1902, 1904), which suggests that the initial lymph sacs are derived by budding from embryonic veins and that the primitive lymphatics spread out throughout the body to form the lymphatic network. In contrast, Huntington and McClure (1910) have indicated in the "centripetal" model that embryonic lymph sacs arise in the mesenchymal tissue independently of the blood vascular system and that the primitive lymphatics connect later with the veins. Several studies have recently indicated the existence of independent lymphangioblasts in the early wing buds, limb buds, and allantoic membrane of the bird (He *et al*., 2003; Papoutsi *et al*., 2001; Schneider *et al*., 1999; Wilting *et al*., 2001, 2003). However, the contribution of the lymphangioblasts, which are independent of the blood vessels, to the development of the mammalian lymphatic network so far remains unclear.

E. Podoplanin

Podoplanin is a mucin-type transmembrane glycoprotein that was first identified on glomerular podocytes (Breiteneder-Geleff *et al*., 1997). The expression of podoplanin has been further demonstrated in osteoblastic cells

(Wetterwald *et al*., 1996), type I pneumocytes (Ramirez *et al*., 2003), and lymphatic endothelial cells (Breiteneder-Geleff *et al*., 1999; Hirakawa *et al*., 2003; Kriehuber *et al*., 2001; Schacht *et al*., 2003) (Fig. 2e).

During the development of the murine vascular system, podoplanin, as well as VEGFR-3 and LYVE-1, is expressed in the endothelial cells of the cardinal vein, and later in lymphatically committed cells (Schacht *et al*., 2003). While podoplanin is restricted to the lymphatic endothelial cells after birth, the upregulation of its expression in adult blood vascular endothelial cells due to ectopic expression of Prox1 has been proved (Hong *et al*., 2002).

Neonatal podoplanin-deficient mice show normal development of blood vasculature, but cutaneous lymphedema is associated with impaired lymphatic transport and an abnormal pattern and dilation of the lymphatic vessels in intestine and skin (Schacht *et al*., 2003). Podoplanin is considered to play a role in migration, adhesion, and tube formation of lymphatic endothelial cells (Schacht *et al*., 2003).

IV. Organization of Lymphatics in the Intestinal Muscle Coat

The gastrointestinal lymphatic system consists of interconnected networks of lymphatic vessels. The earlier descriptions of the lymphatic network were by Frey (1863), Mall (1888), Ranvier (1896), Shimizu (1932), and Grau and Schluns (1962). Later contributions made with SEM and histochemistry established the structural organization of the lymphatic network in the gut and provided further details (Ohtani, 1987, 1989, 1992; Ohtani and Murakami, 1992; Ohtani and Ohtsuka, 1985; Shimoda, 1998; Shimoda *et al*., 1996, 1997, 1998; Sugito *et al*., 1996; Ushiki, 1990; Yamanaka *et al*., 1995).

One characteristic of the gastrointestinal lymphatic network is that it is, in effect, independent of the network of blood vessels. The lymphatic networks presumably serve as a reservoir for fluid transport and prevent lymph stasis caused by the contraction of the intestine.

We have used the topography of the lymphatic network in the muscle coat of the small intestine as the archetype for our description, based on our histochemical examinations using whole-mount preparations of the gut of rodent, monkey, and human (Shimoda *et al*., 1996, 1997, 1998; Shimoda, 1998). The lymphatic network in the intestinal muscle coat is composed of larger and thinner lymphatic vessels that are distributed in the circular and longitudinal muscle coats, throughout the connective tissue layers between the muscle coats (myenteric layer), and between the external muscle and the serosal mesothelium (subserous layer) (Fig. 3A). The myenteric layer in

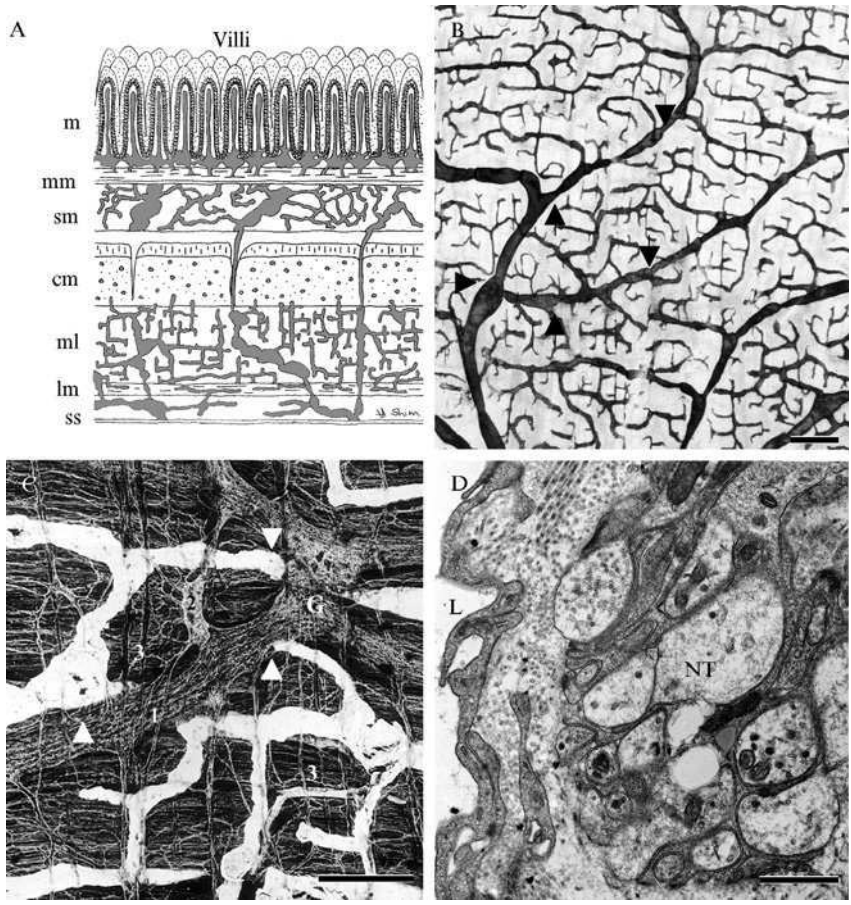

FIG. 3 Organization of the lymphatic network in the monkey small intestine. (A) A schematic drawing of the intestinal lymphatic network based on our histochemical study. cm, circular muscle layer; lm, longitudinal muscle layer; m, mucosa; ml, myenteric layer; mm, muscularis mucosae; sm, submucosa; ss, subserosa. (B) Light micrograph of a whole-mount preparation of the intestinal myenteric layer with 5′-Nase staining. A well-developed network of lymphatic vessels with 5′-Nase activity is shown throughout the myenteric layer. The arrows indicate knotty structures of the large lymphatics. (C) Backscattered scanning electron microscopic image of a whole-mount preparation of the myenteric layer with 5′-Nase and acetylcholinesterase (AChE) double staining. The 5′-Nase-positive lymphatics are strongly highlighted, whereas the AChE-positive nerves are less bright. Some segments of lymphatic capillaries run along primary nerve strands (1; *arrowhead*) and cluster with the blind ends around the myenteric ganglion (G; *arrows*). The lymphatics are intertwined with secondary nerve strands (2) and tertiary nerve components (3). (D) Transmission electron microscopic view of the intestinal myenteric layer. Unmyelinated nerve terminals (NT) are in close proximity to lymphatic endothelial cells. L, lymphatic vessel. Bars: 500 μm (B), 100 μm (C), 1 μm (D).

particular contains a well-developed lymphatic network (Fig. 3B). The network is continuous around the circumference of the gastrointestinal tract and along its length. The essential architecture of the intramuscular lymphatic network is characteristic and well conserved among the species and areas.

The lymphatic vessels in the myenteric and muscle layers are tubulosaccular in shape and run predominantly along the long axes of the muscle bundles to form a dense network featuring a maze- and/or mesh-like distribution with a two-dimensional expanse. The intramuscular and myenteric slender lymphatics start with knob-like blind endings to drain into the larger ones at their various sites in the myenteric layer. The myenteric large lymphatics, which display knotty structures indicative of the locations of valves, extend into the subserosa by obliquely penetrating the longitudinal muscle layer, and connect subserosal collecting tubules leading to the mesenteric ones.

The intramuscular and myenteric lymphatics are plentiful in blind ends at their apical parts, but they are not equipped with periendothelial cells such as smooth muscle cells. As a result, they are regarded as initial lymphatics (lymphatic capillaries). Since the intramuscular and myenteric lymphatics are probably not capable of contraction, the contractile movements of the intestinal muscle coat may regulate lymph flow in this area. The frequent localization of valve structures in the myenteric lymphatic capillaries may play a part not only in the prevention of the reflux of lymph flow, but also in protecting the excessive dilatation and/or oppression of the lymphatic wall. The large lymphatics in the subserosa running parallel to the large blood vessels sometimes display organized periendothelial smooth muscle cells, which correspond to the collecting lymphatics.

Although the lymphatic network is mostly independent of the blood vascular network, certain segments of the lymphatic capillaries are accompanied in part by arterial segments of blood vessels. This suggests that the blood vascular motion supplies the lymphatics with an energy source aiding lymph formation and transport, as well as intestinal muscular motility.

It is well known that the intestinal muscle coat houses an integrated nervous system called the myenteric nerve plexus of Auerbach (Auerbach, 1864). Auerbach (1865) has further described a lymphatic network with the peculiar nerve plexus in the intestinal myenteric layer. The myenteric nerve plexus takes the form of a meshwork consisting of ganglia, primary nerve strands (connectives), secondary nerve strands, and tertiary nerve components (Furness and Costa, 1987; Kobayashi *et al*., 1986).

The lymphatic network interlaces with the neural meshwork throughout the myenteric layer (Shimoda *et al*., 1998). The initial lymphatics often tend to run along the primary thick nerve connectives and cluster around the ganglia with their blind endings to make the peculiar maze-like distribution within meshes formed by the ganglia and internodal nerve strands (Fig. 3C).

The lymphatic capillaries presumably provide the myenteric nerves with a microenvironment proper to the nerves by regulating the interstitial tissue fluid.

Unmyelinated nerve fibers, which arise from the secondary or tertiary nerve components, are often distributed closely beneath the endothelium of the initial lymphatics (Fig. 3D). Several studies have proposed that the endothelial cells of the lymphatic capillaries may be one of the targets of bioactive molecules including neurotransmitters (Ichikawa *et al*., 1990, 1991a,b, 1993; Ito *et al*., 1989, 1990; Magari, 1983; Magari *et al*., 1979; Unthank and Hogan, 1987). An intimate association of nerve terminals with the myenteric lymphatic capillaries raises the possibility that these nerves might modulate the endothelial activity for producing lymph and/or monitor not only tension changes of the lymphatic wall as mechanoreceptors, but also chemical information around the lymphatics as chemoreceptors, though further studies are required to confirm this hypothesis.

The lymphatic vessels in the gastrointestinal muscle coat vary in caliber from species to species and from one area of the gut to another. The calibers of the lymphatic vessels in mammals are naturally larger in proportion to their body size. While the intramuscular lymphatics in the esophagus and stomach are slightly larger in caliber than those in the small intestine of the same mammals (Shimoda, 1998), the lymphatics in the muscle coat of the large intestine are much larger in caliber than the small intestinal ones. The diversity in caliber of the lymphatics among the portions of the gut might be attributed to tonic and phasic contractile potentials of each muscle coat reacting to forms of the intraluminal contents.

V. Development of Lymphatics in the Intestinal Muscle Coat

The formation of the gastrointestinal lymphatic network is poorly understood, though novel insights into embryonic development of the lymphatic system have been provided by recent research. Since the intestinal muscle coat is richly supplied with extrinsic lymphatic vessels, it is advantageous to examine the formation of the lymphatic network. Our histochemistry for 5′-Nase has demonstrated a microanatomical process of the lymphatic development in the muscle coat of the small intestine (Shimoda *et al*., 2001) (Fig. 4A).

The formation of the lymphatic network starts postnatally in the intestinal muscle coat. In newborns, the lymphatic vessels extend from the mesenteric lymphatics into the intestinal subserous layer, accompanying large blood vessels. The subserous lymphatics run toward the side opposite the mesenterium attachment and occasionally connect to each other by thin tubules.

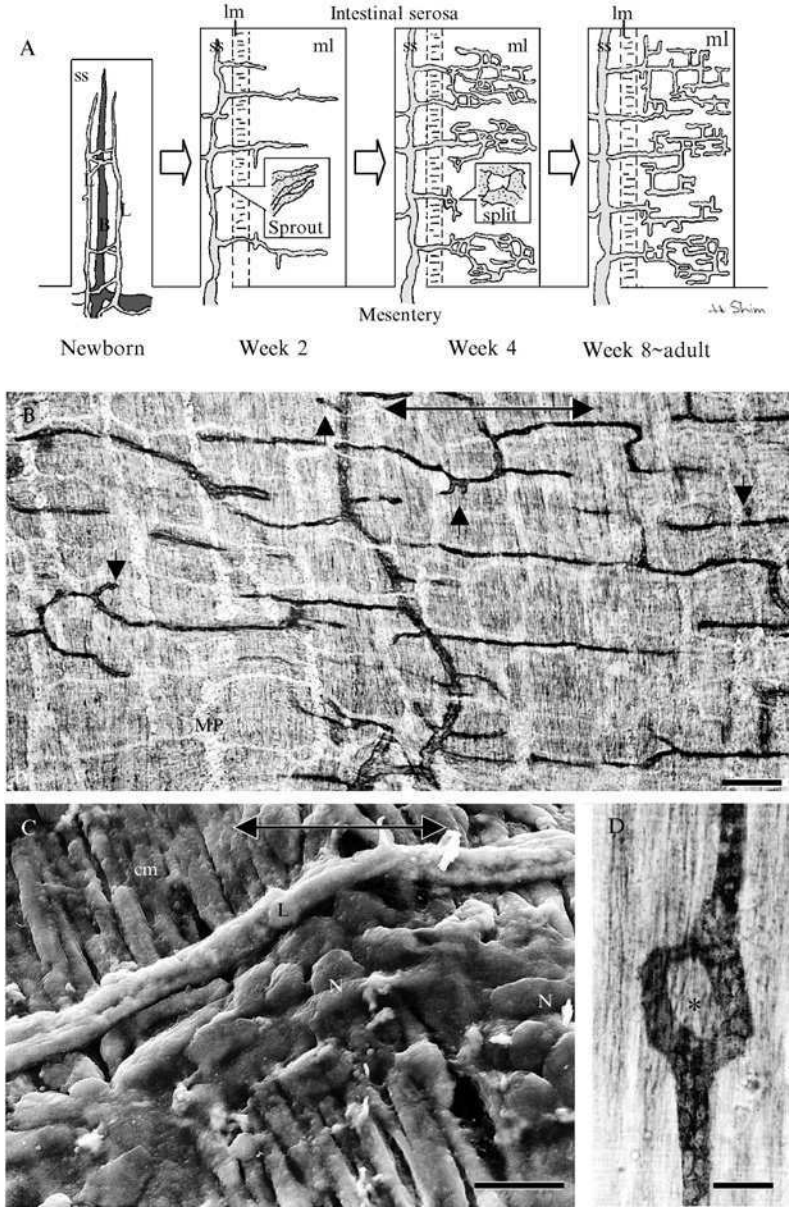

FIG. 4 Development of the lymphatic network in the muscle coat of the rat small intestine. (A) A schematic drawing of the process of lymphatic growth in the intestinal muscle coat based on our histochemical study. B, blood vessel; L, lymphatic vessel; lm, longitudinal muscle layer; ml, myenteric layer; ss, subserosa. (B) Light micrograph of a whole-mount preparation of the muscle coat from postnatal week 2 rat small intestine with 5′-Nase staining. The growing

By postnatal week 2, the subserous lymphatics issue many of lateral processes, which comprise the endothelial cells showing spindle-shaped cytoplasmic projections, *en route*. The processes grow along the longitudinal muscles and then enter the myenteric layer and circular muscle layer, while giving off further sprouts *en route* (Fig. 4B). The terminal portions of the growing lymphatics demonstrate tapered or blunt ends composed of filopodia-like projections of the endothelial cells. The lymphatic endothelial cells are ultrastructurally characterized by an unusual abundance of cell organelles including mitochondria, rough and smooth endoplasmic reticulum, Golgi apparatus, microfilaments, and caveolae, thus indicating a high cellular potential. They also disseminate long cytoplasmic extensions and many microspikes projected into the interstitial spaces, this suggesting their high migratory activity. However, the nature of the lymphatics closed by the endothelial cells is preserved during their growth. The lymphangiogenic vessels are capable of functioning as pathway transporting lymph-like mature vessels in adult tissue, because they contain lymph and immune cells within their lumen.

The growing lymphatics also tend to extend along the developing internodal nerve strands of the nerve plexus in the myenteric layer (Fig. 4C) and along the growing smooth muscle bundles in the muscular layers, as seen in the lymphatic network of the adult intestinal muscle coat. Though the tissue elements possibly navigate migration of the growing lymphatic endothelial cells, the machinery including molecular control is still unknown.

By postnatal week 4, multiple intervascular spaces occur at branching points of the vessels and within the enlarged lymphatic segments due to splitting of the vessels (Fig. 4D). The vascular splitting, also known as intussusceptive vascular growth, is similar to that documented in the postnatal development of blood vessels in some organs (Burri, 1992; Burri and Tarek, 1990; Caduff *et al*., 1986; Patan *et al*., 1992; Van Groningen *et al*., 1991). Subsequently, the intervascular holes grow larger to form a meshwork, and they are partially segregated to extend new lymphatic branches. The growing lymphatics thus increase their vascular bed to develop a dense maze-like network.

Hence, the development of the lymphatic network in the intestinal muscle coat is essentially attributable to the vascular sprouting from preexisting lymphatics and to the subsequent formation of intervascular meshes caused

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5′-Nase-positive lymphatics extend in a circular or longitudinal direction of the intestine and often show sprouting profiles (*arrows*) *en route*. The myenteric nerve plexus (MP) appears as a faintly stained network. (C) Secondary emission scanning electron image of the myenteric layer from postnatal week 2 rat small intestine. The growing myenteric lymphatic (L) extends along the nerve bundle (N). cm, circular muscle. (D) Light micrograph of the myenteric lymphatic from postnatal week 4 rat small intestine with 5′-Nase staining. A small intervascular hole (*asterisk*) is seen in 5′-Nase-positive lymphatic vessel. The double-headed arrow in (B) and (C) indicates the longitudinal direction of the intestine. Bars: 200 μm (B), 10 μm (C), 50 μm (D).

by vascular splitting. The process of postnatal lymphangiogenesis corresponds, in its essential mode, to angiogenesis (Hudlicka and Tyler, 1986; Pardanaud *et al.*, 1989; Risau *et al.*, 1988). However, lymphatic capillaries are substantially composed of only endothelial cells, whereas blood vessels are composed of endothelial cells, mural cells, such as pericytes, and the basement membrane. It remains to be established whether the growing lymphatic endothelial cells require a process to dissolve extracellular matrices, as in blood vessel angiogenesis, to migrate. In addition, whether the circulating or stromal progenitors of endothelial cells participate in the formation of the lymphatic network, as evidenced in blood vascular formation (Asahara *et al.*, 1997; Rafii, 2000), remains unknown. The precise dynamics of the developing lymphatic endothelial cells, involving molecular interaction between the cells and the other tissue elements, in the intestinal wall will be explored in future studies.

VI. Regeneration of Lymphatics in the Intestinal Muscle Coat

A recently developed histochemical method for studying the nature of the lymphatic vasculature has led us to make advances in the research on the functional morphology of the regeneration of lymphatic vessels. An investigation of the intestinal lymphatic regeneration is not only of critical importance for developing lymphangiogenic events in both physiological and pathological conditions, but also helpful in understanding the development of the lymphatic network peculiar to the intestinal tissue.

Several studies have demonstrated lymphatic regeneration in wounded cutaneous tissue (Bellman and Oden, 1959; Clark and Clark, 1932; Paavonen *et al.*, 2000). In contrast, the restoration of the lymphatic network in tissue repair of the gut has so far not been extensively assessed. This section describes the lymphatic regeneration in the muscle coat of the small intestine, essentially based on our study (Shimoda *et al.*, 2004), using an experimental model that undergoes transection of the muscle coat (myectomy) combined with histochemical methods.

This ''myectomy'' operation involves the circumferential removal of the muscle coat containing the inner circular and outer longitudinal muscles with a myenteric layer, subserosa, and serosa (5 mm in length) to excise the intramuscular lymphatic network, but it does not damage the submucosa or mucosa (Fig. 5). The portion operated on is conserved by some bioabsorbable membrane to prevent the area from undergoing any adhesional disorders. No clinical disorders, such as intestinal ileus, have so far, been developed in any experimental models. The myectomy model has proven to be useful in determining the manner of lymphatic regeneration and the

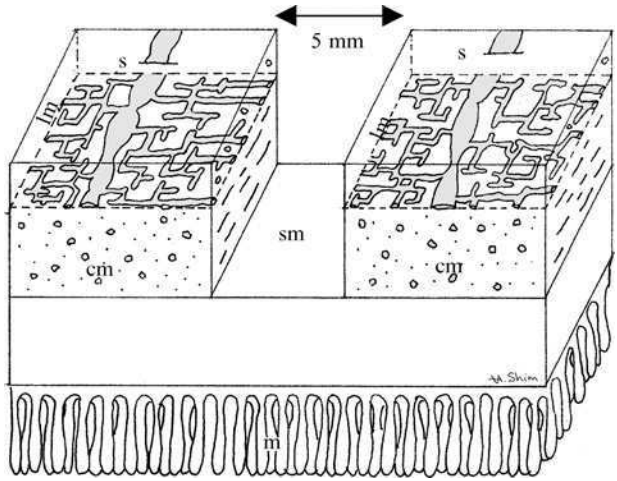

FIG. 5 A schematic drawing of myectomy in our experimental model. The myectomy involves the circumferential removal of 5 mm of the intestinal muscle coat, thus excising the lymphatic network in this area. cm, circular muscle layer; lm, longitudinal muscle layer; m, mucosa; s, serosa; sm, submucosa.

mechanism responsible for the restoration of the intestinal lymphatic network. The experimental data from the small intestine are thought to provide the benchmark for other areas of the gastrointestinal muscle coat, because the organization of the myenteric lymphatic network in the small intestine essentially conforms to that in other portions of the gut.

A. Lymphatic Regeneration

The broad manner of lymphatic regeneration in the intestinal muscle coat, which has been investigated by 5′-Nase histochemistry, is schematically presented in Fig. 6. An ultrastructural examination and immunohistochemistry for VEGFR-3 and Prox1 have confirmed that our defined 5′-Nase-positive vessels are lymphatic vessels in the small intestine. Since the regenerative profiles of lymphatic vessels at oral and anal sides of the operated area are essentially identical, the lymphatics are probably capable of regrowing in either direction of the intestine.

1. Initial Regenerative Stage

Following the transection of the intestinal muscle coat, hemostatic and inflammatory responses occur in the severed tissue stumps, as cutaneous injury. Inflammatory cells are recruited to the injured area after successful

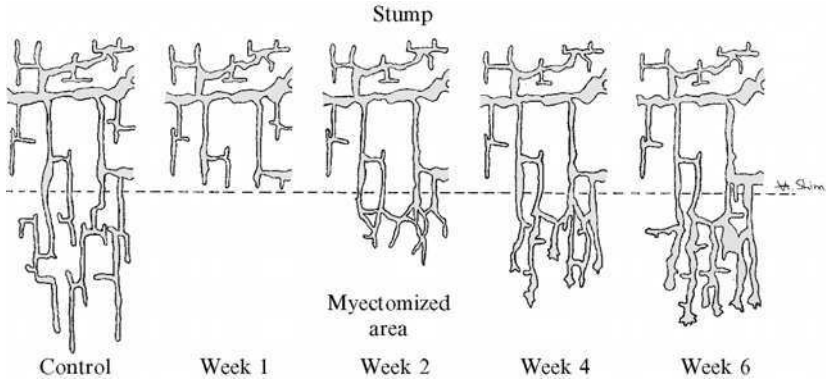

FIG. 6 A schematic drawing of the process of lymphatic regrowth after transection of the rat small intestinal muscle coat based on our histochemical study. The broken line indicates the cut line of the muscle coat.

hemostasis, which are integrated by platelets. Polynuclear leukocytes are recruited in the initial inflammatory phase, and thereafter macrophages are predominant 2–5 days following surgery. Macrophages are involved not only in tissue debridement and extracellular matrix degradation, but also in tissue remodeling through the release of cytokines such as chemotactic growth factors and matrix metalloproteinases (Fine and Mustoe, 1996; Graham *et al.*, 1992; Schaffer and Nanney, 1996). The tissue macrophages further stimulate proliferation and functional activity of fibroblasts to restore the wounded tissue.

At this stage, the newly formed immature tissue includes vigorous ingrowth of blood vascular endothelial cells, neural elements, and smooth muscle cells (Takahashi *et al.*, 2002). Thin blood vessels predominantly emanating from the severed vascular stumps wind their way into the regenerative tissue of the lesion. They thereafter form the blood vascular bed by anastomosing, sprouting and splitting at 7 days after myectomy. Angiogenesis is regarded as being accurately regulated by angiogenic factors and inflammatory cytokines released by macrophages, fibroblasts, and some other cells (Beck and D'Amore, 1997; Fine and Mustoe, 1996; Graham *et al.*, 1992; Hanahan, 1997; Schaffer and Nanney, 1996).

In contrast to blood vascular dynamics, the ingrowth of lymphatic vessels is not observed even at 7 days after surgery. The lymphatic vessels in the intestinal muscular stumps slightly decreased 5′-Nase activity in their endothelial cells, but the immunoreactivities for LYVE-1 and podoplanin are well conserved in them. The lymphatics restore their injured portions by closing their attenuated endothelial flaps for the duration of this stage. However, the

lymphatic endothelial cells are abundant in cell organelles including microfilaments, and project numerous cytoplasmic microspikes into both the intraluminal and abluminal sides; this indicates that the endothelial cells possess high levels of cellular potential to regenerate. This process is actually thought to be important to prepare growth points for lymphatic sprouts. Fibroblasts are often in close proximity to the terminal ends of the lymphatics, this suggesting a close association of the cells with lymphatic regrowth.

2. Early Regenerative Stage

By postoperative week 2, the lymphatic ingrowth into the regenerative tissue finally occurs after the blood vascular regeneration, as in cutaneous wound healing (Bellman and Oden, 1959; Paavonen *et al*., 2000).

Angiogenesis in tissue repair is thought to create a microenvironment proper for lymphatic regeneration, because no lymphatic vessels are distributed in the tissues without a supply of blood vessels in most normal organs. A sufficient blood supply is essential for the reconstruction of tissue, and the recovery of lymphatic vasculature is also requisite for practical tissue regeneration, because an impairment in the lymphatic function results in tissue edema, poor blood perfusion and a poor immune function, and inferior fibrosis. Hence, some biological responses, involving inflammation and tissue edema, via blood vessels invading the regenerative tissue, possibly encourage lymphatic regeneration, although the interactive machinery between regenerative blood and lymphatic vessels is so far obscure.

At 2 weeks after surgery, slender lymphatic vessels with increased 5′-Nase activity issue from the lymphatic stumps to extend into the injured area (Fig. 7A). The newly formed lymphatics grow in a longitudinal or oblique direction to form vascular arcades by anastomosing with each other in the lesion (Fig. 7A). These lymphatic arcades presumably serve as growth points in lymphangiogenesis, as in blood capillary growth (Rodin and Fujita, 1989), because many new sprouts with tapered tips further emanate from the arcades (Fig. 7A). By postoperative week 4, the lymphatics arising from the newly formed vascular arcades gradually grow larger and varicose in parts, in proportion to their extension (Fig. 7B), and often form terminal expansions, some of which provide further growth points for lymphatic extension (Fig. 7B).

The lymphatic arcades and terminal expansions are three-dimensionally composed of highly elongated endothelial cells without an investment of periendothelial cells, such as smooth muscle cells (Fig. 8A). The endothelial cells predominantly connect to each other by overlapping and/or interlacing, but in some parts small gaps are left between them (Fig. 8A). Ultrastructurally, the cells, which are rich in cell organelles including actin filaments,

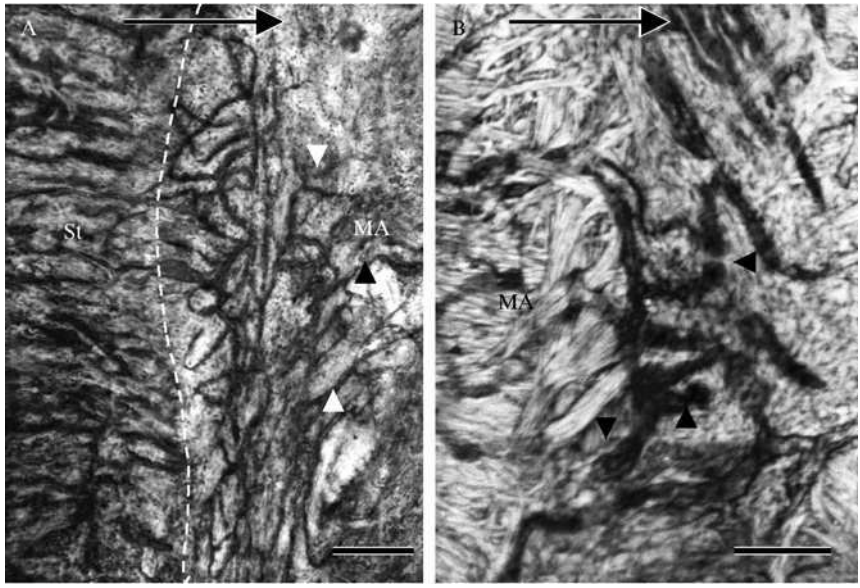

FIG. 7 Light micrographs of whole-mount preparations of the operated region from the postmyectomized week 2 (A) and 4 (B) rat intestines with 5′-Nase staining. (A) Many slender lymphatics with 5′-Nase activity extend from the severed stump (St) into the myectomized area (MA), taking a circular or longitudinal course. Regrowing lymphatics form vascular arcades and issue sprouts (*arrowheads*). (B) The regrowing lymphatics with 5′-Nase activity show thick varicose profiles. The arrowheads indicate the terminal expansions of regrowing lymphatics. MA, myectomized area. The arrow and broken line in (A) and (B) indicate the direction of regeneration and cut line of the muscle coat, respectively. Bars: 300 μm (A), 200 μm (B).

extrude filopodium-like projections into the interstitial spaces at the vascular tips, but dispose neither continuous basal lamina nor periendothelial cells along them (Fig. 8B). These unusual morphological features imply high migratory activity contributing to the establishment of new vascular channels. The vigorous migration of the endothelial cells possibly forms gaps between them.

It is interesting to note the occurrence of regenerating fibroblasts accompanied by the regrowing lymphatics near their distal ends (Fig. 8B). The fibroblasts being in close proximity to the lymphatics are considered to supply not only a pathway for the lymphatic extension but also some mechanics for controlling lymphatic regrowth.

Lymphatic regrowth in the intestinal muscle coat is, therefore, essentially attributable to vascular sprouting from preexisting lymphatics, as in postnatal intestinal development (Shimoda *et al.*, 2001) and lymphangiogenesis in wounded cutaneous tissue (Hong *et al.*, 2004b; Paavonen *et al.*, 2000; Saaristo *et al.*, 2004). However, the possibility that lymphatic regeneration

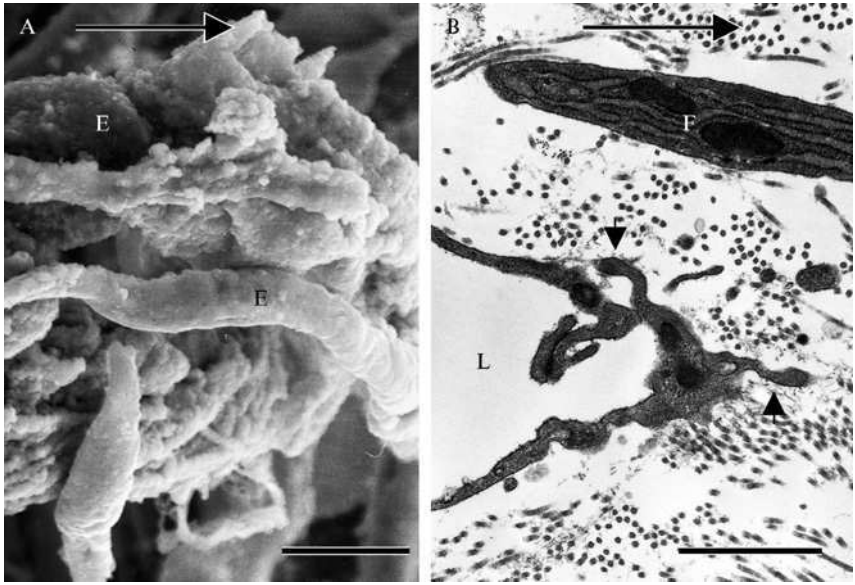

FIG. 8 Secondary emission scanning (A) and transmission electron microscopic (B) views of regrowing lymphatics in the myectomized area from the postmyectomized week 4 rat small intestine. (A) An expanded end of the lymphatic is composed of elongated endothelial cells (E) overlapping and/or interlacing with each other. (B) The endothelial cells of the lymphatic (L) are abundant in the thin filaments and show abluminal cytoplasmic microprojections (*arrows*) at the terminal tip. A fibroblast (F) is also seen in close proximity to the lymphatic tip. The large *arrow* in (A) and (B) indicates the direction of regeneration. Bars: 10 μm (A), 1 μm (B).

also involves a contribution of endothelial progenitor cells, as in angiogenesis, and/or lymphatic precursors descendent from preexisting veins (Wigle and Oliver, 1999; Wigle *et al.*, 2002) or mesenchymal stroma (Wilting *et al.*, 2003), as seen in their embryonic development, cannot be ruled out.

3. Late Regenerative Stage

At 6 weeks after surgery, the regrowing lymphatics are often equipped with knotty structures *en route* and knob-like blind ends at their leading tips to recover the structure proper to lymphatic capillaries (Shimoda *et al.*, 1997, 2001). They develop an irregular network in the lesion, interlacing regenerating smooth muscle bundles (Fig. 9A). While some leading tips of the regrowing lymphatics terminate as blind ends in both subserosal and submucosal sides of the musculature, the others further protrude small sprouts, indicating lymphatic regrowth (Fig. 9B). Hence, lymphatic regeneration in the intestinal muscle coat progressively advances with some manner of

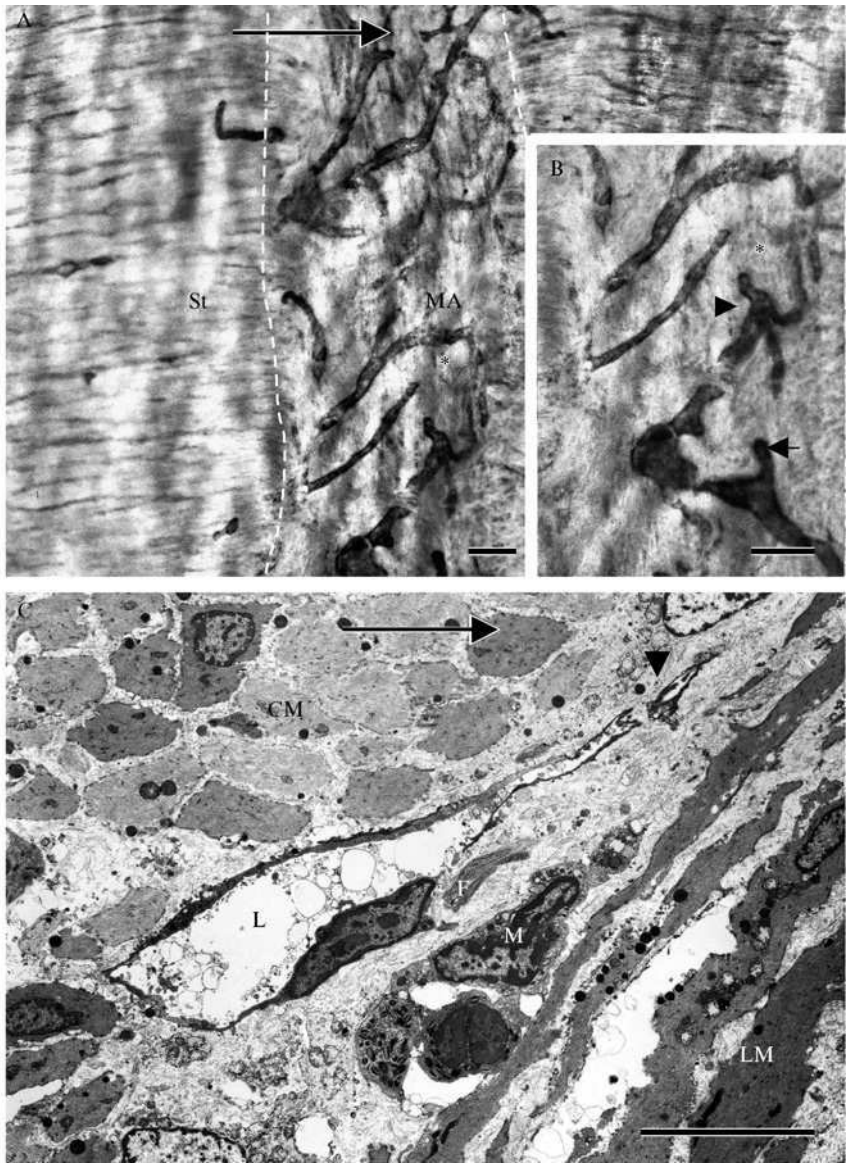

FIG. 9 Light micrographs of the operated area stained for 5′-Nase and transmission electron microscopic view of a regrowing lymphatic from the postmyectomized week 6 rat small intestine. (A) Many large regrowing lymphatics with 5′-Nase activity spread in the myectomized area (MA), interlacing with regenerative smooth muscle bundles. The broken lines indicate cut lines of the muscle coat. St, stump. (B) A higher magnification of the area indicated with an asterisk in (A). Knob-like blind end (*arrow*) and terminal expansion with several offshoots (*arrowhead*) are seen at the tips of the regrowing lymphatics. (C) A regrowing lymphatic (L)

vascular remodeling, whereas regrowing lymphatic vessels in cutaneous tissue repair have been reported to result in regression at 2 weeks after injury in cases without any exogenous measures promoting lymphangiogenesis (Paavonen *et al.*, 2000). The reasons for the discrepancy regarding the manner of lymphatic regeneration between cutaneous wound healing and intestinal muscular repair remains unknown. Lymphatic regeneration after tissue injury, however, might be subject to differences in the characteristics of the repaired tissue between the organs, because the intestinal muscle coat has an integrated motor activity in contrast to cutaneous tissue.

At this stage, caveolae on the endothelial cells and an enlargement of the vascular lumen filled with lymph become prominent in the regrowing lymphatics (Fig. 9C), and the cells recover significant 5′-Nase activity. However, the regrowing lymphatics are not endowed with either basal lamina or periendothelial cells, as are normal lymphatic capillaries. These findings suggest that the lymphatics develop into morphologically and functionally mature ones in proportion to the progression of their regeneration.

As a result, this myectomy model holds promise for accurately analyzing the mechanism responsible for the reconstruction of the lymphatic network due to the fact that the lymphatic regeneration in this model is progressive with vascular maturation.

B. Mechanism of Lymphatic Regeneration

VEGF-C is a major functional molecule that controls lymphangiogenesis by activating the tyrosine kinase-linked receptor VEGFR-3 (Jeltsch *et al.*, 1997; Veikkola *et al.*, 2001). A number of studies have described the significance of molecular signaling in lymphangiogenesis during embryonic development (Dumont *et al.*, 1998; Kukk *et al.*, 1996; Persaud *et al.*, 2004), inflammation (Baluk *et al.*, 2005; Cursiefen *et al.*, 2004) and tumor progression (He *et al.*, 2002, 2004; Karpanen *et al.*, 2001; Mandriota *et al.*, 2001; Shimizu *et al.*, 2004; Skobe *et al.*, 2001; Stacker *et al.*, 2001). The expression of VEGF-C has been demonstrated in a variety of cell types, as mentioned in Section III. B. During embryonic lymphangiogenesis, VEGF-C is considered to be a paracrine factor modulating regular lymphatic development (Karkkainen *et al.*, 2004). Furthermore, Saaristo *et al.* (2004) have recently indicated that adenoviral VEGF-C gene transfer at the cutaneous incision edges induces lymphangiogenesis and the restoration of the lymphatic flow across wounds. However,

lined with attenuated endothelial cells contain lymph within the lumen. A fibroblast (F) is also seen in close proximity to the lymphatic. An *arrowhead* indicates the terminal tip of the lymphatic. CM, circular muscle; LM, longitudinal muscle; M, macrophage. The arrow in A and C indicates the direction of regeneration. Bars: 2 mm (A), 200 μm (B), 20 μm (C).

the involvement of VEGF-C in tissue so far remains poorly understood. The spatial expression of the molecule in the lymphatic regrowth during tissue regeneration has been provided by our histochemical examination on the myectomy model of the intestinal muscle coat (Shimoda *et al*., 2004).

During tissue repair in the intestinal muscle coat, VEGF-C has been demonstrated to be preferentially expressed in a subpopulation of regenerating interstitial cells being in close proximity to the regrowing lymphatic vessels with 5′-Nase activity (Fig. 10), but not in regenerative smooth muscles and blood vascular endothelial cells. The cells with VEGF-C transcripts detected by *in situ* hybridization technique have further shown an increase in the cell density and intensity of the mRNA signals in proportion to tissue regeneration. These results imply the upregulation of VEGF-C in the stromal cells during tissue repair in the intestinal muscle coat and the functional significance of the cells for lymphatic regeneration.

The ultrastructural finding that fibroblastic cells are in close proximity to the leading tips of the regrowing lymphatics in the regenerative area may also indicate a presumable interaction between the stromal cells and regenerating lymphatic vessels. The regenerative interstitium, therefore, is considered to play a critical role in intestinal intramuscular lymphangiogenesis.

In contrast, VEGFR-3, the cognate receptor for VEGF-C, is definitely localized on the cell membrane of regenerating lymphatic endothelial cells. These findings suggest that a subpopulation of stromal cells regulates lymphatic regeneration by a paracrine action of VEGF-C and through the VEGF-C/VEGFR-3 signaling pathway during tissue repair in the small intestinal muscle coat.

VEGF-A, a representative angiogenesis factor, is known to be upregulated in wounded cutaneous tissue to promote angiogenesis associated with granulation tissue (Arnold and West, 1991; Singer and Clark, 1999). Although

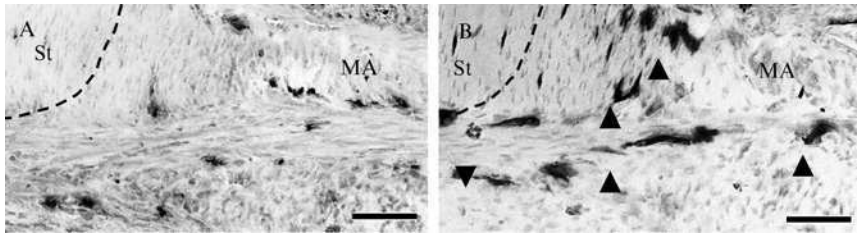

FIG. 10 Light micrographs of *in situ* hybridization for VEGF-C mRNA (A) and 5′-Nase staining (B) on serial tissue sections from the postmyectomized week 2 rat jejunum. (A) Intense signals for VEGF-C mRNA are seen in many stromal cells near the regions (*arrowheads*), in which 5′-Nase-positive lymphatics are seen in (B), in the myectomized area (MA). St, stump. (B) Many 5′-Nase-positive lymphatics are seen in the myectomized area (MA). St, stump. The broken line in (A) and (B) indicates the cut line of the muscle coat. Bars: 100 μm.

VEGF-A has generally not been considered to be a lymphangiogenic factor, a recent study has found that chronic transgenic delivery of VEGF-A potently promotes the growth of lymphatic vessels in cutaneous wound healing and that the lymphangiogenic effect of VEGF-A is mediated by VEGFR-2 expressed on wound-associated lymphatic endothelial cells (Hong *et al.*, 2004b). VEGF-A has been demonstrated to be predominantly expressed in epidermal keratinocytes at the wound edge and in the newly formed epidermis during cutaneous tissue repair (Brown *et al.*, 1992; Nissen *et al.*, 1998). In addition, in a suture-induced inflammatory corneal model, VEGF-A stimulates lymphangiogenesis via recruitment of bone marrow-derived macrophages that release VEGF-C/VEGF-D (Cursiefen *et al.*, 2004).

In our myectomy model, VEGF-A is expressed in a subpopulation of stromal cells and blood vessels, and its receptor, VEGFR-2, is localized both in blood and lymphatic vessels in the intestinal wound tissue at an early regenerative stage (unpublished observations). Although VEGF-A is also capable of promoting lymphatic regrowth in the intestinal muscle coat via VEGFR-2, it remains to be investigated, in future studies, how molecular signaling correlates with lymphatic regeneration.

On the other hand, another lymphatic endothelial marker, 5′-Nase, used to demonstrate the manner of regrowth of the lymphatics following myectomy, is also upregulated in the endothelial cells of the regenerating lymphatics. Since 5′-Nase serves membrane transport of the endothelial cells as an ectoenzyme, upregulation of the enzyme in the regenerating lymphatic vessels presumably correlates with the functional maturation of the lymphatics. However, the role of 5′-Nase in lymphatic regeneration remains to be elucidated.

VII. Concluding Remarks

Successful tissue repair requires lymphatic regrowth and reconstruction of the functional lymphatic vascular system, because the impairment of lymphatic function results in tissue edema, poor blood perfusion and immune function, and inferior tissue regeneration. A recent identification of the lymphatic endothelial markers has provided new insight into the process and molecular mechanisms underlying the lymphatic regeneration in cutaneous wound healing (Hong *et al.*, 2004b; Paavonen *et al.*, 2000; Saaristo *et al.*, 2004). In contrast, since lymphatic regeneration in tissue repair of the gut is starting to be elucidated, a number of questions remain to be answered regarding lymphatic regeneration in tissue restoration of the gastrointestinal muscle coat.

Our study using a myectomy model demonstrates lymphangiogenesis in the regenerative tissue of the intestinal muscle coat with fractional information of the regulatory mechanism. However, we still do not understand the detailed molecular mechanism involved in intestinal lymphatic regeneration. A spatiotemporal expression of the functional molecules related to lymphatic growth during intestinal tissue repair and an association of the molecules with the regenerative dynamics of the tissue elements including the inflammatory response must be defined.

Recent studies have indicated that several functional molecules, which have been studied in research in the field of blood vessel growth, are related to the formation and function of lymphatic vessels. The second member of the angiopoietin family, angiopoietin-2, is considered to be involved in blood vessel remodeling by regulating vascular sprouting and regression (Goede *et al*., 1998; Hackett *et al*., 2000; Maisonpierre *et al*., 1997; Stratmann *et al*., 1998; Vajkoczy *et al*., 2002; Visconti *et al*., 2002; Yancopoulos *et al*., 2000). Angiopoietin-2 has further been shown to be required for lymphatic development and patterning by means of its deficient mice (Gale *et al*., 2002).

FOXC2/MFH-1 is a member of the forkhead family of transcription factors and is essential for the formation of cardiovascular and genitourinary systems (Iida *et al*., 1997; Kanzaki-Kato *et al*., 2005; Winnier *et al*., 1997, 1999). It has been shown that mutations in the FOXC2 transcription factor cause a major form of hereditary lymphedema, the lymphedema-distichiasis syndrome (Kriederman *et al*., 2003). This molecule is shown to be responsible for the morphogenesis of lymphatic valves and the lymphatic network being free from mural cell investment with the cooperation of VEGFR-3 (Petrova *et al*., 2004). It is possible that the molecules correlate with reformation of the lymphatic network in the intestinal muscle coat.

Further functional molecules contributing to the establishment of profiles of lymphatic vessels will be elucidated in future studies. Such novel discoveries will also lead us to an understanding of the precise molecular mechanism needed for the reconstruction of the intestinal muscular lymphatic network.

The lymphatic network in the muscle coat is well organized with other tissue elements including the enteric nerve plexus in the normal gastrointestinal tract (Auerbach, 1865; Shimoda *et al*., 1998). The lymphatic regeneration in the intestinal muscle coat progressively advances in proportion to tissue repair, but the newly formed lymphatic vessels still do not recover their original network configuration, as seen in normal conditions, even at the later regenerative stage. Since the integrated function of the lymphatic system is supposed to be intimately associated with the structural organization peculiar to the tissues of the organ, it remains controversial as to whether the reconstructed intestinal lymphatic network exerts its own physiological function. It may require some molecular devices to establish a sufficient

reorganization of the regenerative tissues involving the lymphatic network during tissue repair of the gastrointestinal tract. Further analyses of the development and restoration of the lymphatic network in the intestinal muscle coat will allow us to address this issue, and provide the basis of the structural and molecular dynamics needed for the functional recovery of the gut following surgery involving transplantation.

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Calcium Homeostasis in Human Placenta: Role of Calcium-Handling Proteins

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The human placenta is a transitory organ, representing during pregnancy the unique connection between the mother and her fetus. The syncytiotrophoblast represents the specialized unit in the placenta that is directly involved in fetal nutrition, mainly involving essential nutrients, such as lipids, amino acids, and calcium. This ion is of particular interest since it is actively transported by the placenta throughout pregnancy and is associated with many roles during intrauterine life. At term, the human fetus has accumulated about 25–30 g of calcium. This transfer allows adequate fetal growth and development, since calcium is vital for fetal skeleton mineralization and many cellular functions, such as signal transduction, neurotransmitter release, and cellular growth. Thus, there are many proteins involved in calcium homeostasis in the human placenta.

KEY WORDS: Bone mineral content, Calcium-binding proteins, Calcium channels, Calcium-handling proteins, Human placenta, Syncytiotrophoblast.

I. Introduction

During pregnancy, the placenta represents the establishment of an intimate connection between mother and fetus that is specific to mammals. The placenta is a special organ that allows the fetus to develop within the protective maternal organism. It is a unique, autonomous, and transient organ that is vital for the maintenance of pregnancy and maternal-fetal exchanges. It is the most variable organ in structure and function in mammals. Moreover, there is

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probably no other organ that exhibits such extensive species differences. All placental types share one structural component, namely the existence of two separate circulatory systems: the maternal system and the fetal system. In addition, the placenta plays different roles in fetal growth and development, and ensures different functions, such as gas exchange (later lung function), excretion, water balance, and pH regulation (later kidney function). It also possesses catabolic and resorptive functions (later the gut function), synthesizes and secretes many hormones, such as most endocrine gland hormones, possesses many of the metabolic and secretory functions of the liver, hematopoiesis of the bone marrow (during early stages of pregnancy), and heat transfer of the skin, and finally has immunological functions, such as its role in the maternal tolerance of fetopaternal antigens (Benirschke and Kaufmann, 2000). There are also different types of placentation that are categorized according to the number and types of layers between the maternal and fetal circulation: epitheliochorial, syndesmochorial, endotheliochorial, and hemochorial. In humans, the placental structure is hemomonochorial, signifying that maternal blood comes into direct contact with fetal blood through the placenta, allowing an intimate contact, via the syncytiotrophoblast, between embryo and nutrients (Leiser and Kaufmann, 1994; Malassine, 2001).

During pregnancy, fetal calcium metabolism differs from that of adults in several ways, reflecting specific fetal needs (Kovacs *et al.*, 2001a). During the third trimester of pregnancy, about 80% of total fetal calcium is accumulated. It is actively transported across the human placenta at a high rate of 140 mg/kg/day (Salle *et al.*, 1987). At term, the human fetus has accumulated about 25–30 g of calcium. This transfer allows adequate fetal growth and development, since calcium is vital for fetal skeleton mineralization (Pitkin, 1983) and many cellular functions, such as signal transduction, neurotransmitter release, and cellular growth. Fetal blood calcium concentration is higher than maternal concentration, resulting in a maternal-fetal calcium gradient that is maintained by the presence of parathyroid hormone-related peptide (PTHrP)[1] (Kovacs, 1995; Rodda and Moseley, 1993; Rodda *et al.*, 1988) in the uteroplacental environment. Placental calcium transfer mainly involves the

[1]Abbreviations: AIIt, [(S100A10)2(annexin A2)2] complex; ATP, adenosine triphosphate; BBM, brush border membrane; BPM, basal plasma membrane; Ca^{2+}, calcium ion; CaBP, calcium-binding protein; CaBP-9k, calbindin-9k; CaBP-28k, calbindin-28k; CaMKII, calmodulin kinase II; CRF, corticotropin-releasing factor; DAG, 1,2-diacylglycerol; DNA, deox, estrogen response element; hCG, human chorionic gonadotropin; hPL, human placental lactogen; HVA, high-voltage activated; IP_3, inositol-1,4,5-triphosphate; IUGR, intrauterine growth retardation; LVA, low-voltage activated; mRNA, messenger ribonucleic acid; Na^+, sodium ion; NCX, Na^+/Ca^{2+} exchanger; NPY, neuropeptide Y; $nVDRE_{RP}$, negative vitamin D response element; 25-$(OH)D_3$, hydroxylated vitamin D_3; 25-$(OH)D_3$-1α-(OH)ase; 25-hydroxyvitamin D_3 1α-hydroxylase; 1,25-$(OH)_2D_3$, 1,25-dihydroxylated vitamin D_3, active form of

syncytiotrophoblast, a polynucleate structure characterized by the presence of a brush border membrane (BBM) facing the maternal circulation and a basal plasma membrane (BPM) facing the fetal circulation. The syncytiotrophoblast expresses many proteins having specific functions such as the handling of cellular calcium. These proteins have been extensively studied in recent years, and among them, many types of calcium channels, intracellular calcium-binding proteins (CaBPs), sodium (Na^+)/calcium (Ca^{2+}) exchangers (NCX), and plasma membrane calcium-ATPases (PMCA) are found. In human placenta, many proteins are known to be involved in calcium regulation and homeostasis; however, their specific functions are still unknown.

In addition, some factors have long been associated with decreased fetal calcium accretion during pregnancy. In fact, lower fetal bone mineral content (BMC) was observed in the presence of some pathologies, such as nontreated diabetes, intrauterine growth retardation (IUGR), and preeclampsia (PE); in some cases, fetal BMC is influenced by maternal nutrition. Thus, this chapter will discuss the placenta and proteins associated with calcium handling and homeostasis during pregnancy.

II. The Human Placenta

A. Implantation and Invasion

The maternal-embryo connection is realized by the placenta.The development of the placenta begins as early as when the fetal membranes establish stable contact with the maternal uterine mucosa. Placental formation is the first priority of the embryo, since it is essential for the embryo's subsequent development. Following conception, a series of asymmetrical divisions creates a mass of totipotent cells, named the morula, composed of about 16–32 fetal cells (blastomeres), and later forms a blastocyst (32–64 blastomeres). Blastocyst formation occurs after the embryo has moved from the oviduct. Each blastomere interacts with its neighbors in a nonadhesive protective

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vitamin D3; PCR, polymerase chain reaction; PE, preeclampsia; pGH, placental growth hormone; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PMCA, plasma membrane Ca^{2+}-ATPase; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; PTHrP(67–84), mid-fragment of PTHrP; ROC, receptor-operated calcium channels; RT-PCR, reverse transcriptase-polymerase chain reaction technique; RXR, retinoic X receptor; siRNA, silencing of RNA; SMOC, second messenger-operated calcium channels; TCTP, translationally controlled tumor protein; TR, transient receptor potential; VDCC, voltage-dependent calcium channel; VDR, 1,25-dihydroxyvitamin D_3 receptor; VDRE, vitamin D response element, vitamin D_3, cholecalciferol.

coating known as the zona pellucida, involving homotypic cell surface adhesion molecules, such as E-cadherin. This process is named compaction. The first differentiation event occurs after compaction of the morula gives rise to trophoblasts. The outer layer facing the maternal tissue is transformed to trophoblast cells (trophectoderm or primitive syncytium) by fusion of neighboring trophoblast cells, while cells of the inner layer of the blastocyst wall are composed of a group of cells that segregates at one pole, called an inner cell mass (ICM); these cells, which remain temporally unfused, are called primitive mononuclear cytotrophoblasts. The trophoblast is the precursor of the fetal membranes and placenta. It represents the specialized placental epithelial cells that directly connect the embryo to the uterus. This process involves the proliferation, invasion, and differentiation of trophoblast cells, which are the placental stem cells. The latter differentiation of the ICM gives rise to the embryo, the umbilical cord, and the amnion (Cross *et al.*, 1994). Moreover, both embryoblast-derived mesenchyme and embryoblast-derived blood vessels contribute to the formation of the placenta (Benirschke and Kaufmann, 2000). Whitin 6–7 days after fertilization, the trophoblasts initiate the implantation into the maternal endometrium. The formation of the placenta is the result of an active dialogue between maternal cells in the decidua of the uterus and fetal trophoblasts. Estrogens (from follicles) and progesterone (from corpora lutea) are involved in modifications of the uterus during the preimplantation period, and both prime the uterus for implantation (White *et al.*, 1994).

Different types of implantation exist, having different invasive capacities. In the noninvasive types of implantation, such as epitheliochorial placentation (e.g., in pig, horse, and some ruminants), implantation is arrested at the stage of attachment and invasion with destruction of the epithelial surface does not occur. In contrast, in most mammals and humans, implantation is invasive and involves transformation of the uterine stroma (the decidual cell reaction), recruitment of inflammatory and endothelial cells, transepithelial invasion of trophoblasts into the endometrium, and apoptosis of the uterine epithelium (Weitlauf, 1988). During attachment and after invasion of the endometrial epithelium, the trophoblastic cells of the implanting embryonic pole of the blastocyst show increased proliferation that results in double layered trophoblasts (Heuser and Streeter, 1941). Thereafter, the syncytial structure penetrates between epithelial cells and rapidly expands into the maternal stroma, but this expansion must result from the differentiation of underlying cytotrophoblast cells, since mitoses are never seen in syncytium. Trophoblast cells are extremely invasive and have the capacity to spread throughout the uterus if the invasion process is unchecked (Weitlauf, 1988). The endometrium also controls trophoblast invasion by secreting factors (cytokines and protease inhibitors) that modulate trophoblast invasion.

Thus, normal implantation and placentation involve a balance between regulatory gradients created by both the trophoblasts and endometrium (Kliman, 1998). About day 9 postconception, lacunae appear in the syncytium and these rapidly enlarge and fuse to communicate with each other. A potential uteroplacental circulation could occur when maternal venous capillaries are eroded by the syncytium so that blood can infiltrate into the lacuna system. This lacuna will subsequently form the intervillous space of the placenta. Thus, following the establishment of close contact between the trophoblasts and uterus, the process of placentation begins (Loke and King, 1993).

B. Placental Formation

In humans, about 21 days postconception the definitive chorionic villous structure of the placenta becomes apparent (Benirschke and Kaufmann, 2000; Loke and King, 1993). With progression of placental formation, two pathways of differentiation lead to the formation of two distinct populations of trophoblast cells: the villous (floating) and the extravillous (anchoring) phenotypes (Benirschke and Kaufmann, 2000; Loke and King, 1993). These two different pathways will be present separately because both trophoblast phenotypes have distinct functions and the types of maternal cells with which they come into contact are different. With the progression of pregnancy, the villous trophoblasts cover all the chorionic villi of the definitive placenta and are related to the placental exchange function of gas and nutrients from mother to fetus. The only maternal cells that are in the presence of villous trophoblast cells are those found in the circulation, while the extravillous trophoblasts invade the maternal uterine wall as far as the myometrium and are in contact with many maternal cell types, since they infiltrate uterine tissues (Loke and King, 1993).

In the villous trophoblast (floating) phenotypes, proliferative trophoblast stem cells remain attached to membranes surrounding the stromal cores, forming a monolyer of epithelial cells. The differentiation and fusion of these cytotrophoblast cells lead to the formation of a syncytiotrophoblast that covers the entire surface of the villous epithelium. This epithelium surrounds a core of connective tissue, including macrophages, fetal vessels, and fibroblasts. Thereafter, villous development results in the appearance of buds of cytotrophoblasts growing into the primitive syncytium. With increasing length and diameter, these buds, called primary villi, are converted (around 5 weeks of pregnancy) into secondary villi when mesenchymal cells, derived from the extraembryonic mesenchyme layer of the primary chorionic plate, invade the solid cytotrophoblast buds. Fetoplacental blood vessels arise from

the mesenchyme of the central stroma of the villi. Thus, the embryonic vessels link with vessels in the mesoderm of the chorionic plate, and ultimately with vessels in the fetus so that by the fourth week of pregnancy the fetoplacental circulation is established (Loke and King, 1993). The appearance of the first embryonic blood vessels in the mesenchymal core (around 6 weeks of pregnancy) marks the development of the tertiary villi (or terminal villi). Thereafter, until term, all fetally vascularized villi (mesenchymal villi, immature intermediate villi, stem villi, mature intermediate villi, and terminal villi) are classified tertiary villi. Thus, at the end of the first trimester of pregnancy, rapid extension and branching of the villi result in the villous tree characteristics of the mature placenta (Benirschke and Kaufmann, 2000; Kingdom and Kaufmann, 2000; Loke and King, 1993). The terminal villi are the predominant villous type during the second half of pregnancy (Kaufmann and Scheffen, 1998). They are considered to be the functional units of the placenta across which the majority of transfer takes place. In these terminal villi, there exist two cell layers separating maternal blood from fetoplacental capillaries, the syncytiotrophoblast. The villi become smaller as the pregnancy progresses, the layer of cytotrophoblast cells appears less prominent as the cells widely separate, and the syncytiotrophoblast becomes more irregular and narrow with clustering of the nuclei. During the last trimester of pregnancy, the development of the terminal villi increases exponentially, and according to Luckhardt *et al.* (1996) the surface area for placental exchange in terminal villi reaches about 13 m^3. Thus, briefly, the syncytiotrophoblast represents a true syncytium, is a unique structure in humans, and is the transporting epithelium of the placenta. While cytotrophoblast cells are the stem cells of the syncytium, they ensure the growth and the regeneration of the syncytiotrophoblast. During pregnancy, the syncytiotrophoblast is continuously renewed and expelled in the maternal circulation following a well-orchestrated process of necrosis and/or apoptosis (De Falco *et al.*, 2005; Morrish *et al.*, 2001). Finally, the villous syncytiotrophoblast synthesizes and secretes the majority of placental hormones. It synthesizes steroids (progesterone and estrogens) (Albrecht and Pepe, 1990), polypeptide hormones [human chorionic gonadotropin (hCG), human placental lactogen (hPL), prolactin, relaxin, placental growth hormone (pGH), neuropeptide Y (NPY), and many others (Frame *et al.*, 1979; Ishimaru, 1971; Krieger, 1982).

In the extravillous trophoblast (anchoring) phenotypes, the invasive cytotrophoblast cells originate from the anchoring villi attached to the uterine wall during the first week of pregnancy. These trophoblasts, as their name indicates, are made up of all the trophoblasts found outside the villi. Extravillous cytotrophoblast cells are highly migratory, proliferative, and invasive; they are related to tumor cells. However, contrary to tumor cells, their formation represents a physiological differentiation process conjointly

controlled by decidual cells, the trophoblast cells themselves and many diffusible factors within the placental bed itself (Bischof *et al.*, 2000; Lala and Hamilton, 1996). This trophoblast invasion necessitates the implication of proteolytic enzymes capable of digesting the extracellular matrices of the uterine wall. In this process serine proteases, cathepsins, and metalloproteineases have been involved (Westermarck and Kahari, 1999). From the primitive syncytium, at some sites, a subpopulation of cytotrophoblast cells residing at the villous basement breaks through the syncytium and forms a multilayered column of nonpolarized cells resulting from local proliferation. The formation of these columns physically connects the placenta to the uterine wall and gives rise to the highly invasive and migratory cytotrophoblasts. These extravillous cytotrophoblasts follow two pathways of differentiation: interstitial trophoblasts and endovascular trophoblasts. The interstitial trophoblasts invade the placental bed, while the endovascular ones also invade the uteroplacental vessels. The cytotrophoblast cell columns have the capacity to fuse to neighboring columns to form a cytotrophoblast shell, and invading trophoblasts arise from this structure. By 8 weeks of pregnancy, the interstitial cytotrophoblast cells invade the decidualized endometrium and reach the first third of the myometrium (Pijnenborg *et al.*, 1981). They can move around the arteries in both decidua and superficial myometrium and could fuse to form multinuclear trophoblast giant cells in the placental bed and myometrium. These cells can be regarded as the terminally differentiated structure of the extravillous pathway (Pijnenborg *et al.*, 1981). The differentiation of invasive trophoblasts into multinucleated placenta bed giant cells could reflect the correct trophoblast differentiation process. In the second half of pregnancy, there is no further penetration of trophoblasts into the myometrium, although vascular transformation appears to continue throughout gestation (Pijnenborg, 1994).

For the endovascular trophoblasts, cells invade the uterine wall spiral arteries to provide the blood supply necessary for the growing fetus. These cells adopt a vascular phenotype by transformation of their adhesion molecules, so as to mimic that of endothelial cells (Zhou *et al.*, 1997). This transformation is a remarkable phenomenon whereby nonneoplasic cells invade and destroy an artery. Thus, endovascular trophoblast cells replace the vascular smooth muscle cells and endothelium, and form distended flabby vessels to provide a large volume of blood into the maternal-fetal interface. This transformation occurs within the first trimester of pregnancy. During this period, the main function of these trophoblasts is to prevent arterial blood from entering the intervillous space at high pressure, acting as valves (Hustin, 1992). This also protects the conceptus from excessive high oxygen level exposure during this critical stage of development (Burton *et al.*, 1999; Hustin *et al.*, 1990). Another invasion period occurs after 16–18 weeks

of pregnancy allowing endovascular trophoblast cells to invade the deep myometrium and its vessels (Pijnenborg *et al.*, 1983). Thereafter, during the two other trimesters, these endovascular trophoblasts contribute to the lining of the arteries supplying the intervillous space (Loke and King, 1993).

III. Placental Transport Functions

A. Generality

During fetal life, the placenta represents the site of exchange between the mother and the fetus. In humans, it is a hemochorial type, composed of a fetal side represented by the chorionic plate containing the highly branched villous trees, the umbilical cord, formed by two arteries and one vein, and a maternal side mainly composed of the decidua basalis. The function of the anchoring villi is to attach the placenta to the uterine wall via the decidua basalis, and these villi are surrounded by the intervillous space in contact with maternal blood. Maternal blood reaches the intervillous space at high pressure via the endometrial arteries, floats around the villous trees, and is returned to the maternal circulation via endometrial veins. The placenta provides oxygen and nutrients to the fetus via the umbilical vein, and it disposes of oxygen-poor blood and fetal waste products into the maternal circulation via two umbilical arteries. The transport functions of the placenta are self-evidently vital to intrauterine normal fetal development, and the syncytiotrophoblast, an epithelium having an area surface that expands faster with progression of pregnancy, is the transporting epithelium in the placenta. Thus, the maternal-fetal exchange of nutrients (oxygen, glucose, amino acids, lipids, water, ions and minerals, vitamins, etc.) and the waste products (CO_2, metabolic waste, ions, lipids, etc.) of fetal metabolism are dependent on an adequate uteroplacental and fetoplacental circulation, a well-balanced and adequate maternal nutrition, and, finally, adequate working of the placental exchange barrier itself (Ayuk *et al.*, 2000).

B. The Syncytiotrophoblast

The syncytiotrophoblast is first formed during implantation (Benirschke and Kaufmann, 2000; Boyd and Hamilton, 1970), remains a kind of steady-state structure at the villous maternal-fetal interface throughout pregnancy (Huppertz *et al.*, 1999), and represents the most important maternal-fetal barrier (Boyd and Hamilton, 1970; Malassine and Cronier, 2002). Many electronic microscopic studies showed that the syncytiotrophoblast is a single

continuous structure (Bargmann and Knoop, 1959; Boyd and Hamilton, 1970; Schiebler and Kaufmann, 1969; Wang and Schneider, 1987). The continuous incorporation of villous trophoblast stem cells (cytotrophoblast cells), by syncytial fusion, into the syncytiotrophoblast is of vital importance for the growth and the survival of the syncytiotrophoblast layer of the human placenta, since this multinuclear layer has no generative potency (Boyd and Hamilton, 1966; Richart, 1961). The syncytiotrophoblast is a polynucleated structure characterized by the presence of a BBM at its apical face (facing maternal blood) and a BPM that is apposed to the trophoblast basement membrane and villous core (Sideri *et al.*, 1983). The maternal surface of the syncytiotrophoblast is covered by microvilli creating a huge maternal-fetal contact zone. This structure significantly increased the transport capacity of syncytiotrophoblasts. At the end of pregnancy, the presence of microvilli increases the total villous surface area to about 92 m^2 (Teasdale and Jean-Jacques, 1985, 1986). Many studies demonstrated the importance of the microvillous structure of the syncytiotrophoblast in the transporting functions of the placenta (Al-Zuhair *et al.*, 1983, 1987; Boyd *et al.*, 1968; Herbst and Multier, 1970). Thus, the syncytiotrophoblast is a multifunctional organ; its main functions are absorption, exchanges, and specific hormonal secretion. We will not review all the specific functions of the syncytiotrophoblast, but rather will discuss some of its functions, specifically calcium homeostasis.

C. Transport Mechanisms

All cellular membranes are composed of a phospholipid bilayer relatively impermeable to densely charged and hydrophilic molecules, reducing the exchange potential of the barrier. In human placenta, the syncytiotrophoblast exchanges are bidirectional and asymmetric, and can be ensured by concentration gradients or active transport systems (Schneider, 1991). The human placenta has the potential to transform stock or distribute some elements that are essential to fetal growth and development. Consequently, maternal-fetal exchanges are complex mechanisms that could be affected by many intrinsic factors, such as substrate and cell protein interactions with transporters, or by extrinsic factors, such as hormones. In brief, there are about three mechanisms by which solutes may be transported across the epithelium, such as syncytiotrophoblast: diffusion, facilitated and active transports, and endocytosis/exocytosis (Faber and Thornburg, 1986; Garnica and Chan, 1996).

Lipid-soluble molecules can rapidly dissolve in the lipid bilayer of the syncytiotrophoblast plasma membranes to reach the fetal blood flow by diffusion. Briefly, the diffusive transfer of these solutes is flow limited, while the rate of diffusive transfer of hydrophilic molecules is much slower than for

the lipophilic molecules, is dependent on membrane permeability, and is considered membrane–limited. It has also been reported that some solutes cross the placental barrier by paracellular diffusion, meaning that molecules bypass plasma membranes of the syncytiotrophoblast by moving through extracellular water pores or channels (Bain *et al.*, 1990; Thornburg *et al.*, 1988; Willis *et al.*, 1986). However, based on the morphological structure of the syncytiotrophoblast, the presence of paracellular diffusion is not obvious. But some studies suggest that in syncytium, small areas of denudation formed by fibrin-containing deposits at discontinuities in the syncytiotrophoblast could be used as a paracellular route (Brownbill *et al.*, 1995; Edwards *et al.*, 1993). Also, using perfused placental cotyledons, it has been reported that small hydrophilic solutes, such as calcium and chloride ions, can cross the placental barrier by paracellular diffusion, and this route accounts for about 80% of the total transferred (Doughty *et al.*, 1996; Stulc *et al.*, 1994). Many other solutes, such as glucose or amino acids, cannot diffuse through the placental barrier. To reach the fetal circulation, they should be transported across the bilayer using transporters. The facilitated transport is a transport that is assisted by a transporter protein, does not require energy, and uses an electrochemical gradient that is already established, while the active transport occurs against an electrochemical gradient and necessitates the use of energy. This process could be divided into two pathways, one that directly uses adenosine triphosphate (ATP) to provide energy for the transport and another that uses a gradient that is generated by an ATP-consuming system, such as sodium-potassium-ATPase (Na^+/K^+-ATPase). Finally, the endocytosis mechanism is a process in which a part of the plasma membrane is invaginated with the transported molecules, forming an intracellular vesicle. Thereafter, the vesicle fuses with the opposite plasma membrane (exocytosis) and releases its content into the extracellular compartment. This mechanism is often associated with cell surface receptors (Birn *et al.*, 2005; Hammes *et al.*, 2005; Jeon and Blacklow, 2005).

Fetal development is ensured by the continuous availability of metabolites (solutes) from the maternal circulation crossing the placenta (Battaglia and Meschia, 1988; Smith *et al.*, 1992). Quantitatively, glucose and amino acids are the most important nutrients crossing the placenta, but there are also other nutrients, such as lipid components, having a low transfer rate, that are vital for fetal development. Briefly, glucose transport is facilitated by transfer proteins according to a concentration gradient between mother and fetus, while amino acids also use transporters, but need energy to cross the placental barrier (Hay, 1994). Fatty acids use many routes to cross the placental barrier; they can diffuse through the bilayer or be actively transported across the syncytiotrophoblast (Lafond *et al.*, 1994, 2000). Mineral ions are also vital for fetal survival, and one of them is calcium. However, as

of now, the specific regulation and transport of calcium in the human placenta are still under debate. In this review, we will present most of the proteins involved in the handling of this ion during its route through the syncytiotrophoblast.

D. Calcium Homeostasis during Pregnancy

1. Maternal Calcium Homeostasis in Pregnancy

Fetal growth during pregnancy represents a major challenge for maternal calcium economy. In healthy pregnancies, an adjustment to maternal calcium homeostasis begins early, although maximal fetal demand for calcium appears during the third trimester. Such adaptations in maternal calcium homeostasis involve increased intestinal absorption (about two times), decreased renal excretion, and increased resorption from the skeleton (Hosking, 1996). Vitamin D_3 (cholecalciferol) is hydroxylated in the liver to form 25-$(OH)D_3$. Following that, in the kidneys, this molecule is hydroxylated a second time by the enzyme [25-$(OH)D_3$-1α-(OH)ase] (CYP27B1) to generate the active form, 1,25-$(OH)_2D_3$. This latter reaction is controlled by parathyroid hormone (PTH) in many tissues (Brenza and DeLuca, 2000; Kong *et al.*, 1999). In hypocalcemia, 1,25-$(OH)_2D_3$ is the major regulator of intestinal calcium absorption, while it acts to increase blood calcium in conjunction with PTH in kidney and bone (Hosking, 1996). The hypercalcemic effect of PTH and 1,25-$(OH)_2D_3$ is controlled by calcitonin. Elevated intestinal calcium absorption in pregnant woman is mediated by an increase in 1,25-$(OH)_2D_3$. During pregnancy, the maternal concentration of 1,25-$(OH)_2D_3$ remains elevated (Cross *et al.*, 1995). This increase appears to be caused by increased production from kidney, decidua, and placenta rather than decreased clearance. In addition, adaptation of calcium metabolism in pregnant women occurs without the increase in PTH that is required in nonpregnant states to elevate serum calcium (Ardawi *et al.*, 1997). It has been suggested that PTHrP, which dramatically increases during pregnancy, can inhibit PTH secretion by exhibiting PTH-like effects on PTH1R receptors (Gallacher *et al.*, 1994). PTHrP, probably originating from fetal, placental, and mammary tissues, is regarded as the prime candidate for the regulation of calcium and bone metabolism in pregnancy (Ardawi *et al.*, 1997; Ferguson *et al.*, 1992, 1994; Wysolmerski and Stewart, 1998). However, serum PTH and 1,25-$(OH)_2D_3$ levels can still increase in response to low serum calcium in healthy pregnant women with a low intake of dietary calcium (Vargas Zapata *et al.*, 2004). This indicates that the normal PTH response to increased calcium demand may remain intact in pregnancy. In contrast, the function of calcitonin in pregnancy appears more variable (Prentice, 2000).

2. Calcium in Fetal Development

The calcium ion is vital for fetal skeletal mineralization (Pitkin, 1983; Salle *et al.*, 1987) and many cellular functions. Thus, increasing amounts of calcium must be transported to the fetus across the human placental trophoblast epithelium to support fetal growth throughout gestation, especially during the third trimester when the majority of calcium is deposited in fetal bone. To attain the required amount of calcium and regulate the fetal blood calcium level, the fetus uses placenta, kidneys, bone, and intestine (Kovacs and Kronenberg, 1997). Fetal blood calcium concentration is higher than maternal concentration (Garel and Barlet, 1976). This increase is mainly due to an elevation of the ionized calcium fraction (Delivoria-Papadopoulos *et al.*, 1967). Thus, this increase results in a maternal-fetal calcium gradient that is maintained by the presence of PTHrP (Kovacs, 1995; Rodda and Moseley, 1993; Rodda *et al.*, 1988) in the uteroplacental environment. In humans, fetal hypercalcemia is documented as early as 15 weeks of gestation (Moniz *et al.*, 1986) and at about 33 weeks of gestation as evidenced at delivery of preterm singleton infants (Delvin *et al.*, 1982). This effect may be regulated indirectly by fetal PTH and PTHrP, since both act in the regulation of fetal blood calcium (Kovacs *et al.*, 2001a,b). Fetal parathyroid glands are able to synthesize PTH as early as the tenth week of pregnancy (Leroyer-Alizon *et al.*, 1981) and its detection in fetal blood likely arises from a fetal source since PTH does not cross the placenta (Dorey and Gell, 1975). Recent studies demonstrate the importance of this hormone in fetal bone mineralization (Kovacs *et al.*, 2001b; Miao *et al.*, 2002). Kovacs *et al.* (2001a) demonstrated that fetal mice lacking parathyroids and PTH (*Hoxa3 null*) have no modification in their morphology or gene expression in their skeletal growth plates, but their skeletal mineralization and blood calcium are significantly reduced. Moreover, in double mutant fetuses (*Hoxa3 null/PTHrP null*), combined loss of PTH and PTHrP caused fetal growth restriction, limb shortening, greater reduction of fetal blood calcium, and reduced mineralization (Kovacs *et al.*, 2001b).

PTHrP is an oncofetal hormone produced throughout fetal life by the fetal parathyroid glands, placental syncytiotrophoblast (Abbas *et al.*, 1990; Emly *et al.*, 1992), amnion (Bowden *et al.*, 1994), chorion (Bowden *et al.*, 1994; Ferguson *et al.*, 1992), and umbilical cord (Ferguson *et al.*, 1992). It is largely found in amniotic fluid and has stimulatory effects on epithelial growth and differentiation, and paracrine effects on placental vascular tone and calcium transfer (Farrugia *et al.*, 2000). The widespread expression of PTHrP mRNA during embryogenesis in extraembryonic and embryonic tissues in mice also suggests the involvement of the peptide in multiple growth and differentiation processes (Karperien *et al.*, 1996). Its concentration increases throughout pregnancy (Ardawi *et al.*, 1997). Alternative splicing of its gene

generates at least three different mRNAs that code for three different proteins having distinct C-terminal regions. Each PTHrP molecule generates at least three different bioactive products, each of which has its own physiological properties (Maioli *et al.*, 2004). The human PTHrP gene possesses a DNA element ($nVDRE_{RP}$) homologous to the negative vitamin D response element (VDRE) in the human PTH gene. Unlike the canonical VDRE, this $nVDRE_{RP}$ bound to the vitamin D receptor (VDR) but not to retinoic X receptor α (RXRα) (Nishishita *et al.*, 1998). Binding of monomeric VDR to this element was inhibited by 1,25-$(OH)_2D_3$ (Nishishita *et al.*, 1998). Thus, like PTH, PTHrP stimulates synthesis of 1,25-$(OH)_2D_3$ (Wysolmerski and Stewart, 1998) and expression of the PTHrP gene is, in turn, repressed by 1,25-$(OH)_2D_3$ (Inoue *et al.*, 1993).

Placental calcium transfer mainly involved syncytiotrophoblast, but its physiological and biochemical regulation in human syncytiotrophoblast is still unknown. Our actual understanding of this placental calcium exchange is based mainly on physiological observations, but real components and mechanisms controlling this calcium transport are still poorly identified. Recently, a new family of calcium-selective ion channels has emerged that is expressed in epithelium involved in calcium transfer, such as kidney and intestine (Hoenderop *et al.*, 1999; Peng *et al.*, 1999). It is postulated that these new epithelial calcium channel/transporter channels, named TRPV5 and TRPV6 (formerly CaT2 and CaT1, respectively), are the prime targets for hormonal control of active transcellular calcium in these epithelia (Hoenderop *et al.*, 2002a,b). Moreover, for many years, numerous CaBPs have actively been studied, because it is suspected that they play an important role in the cellular calcium buffering and shuttling capacity. Also several studies showed that CaBPs might directly or indirectly regulate PMCA activity, an important enzyme involved in calcium extrusion from the syncytiotrophoblast and/or the NCX.

E. Factors Involved in Transplacental Calcium Movement

Cellular calcium entry is critical for the development and/or physiology of all cells. A diversity of channels is responsible for calcium influx in different cell types, and there are similarities in mechanisms of calcium influx among highly divergent cell types, such as excitable versus nonexcitable cells. Transcellular calcium transport in polarized epithelial cells, such as in kidney and placenta, is of vital importance for overall calcium homeostasis. Our research team demonstrated the presence of a voltage-dependent calcium channel (VDCC) in syncytiotrophoblast, a nonexcitable cell type (Robidoux *et al.*, 2000a,b), and showed that these channels are mostly involved in the hormonal secretion

of hCG and corticotrophin-releasing factor (CRF) (Robidoux *et al.*, 2000a,b). However, we failed to demonstrate their role in basal calcium transport in placental syncytiotrophoblast (Moreau *et al.*, 2002b) and BeWo cells, a choriocarcinoma cell line (Moreau *et al.*, 2001). Thereafter, we demonstrated the role of TRPV5 and TRPV6 in syncytiotrophoblast calcium entry (Moreau *et al.*, 2002a,b, 2003b). In addition, since it is well know that the maternal-fetal calcium gradient is maintained by the presence of PTHrP in the fetal–placental environment, this hormone could be greatly involved in the regulation of placental calcium homeostasis and transfer. However, the mechanistic pattern regulating the transcellular calcium movement is still unknown. The following sections will describe the different cellular components known to participate and to allow this physiological process in other epithelial cells, such as VDCC, TRP, and other channels, intracellular calcium sequestration and movement (CaBPs), and calcium extrusion (PMCA and NCX). In the following section, we will describe the many proteins involved in the regulation of transplacental calcium movement from mother to fetus.

1. Calcium Channels

Various categories of calcium channels have been found to coexist in human plasma membranes (Parekh and Putney, 2005). There are (1) the VDCC that are mainly found in excitable cells, such as nerve and muscle, and are largely excluded from nonexcitable cells, with some exceptions, such as placenta; (2) the receptor-operated calcium channels (ROC), mostly found in excitable cells, and known to open rapidly following the binding of a specific extracellular ligand (often a neurotransmitter) to its receptor; (3) the second messenger-operated calcium channels (SMOC) that are less distributed and expressed in both excitable and nonexcitable cells; and finally (4) the store-operated calcium channels (SOC) that are distributed in all eukaryotic cells from yeast (Locke *et al.*, 2000) to humans (Partiseti *et al.*, 1994). In this review, we will mainly discuss VDCC and ROC, since both exhibit a physiological role in human placenta.

a. Voltage-Dependent (or Gated) Calcium Channels (VDCC) Many physiological processes, such as gene transcription, muscle contraction, hormone secretion, and neurotransmitter release, are regulated by an intracellular calcium influx, resulting from the activation of VDCC (McRory *et al.*, 2001). These channels are transmembrane proteins found in all excitable cells and some nonexcitable cells. The existence of the VDCC was initially reported by Hagiwara *et al.* (1975). Since then, noticeable advances have been made regarding the electrophysiology and molecular biology of VDCC. These channels can be divided into two subgroups based on their activation

threshold, low-voltage activated (LVA) and high-voltage activated (HVA), and each subgroup has its own gene. The threshold activation of calcium currents through HVA calcium channels occurs at −40 to −10 mV, while the threshold activation of the currents through LVA channels occurs at lower membrane potentials of −60 to −70 mV. The HVA channels encompass L-type, N-type, P/Q types, and R type, the latter also occasionally classified as intermediate voltage-activated (IVA) channels, since they are activated at relatively depolarized potentials (>−40 mV) (Stea *et al.*, 1995; Yamakage and Namiki, 2002). LVA calcium channels consist only of T-type calcium channels (Huguenard, 1998).

i. L-Type VDCC The L-type VDCC are widely distributed in many excitable cell types (Yamakage and Namiki, 2002) and in nonexcitable ones, such as human trophoblast cells (Cemerikic *et al.*, 1998; Robidoux *et al.*, 2000b). These channels are heteromultimeric proteins composed of five different polypeptide subunits that form the ion pore unit. The α_1 subunit (175 kDa) forms the ion pore channel. Many α_1 subunit isoforms are found [α_{1C} (for cardiac), α_{1D} (for neuronal), α_{1F} (for retina), and α_{1S} (for skeletal)]. They all belong to the Ca_v1 family. This subunit represents the voltage sensor and contains the binding sites for various drug and channel modulators (Barritt, 1999; Beurg *et al.*, 1999; Ellis *et al.*, 1988; Tanabe *et al.*, 1987). Thus, the properties of the VDCC are largely conferred by the pore-forming α_1 subunit (Perez-Reyes and Schneider, 1995). There are also the auxiliary α_2 subunit (143 kDa), which does not contain any high affinity binding sites (Ellis *et al.*, 1988), and four low-molecular-weight subunits β (54 kDa) (β_{1A}, β_{1B}, β_2, and β_3) (Birnbaumer *et al.*, 1998; Freise *et al.*, 1999). The presence of the β subunit, and its association with the α subunit, is the rate-limiting step in VDCC function, and it modifies the properties of the channel complex by allosteric modulation of the α_1 subunit function and by chaperoning the translocation of the α_1 subunit to the plasma membrane (Gerster *et al.*, 1999; Serikov *et al.*, 2002; Wakamori *et al.*, 1993). The α_1 and β subunits contain phosphorylation sites for cAMP-dependent protein kinase. There is also one γ subunit (30 kDa) (Jay *et al.*, 1990) and one δ subunit (27 kDa) (Takahashi *et al.*, 1987). These channels are also characterized by their sensitivity to specific blockers such as dihydropyridines, phenylalkylamines, and benzodiazepines (Kameyama and Kameyama, 1996; Reuter, 1983). The presence of many glycosylated sites in the α_2, γ, and δ subunits indicates their extracellular localization (Takahashi *et al.*, 1987). This section will mainly focus on the L-type and T-type channels, since their presence has been demonstrated in the placenta (Cemerikic *et al.*, 1998; Meuris *et al.*, 1994; Petit and Belisle, 1995; Robidoux *et al.*, 2000b). In human placenta, our research team reported the presence of both mRNA of α_{1C} and α_{1D} subunits in cytotrophoblast cells and syncytiotrophoblast, while only α_{1S} is present in BeWo cells (Moreau *et al.*, 2003a). We also reported that the basal calcium

uptake in BeWo cells is inhibited by lowering the pH of the incubation medium, is voltage–independent, and is not influenced by L-type VDCC and capacitative calcium conductance modulators (Moreau *et al.*, 2001). In addition, in human trophoblast cells, the presence of L-type VDCC modulators (Bay K8644 and nitrendipine) in the incubation medium had no effect on basal calcium uptake, suggesting that the process is mainly voltage–independent and does not involve these channels (Moreau *et al.*, 2002b). In addition, these channels were also studied in relation to their potential implication in the hormonal secretion of trophoblast cells. Robidoux *et al.* (2000b) showed that incubation of the trophoblasts with KCl, a depolarizing stimulus, or with Bay K8644, an L-type VDCC agonist, led to an increase in CRF secretion. They also suggest that Calmodulin Kinase II (CaMKII) and protein kinase C (PKC) seem to be potential modulators or mediators of these calcium effects on CRF secretion. In addition, Cemerikic *et al.* (1998) reported that the release of hCG is an opioid-regulated placental function that depends on extracellular calcium and the modality of its influx via L-type VDCCs. All these data suggest that these L-type VDCCs are mostly involved in placental hormonal regulation, in opposition to their potential role in trophoblast cell calcium uptake.

ii. T-Type VDCC T-type VDCC was initially found in guinea pig neurons (Somlyo and Somlyo, 1994) and is expressed in many organs, including nervous tissue, heart, kidney, and sperm. These channels are also expressed in various arteries and veins with a distribution different from that of L-type VDCC and are believed to participate in the regulation of the microcirculation (Hayashi *et al.*, 1996, 2005). They were also expressed in the smooth muscle of bronchi, ileum, colon, bladder, and uterus, were associated with the secretion of various hormones (Barrett *et al.*, 1995; Bhattacharjee *et al.*, 1997; Wagner *et al.*, 1998; Wen *et al.*, 2000), and were linked to functions such as smooth muscle contraction, myoblast fusion, and fertilization (Perez-Reyes, 2003). Usually, the electrophysiological properties of currents recorded from various cell types for this channel are similar, but their inactivation and their pharmacology present some differences. This heterogeneity can be explained in part by the existence of three T-type channels that are encoded on separate genes, α_{1G}, α_{1H}, and α_{1I} (Cribbs *et al.*, 1998; Lee *et al.*, 1999b; Perez-Reyes *et al.*, 1998), which all belong to the Ca_v3 family. Electrophysiological studies of recombinant channels show that expression of rat α_{1G}, human α_{1H}, and rat α_{1I} subunits in HEK-293 cells results in a robust calcium inward current. Both similarities and marked differences are found between their biophysical properties. Currents induced by α_{1G} show the fastest activation and inactivation kinetics, while the α_{1H} and α_{1I} currents activate and inactivate up to 1.5- and 5-fold more slowly, respectively. No differences in the voltage dependence of steady-state inactivation are detected (Klockner *et al.*, 1999). These channels are also sensitive to nickel,

but only α_{1H} currents were sensitive to low micromolar concentrations (IC_{50} = 13 μM); much higher concentrations were required for α_{1I} and α_{1G} (IC_{50}= 220–250 μM) (Lee *et al.*, 1999a). It was concluded that nickel is a selective blocker of α_{1H} currents, while the higher concentration needs of α_{1G} and α_{1I} to be blocked will also affect HVA channels. Actually, many compounds have been reported to inhibit the T-type VDCC, but most of them also affect other ion channels and transporters, and no specific inhibitor of the channel has yet been established (Heady *et al.*, 2001). In addition, according to its localization and associated function, actual studies focus mainly on the development of novel therapeutic agents for cardiovascular, neuronal, and endocrine disorders.

There are few studies on the presence of T-type VDCC in the placenta (Couderc *et al.*, 1995), and a transcript of α_{1H} was demonstrated by Northern blot in this tissue (Williams *et al.*, 1999). Thus little is known about its function in human placenta; however, studies showed that T-type expression can be induced by chronic hypoxia, and that the regulation by oxygen tension is specific for these channels, thus these channels may be differentially regulated in PE.

b. Store-Operated Calcium Channels (SOC) Calcium influx in nonexcitable cells regulates such diverse processes as exocytosis, contraction, enzyme control, gene regulation, cell proliferation, and apoptosis (Parekh and Penner, 1997). In intracellular calcium homeostasis, the endoplasmic reticulum (ER) is a multifunctional organelle involved in a long list of interdependent processes. Many of them require an increase in cytoplasmic calcium and involve calcium depletion of the ER. The trigger for SOC activation is decreased calcium in the lumen (Parekh and Penner, 1997; Putney and Bird, 1993). Thus, it is believed that the maintenance of ER calcium levels is the major function of this calcium channel (Ma *et al.*, 2000); this process was originally called capacitative calcium entry. The induction of depletion of calcium pools commonly induced activation of a sustained calcium inward current that is highly selective for calcium, over barium, strontium, and manganese. This calcium current is named I_{CRAC} (calcium release-activated calcium). It is not voltage–dependent and inwardly rectifying. It may be the mechanism by which electrically nonexcitable cells maintain raised intracellular calcium levels and refill their empty calcium stores after receptor stimulation (Hoth and Penner, 1992; Parekh and Penner, 1997; Zweifach and Lewis, 1993). In many nonexcitable cells, a major pathway for calcium entry involves the stimulation of phospholipase C (PLC). The activation of this effector by an agonist, commonly through the $G_{q/11}$ protein, induces the production of 1,2-diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3), which induce the release of calcium from intracellular IP_3-sensitive

stores. Following calcium release, channels on the plasma membrane are activated to allow the entry of calcium to replenish stores and maintain the intracellular calcium level (Kiselyov and Muallem, 1999; Putney, 2001). Actually, in human syncytiotrophoblast, various calcium channels are thought to be involved in this process, and recently members of the transient receptor potential (TRP) family were thought to be involved. A significant number of mammalian homologs of TRP have been found, and the TRP subfamily includes about 20 related channels (Harteneck *et al.*, 2000). They are classified into three major subfamilies: TRPC (for canonical), TRPV (for vanilloid), and TRPM (for melastatin).

i. TRPC The TRPC subfamily showed the highest level of sequence homology with the *Drosophila* TRP. Seven related members of the TRPC family have been found, from 1 to 7, that are encoded by seven different genes. TRP1 (Liu *et al.*, 2000), 4 (Philipp *et al.*, 2000), and 5 (Sossey-Alaoui *et al.*, 1999) genes are thought to encode the SOC channel; TRPC6 and TRPC7 genes are thought to encode ROC and are directly activated by IP_3 or DAG (Zhang and Saffen, 2001); the TRPC3 gene seems to be an SOC or an ROC (Kiselyov *et al.*, 1998; Zhu *et al.*, 1996, 1998); the TRPC2 gene is a pseudogene (Wes *et al.*, 1995). By analogy with other similar channels, a functional TRPC channel might be formed by the association of four TRPC proteins (Hofmann *et al.*, 2002), and found as homodimers or heterodimers (Hofmann *et al.*, 2002). However, the function and regulation of these channels are still unclearly defined, because different research teams produced a wide variety of results in interpretation and conclusions (Putney, 2005). Briefly, it was suggested that there are three possible modes of action for these channels. (1) TRPC channels are sequestered in a vesicular compartment, and following stimulation with growth factor, they are translocated to the plasma membrane. (2) They can be activated by DAG following the activation of PLC by the G-protein-coupled receptor (GPCR). In this pathway, the activation of tyrosine kinase, Src, is solicited and is negatively regulated by PKC. (3) The production of IP_3 following PLC activation induces the activation of the IP_3 receptor (IP_3R), causing release of calcium from the ER, and consequently the activation of TRPC channels, via the SOC pathway (Putney, 2005). Thus, these channels present a variety of activation modes and regulation. Riccio *et al.* (2002) first demonstrated the expression of TRPC mRNA in human placenta, and extensively studied all TRPC members. They showed that the highest expression level was observed for TRPC6, while the expression level was low for TRPC1 and 4, and very low for TRPC3 and 5. No expression was found for TRPC7. Interestingly, Clarson *et al.* (2003) evaluated the presence of TRPC channels (PCR, Western blot, and immunocytochemistry) in human placenta for different stages of pregnancy (first trimester, second trimester, and term)

and the presence of SOC entry in the placenta. They showed that SOC may play an important role in calcium entry into syncytiotrophoblast at term, but not in first trimester placenta. For the mRNA encoding TRPC1, 3, and 6, strong signals were obtained for all three placental groups. For TRPC5, a weak signal was observed for all groups, while for TRPC4, a faint signal was observed for the first trimester and a strong signal for the second trimester and term. At the protein level, from Western blot, only TRPC3 and 6 were detected in term placental homogenates. While, by immunocytochemistry, both TRPC3 and 4 were detected in villous cytotrophoblast cells of the first trimester, with faint staining in the syncytiotrophoblast cytosol and no apparent staining in BBM. By contrast at term, TRPC3 and 4 were clearly located in the syncytiotrophoblast, in both BBM and BPM of human placenta. They also showed that the TRPC6 protein is confined to the syncytiotrophoblast cytosol during the first trimester, while it is mainly expressed in the syncytiotrophoblast at term (Clarson *et al.*, 2003). Based on these results, they concluded that SOC entry may be an important route for calcium entry into the syncytiotrophoblast and that TRPC channels may underline this mechanism.

ii. TRPV5 and TRPV6 In the past 5–6 years, a new family of calcium-selective ion channels, expressed in epithelium involved in calcium transfer, has emerged (Hoenderop *et al.*, 1999; Peng *et al.*, 1999). These channels were recently classified as calcium selective channels of the transient receptor potential (TRP)-vanilloid (V) family. There are two highly homologous members, TRPV5 and TRPV6, which are juxtaposed on human chromosome 7q35, and their genes comprise 15 exons (Hoenderop *et al.*, 2001; Peng *et al.*, 2001a). TRPV5 was first identified in rabbit kidney and TRPV6 in rat small intestine. Thereafter they were identified in many other tissues and species (Hoenderop *et al.*, 2001; Moreau *et al.*, 2002a,b; Qiu and Hogstrand, 2004). Both have a molecular mass of about 83 kDa and are composed of about 730 amino acids. The amino acid sequences of both TRPV5 and TRPV6 are 76% identical (Weber *et al.*, 2001). These channels consist of six transmembrane domains and one putative pore-forming region between transmembrane domains 5 and 6. They have two long cytosolic NH_2 and COOH tails containing ankryin repeats (Hoenderop *et al.*, 2003). The molecular determinant of the calcium selectivity and the permeation of both channels reside at a single aspartate residue (D542 for TRPV5 and D541 for TRPV6) present in the pore-forming region (Nilius *et al.*, 2001; Voets *et al.*, 2003). Immunoblotting studies revealed that the TRPV5 and TRPV6 complexes migrate with a molecular mass of about 400 kDa, corresponding to a tetrameric structure. Moreover, immunoprecipitations using membrane fractions from oocytes coexpressing TRPV5 and TRPV6 demonstrated that both channels can form heteromeric complexes (Hoenderop *et al.*, 2003).

Expression of all possible heterotetrameric TRPV5/6 complexes in HEK293 cells modulates the activity of these channels according to calcium-dependent inactivation, barium selectivity, and pharmacological block. It was concluded that calcium-transporting epithelia coexpressing TRPV5 and TRPV6 can generate a pleiotropic set of functional heterotetrameric channels with different calcium transport kinetics (Hoenderop *et al.*, 2003). The order of sensitivity for the TRPV5/6 pore for divalent ion is calcium > barium > strontium > manganese, while the inhibition potency order is lanthanum > cadmium > manganese. Both TRPV5 and TRPV6 also share similar electrophysiological characteristics; the major difference resides in their calcium-dependent inactivation, their selectivity for barium, and their sensitivity to ruthenium red, a potent inhibitor of these channels (Hoenderop *et al.*, 2002b; Peng *et al.*, 2003). TRPV6 has a 100-fold lower affinity for ruthenium red than TRPV5 (Hoenderop *et al.*, 2001). Many studies also demonstrated that these channels are regulated by 1,25-$(OH)_2D_3$ (Fleet *et al.*, 2002; Hoenderop *et al.*, 2002a; Peng *et al.*, 2001b). In addition, the gene structure analysis of TRPV5/6 revealed a gene consisting of 15 exons and putative VDREs and an estrogen response element (ERE) in the promoter sequences (Muller *et al.*, 2000b). Thus, because the transcellular calcium movement in epithelial cells is a phenomenon highly coordinated and regulated, it was postulated that these channels could be the prime target for hormonal control of the active transcellular calcium movement (Hoenderop *et al.*, 2002b). In addition, based on their functional properties, high calcium selectivity, and *in vivo* localization, it was suggested that these channels are responsible for the calcium influx involved in epithelium that processes transcellular transport, such as intestine and kidney (Hoenderop *et al.*, 2002b; Peng *et al.*, 2003). In addition, according to results obtained in our laboratory, we also believed that these transporter proteins are involved in the maternal-fetal transplacental calcium movement (Moreau *et al.*, 2002a,b).

In human placenta, the presence of TRPV6 was first demonstrated in 2000 (Peng *et al.*, 2000; Wissenbach *et al.*, 2001). Thereafter, Peng *et al.* (2001a) demonstrated, by real time PCR, that in total human placenta TRPV6 is abundantly expressed as compared to TRPV5 (TRPV6/TRPV5 ratio = 800). We observed similar results by semiquantitative analyses in syncytiotrophoblast (Moreau *et al.*, 2002a,b). We also showed that both TRPV5 and TRPV6 are expressed mainly at the apical surface (BBM) of the trophoblasts (personal observations), suggesting their importance in placental calcium entry. Hoenderop *et al.* (2000) obtained similar results in rabbit kidney, and showed that TRPV5 is colocalized with other proteins involved in calcium transport, such as calbindin-28k (CaBP-28k), NCX1, and PMCA1b. Peng *et al.* (2000) also demonstrated the presence of TRPV5 in rat kidney, showing the colocalization of its mRNA with that of CaBP-28k. It was also

demonstrated that TRPV6 is regulated by PKC and calmodulin (Niemeyer *et al.*, 2001). Our results also demonstrated the presence of both TRPV5 and TRPV6 in human cytotrophoblast cells and syncytiotrophoblast, showing an increase in their mRNA level with differentiation (Moreau *et al.*, 2002a,b). This increase in the mRNA levels of TRPVs is correlated with the increase of calcium uptake with differentiation of cytotrophoblast cells into syncytiotrophoblast. Moreover, we demonstrated that TRPV6 could be directly involved in the calcium entry pathway of human syncytiotrophoblast, since syncytiotrophoblast calcium uptake is significantly reduced by 65% and 35% when these cells were, respectively, incubated in the presence of magnesium or ruthenium red (Moreau *et al.*, 2002b). Wood *et al.* (2001) demonstrated that Caco-2 cells expressed a low level of TRPV6 mRNA in the absence of 1,25-$(OH)_2D_3$, but are rapidly upregulated by treatment with 1,25-$(OH)_2D_3$. Moreover, in 2002, they showed that in addition to an increase in TRPV6 mRNA, 1,25-$(OH)_2D_3$ also increased PMCA and calbindin-9k (CaBP-9k) mRNA levels. Moreover, Barley *et al.* (2001) showed that TRPV6 mRNA levels are closely correlated with PMCA1 mRNA levels in human duodenal biopsies. They raised the interesting possibility that the expression of genes coding the proteins that control uptake (TRPV6) and extrusion (PMCA1) of calcium from the intestinal cell is coordinately regulated. All these findings suggest that induction of TRPV5/6 production may be important for the adaptive upregulation of calcium transport in syncytiotrophoblast. Interestingly, using a 1,25-$(OH)_2D_3$ receptor (VDR) gene knockout model, Kovacs *et al.* (2005) demonstrated that among all offspring of heterozygous mothers ($VDR^{(+/-)}$), there was no alteration in serum calcium, phosphorus, or magnesium, PTH, placental calcium transfer, calcium and magnesium content of the fetal skeleton, and morphology and gene expression in the fetal growth plates. However, specifically in VDR null fetuses, higher 1,25-$(OH)_2D_3$ plasma levels and 25-$(OH)D_3$-1α-(OH)ase mRNA were observed (in kidney, but not in placenta). Also, a slight increase of the placental expression of PTHrP and TRPV6 was observed, while normal levels of CaBP-9k and PMCA were found. They concluded that VDR is not required by fetal mice to regulate placental calcium transfer, circulating mineral levels, and skeletal development. They also suggested that if VDR has any involvement in the regulation of placental calcium transfer, it may have an inhibitory effect that is released by its absence, perhaps by permitting upregulation of PTHrP, which in turn upregulates placental calcium transfer. However, in human placenta, little is known about the regulation of TRPV5 and TRPV6 with respect to calcium transport.

Moreover, recently, in primary trophoblasts in culture, using gene silencing (siRNA), the suppression of the expression of both TRPV5/6 by about 80% significantly reduced trophoblast calcium uptake by about 50%, unambiguously demonstrating the role of these channels in trophoblast

calcium uptake. Moreover, the suppression of these channels did not modify trophoblast differentiation, since hCG secretion was not modified following siRNA treatment. Similar results were also obtained using a JEG-3 cell line (personal observations).

2. The Calcium-Sensing Receptor (CaSR)

The CaSr is a G-protein-coupled receptor with seven transmembrane domains that tightly regulate extracellular calcium levels during human adult life (Brown *et al.*, 1993, 1995; Chattopadhyay *et al.*, 1996). Renal calcium excretion is regulated by CaSR, which also modulates the transepithelial movement of other electrolytes and water (Ba and Friedman, 2004; Ikari *et al.*, 2001). Its mRNA is mostly expressed in the parathyroids and kidneys of adults (Brown *et al.*, 1993; Riccardi *et al.*, 1995), but it is also present in the C cells of the thyroid (Garrett *et al.*, 1995), the central nervous system (Ruat *et al.*, 1995), the placenta (Bradbury *et al.*, 1998, 2002), and some other tissues (Chattopadhyay *et al.*, 1996). In human placenta, the CaSR is expressed in both villous and extravillous regions during first trimester and term placentas (Bradbury *et al.*, 2002). In the villous regions, the CaSR was mainly detected in the syncytiotrophoblast BBM and at lower levels in cytotrophoblast cells. Its presence at the apical surface of the placenta suggests a role for maternal calcium concentration in the control of transepithelial transport between the mother and the fetus. The CaSR was also localized in anchoring trophoblast cells in close proximity to maternal blood vessels, suggesting that the CaSR could be a possible target by which maternal extracellular calcium levels could promote or maintain placentation. It was suggested that this receptor could contribute to the local control of placental calcium movement and to the regulation of placental development (Bradbury *et al.*, 2002).

In addition, using a CaSR gene knockout mice model, Ho *et al.* (1995) showed that heterozygous ($CaSR^{+/-}$) offspring develop normally after birth, but have increased parathyroid glands and serum ionized calcium levels and reduced urinary calcium excretion. In contrast, CaSR null offspring have the highest level of ionized calcium, parathyroid gland hyperplasia, bone abnormalities, and retarded growth and died within 3 weeks of birth. Using the same model, Kovacs *et al.* (1998) demonstrated that fetal disruption of the CaSR gene perturbs its environment, which is usually regulated by PTHrP, because in heterozygous or null fetal mice, the fetal ionized calcium level is increased, resulting in a worsening of fetal hyperparathyroidism and a significant reduction in placental calcium transport. Thus, CaSR disruption may, directly or indirectly, downregulate PTHrP or its effect on the placenta. Thus it is obvious that CaSR is involved in the regulation of transplacental

calcium transfer, but studies are still necessary to clarify its specific role in this physiological process.

3. Calcium-Binding Proteins (CaBPs)

Intracellular calcium is a crucial second messenger in several physiological cell functions and is an essential element for fetal survival. During pregnancy, regulation of fetal calcium homeostasis is ascertained by the placenta. However, presently, little is known with regard to the intracellular machinery of the placenta that maintains calcium homeostasis. Many studies suggest that CaBPs play a cardinal role in regulating or shuttling calcium. Nevertheless, in the placenta, the physiological implication of this is still unknown in many cases. This section discusses current views in this field with an emphasis on the localization, structure, and regulation of CaBPs.

The first CaBPs were initially localized in intestines (Arnold *et al.*, 1975; Harrison *et al.*, 1975), and thereafter in brain (Baimbridge *et al.*, 1982; Taylor, 1977), bones (Christakos and Norman, 1978), pancreas (Morrissey *et al.*, 1975), placenta (Bruns *et al.*, 1978, 1982), and kidney (Hermsdorf and Bronner, 1975; Staun *et al.*, 1984). Usually, these proteins have been found only within cytosol or on membrane facing cytosol (Moncrief *et al.*, 1990). The largest family of CaBPs shares a common calcium-binding structural motif, the EF-hand, named for the E- and F-helices of parvalbumin. The EF-hand domain consists of a helix-loop-helix motif that selectively binds calcium with high affinity (Kretsinger, 1980; Nakayama and Kretsinger, 1994). In fact, the EF-hand gene family evolved from a common ancestor by gene duplication, transposition, and splicing (Moncrief *et al.*, 1990; Nakayama and Kretsinger, 1993) to generate CaBPs that contain from two to eight copies of the EF-hand motif. In the mammalian genome, most genes encoding EF-hand CaBPs are not regrouped, except for those that encode the S100 superfamilly, which are localized in chromosome 1 (Berchtold, 1993; Schafer *et al.*, 1995). Calmodulin is the best studied CaBP from the EF-hand family. It is ubiquitously expressed in all tissues and possesses a multifunctional role (James *et al.*, 1995; Klee and Cohen, 1988). However, most of the other CaBPs show cell- and tissue-specific expression patterns, pointing toward a high specification for these proteins (Heizmann and Hunziker, 1991). Many studies showed that the expression of CaBPs, to mRNA or protein levels, is influenced by steroid hormones, such as 1,25-$(OH)_2D_3$ (Cao *et al.*, 2002; Li *et al.*, 2001b) and estrogens (Jeung *et al.*, 1994; Krisinger *et al.*, 1993). In placental tissue, a direct relation was established between some endocrine factors and the regulation of CaBP expression (An *et al.*, 2003, 2004; Hong *et al.*, 2003). Effectively, some studies reported that placental calcium

transport, a process that involves CaBPs, is regulated by PTHrP (Hershberger and Tuan, 1998; Kovacs and Kronenberg, 1997). Many CaBPs are present in human placenta and their number increases continuously. The following section will address the importance of these proteins in placenta and their potential regulation and functions.

a. The S100 Proteins The S100 proteins represent the largest subfamily within the EF-hand superfamilly, are encoded by a mutigene family, and actually about 20 members have been reported in the human genome (Marenholz *et al.*, 2004; Ravasi *et al.*, 2004). S100A1 to A16 are encoded by genes that are tightly clustered in region 1q21 of human chromosome 1, while the gene is localized on choromosome 21 for S100B, on chromosome 4 for S100P, and finally on chromosome X for CaBP-9k (CALB3) (Marenholz *et al.*, 2004). The S100 proteins are acidic proteins; most have a molecular mass of 9–14 kDa. They are composed of a C-terminal EF-hand domain that contains the canonical calcium-binding loop encompassing 12 amino acids and a noncanonical N-terminal loop encompassing 14 amino acids. The C-terminal domain represents the site for high calcium affinity, while the noncanonical EF-hand domain has lower affinity for calcium (100 times less). Both EF-hand domains are interconnected by an intermediate region, referred to as the hinge region. These proteins form homo- and heterodimers, and sometimes oligodimers, which contribute to their functional diversification. The dimerization of S100 proteins is calcium–dependent in most proteins, and could facilitate their interaction with intracellular target proteins (Heizmann, 1999; Zimmer *et al.*, 1995), such as myosin (Burgess *et al.*, 1984), tubulin (Donato *et al.*, 1989), cytosolic phospholipase A2 (Wu *et al.*, 1997), calcium release channel (ryanodine receptor) of the sarcoplasmic reticulum (Treves *et al.*, 1997), and annexins (Garbuglia *et al.*, 1996). Thus, they have the capacity to influence the activity of many enzymes, the dynamic of cytoskeleton components, cell growth and differentiation, and calcium homeostasis (Donato, 1999, 2001; Schafer and Heizmann, 1996; Zimmer *et al.*, 1995). In addition, some recent studies demonstrate an extracellular role of some S100 proteins, such as S100B (Adami *et al.*, 2004). The S100 proteins are not ubiquitously distributed in tissues, suggesting that they have specific cellular functions (Marinoni *et al.*, 2002). Effectively, these proteins were recently extensively studied because of their specific expression in some tissues and their role in some diseases, such as in cancer (Albuquerque *et al.*, 2003; Zimmer and Van Eldik, 1986), cardiomyopathies (Ehlermann *et al.*, 2000; Remppis *et al.*, 1996), psoriasis (Broome *et al.*, 2003; Semprini *et al.*, 2002), and inflammatory (Foell *et al.*, 2004; Frosch *et al.*, 2000) and neurodegenerative disorders (Li *et al.*, 1998; Sussmuth *et al.*, 2003). S100 proteins also participate in an extensive number of cellular activities such as signal transduction, cellular differentiation, regulation of cell motility and

transcription, and progression of the cell cycle (Schafer and Heizmann, 1996). The following sections detail the S100 proteins present in the human placenta as well as their properties. Moreover, Table I shows the different nomenclature used for these proteins, since different names have been attributed to the same proteins, and summarizes their different functions.

i. S100A1 S100A1 is a cytosolic 10-kDa protein (Most *et al.*, 2004). In perinatalogy, the presence of both S100A and B in amniotic fluid, cord blood, and fetal cerebrospinal fluid is usually associated with severe neonatal problems related to cervical damage (Blennow *et al.*, 1995; Gazzolo *et al.*, 1999). However, few studies focused on the presence of the S100A 1 protein in human placenta. Recently, Wijnberger *et al.* (2002) revealed its presence in many human placental cell types: syncytiotrophoblast, myofibroblasts, smooth muscle cells of the vascular wall, and macrophages. However, the specific role of this protein in the placenta is still unknown.

ii. S100A4 S100A4 is a cytosolic protein of 10 kDa. In mice, its mRNA was detected in many tissues. Nonpregnant uterus and placenta express the highest level of mRNA, while lower levels were observed in embryo and adult kidney, and very low levels were observed in testis and thymus. Moreover, during gestation the concentration of S100A4 mRNA in total placental extract decreases from day 8 to day 10 of pregnancy, and is below the detection level throughout the latter half of gestation. Moreover, the presence of this mRNA in both the fetus and the uterus suggests that its expression in placenta may occur in cells derived from the mother as well as the embryo (Jackson-Grusby *et al.*, 1987). Moreover, it was suggested that S100A4 shares many characteristics with other CaBPs involved in calcium transport in intestine and placenta. It was hypothesized that this protein may participate in calcium uptake in mid-pregnant placenta, in developing embryo, and in other tissues (Jackson-Grusby *et al.*, 1987). In addition, the mRNA of this protein is similar in size and sequence to that of S100A6 (Calabretta *et al.*, 1986), even though their respective mRNAs derive from two distinct genes (Jackson-Grusby *et al.*, 1987).

iii. S100A6 The S100A6 protein is a 10-kDa CaBP, possesses an open frame of 270 nucleotides, and shows 55% homology with the coding sequence of the β-subunit of the S100 protein (Calabretta *et al.*, 1986). In mice, high to moderate mRNA levels were detected in uterus, decidua, and placenta, based on different periods of gestation (Waterhouse *et al.*, 1992). An interesting study also showed that S100A6 is released by mice trophoblast cells in culture, and that it induces the liberation of mouse placental lactogen type II in the culture medium (Thordarson *et al.*, 1991). It was also suggested that the S100A6 protein could play an important physiological role in the regulation of mPL-II secretion. Most recently, Farnsworht and Talamantes (1998) confirmed this hypothesis and showed that mPL-I secretion is not influenced by this CaBP, but that it is a specific secretogogue for mPL-II.

TABLE I
Nomenclature of CaBPs and Their Different Functions

Proteins	Synonyms	Eventual roles
S100A1	S100, S-100α	ND
S100A4	Metastasin, calvasculin, 18A2, CAPL, p9Ka, 42A, pEL98, MTS1, murine placental homologue	May participate in calcium uptake in mid-pregnant placenta, in developing embryo, and in other tissues during pregnancy
S100A6	Calcyclin, CACY, PRA, 2A9, 5B10, CABP	It is a specific secretogogue for placental lactogen-II in mouse
S100A8	Calgranulin A (CAGA), MRP-8, p8, B8Ag, CP-10 (murine), cystic fibrosis antigen (CFAg), 60B8Ag, L1Ag leucocyte L1 light chain (L1L), calprotectin	Could play important roles in the production of placental hormones and the immunoregulation of fetal acceptance Could play a role in the prevention of maternal rejection of the implanting embryo
S100A9	Calgranulin B (CAGB), MRP14, P14, NIF, MIF, 60B8Ag, CGLB, CFAg, L1Ag, LIAg, MAC387	Could play a critical role in the implantation and development of the embryo during the first trimester of pregnancy
S100A10	Annexin II ligand, ANX2L, 42C, ANX2LG, calpactin I CAL1L, CLP11, GP11, p10, light polypeptide (p11)	The S100A10-annexin 2 association could play a crucial role in routing of TRPV5 and TRPV6 to the plasma membrane, favoring calcium entry into cells May play a role in the differentiation process of the placenta and in the function of the mature microvillous structure

S100A11	Calgizzarin, MLN70, S100C	ND
S100B	S100β, S100 beta chain	Could react with many enzyme signaling pathways involved in signal transduction
S-100P	Placental binding protein	ND
Calbindin D9k	Calbindin 3, CALB3, CaBP9k, intestinal CaBP, cholecalcin, CALB9	Could mainly act as an intracellular calcium buffer to favor calcium diffusion throughout the cytosol
Calbindin D28k	Calbindin 1, CALB1, CALB28, CaBP28k	Could act as an intracellular transporter to facilitate calcium movement into the trophoblast cytosol Could play a direct role in calcium stocking in some tissues, such as in fetal growing bone
Calbindin D57k	CaBP57k, mouse 57-kDa Ca^{2+}-binding protein	Could act as a calcium buffer and favors placental calcium transport Could encourage placental trophoblast differentiation
Oncomodulin	Parvalbumin β	Could favor the invasion capacity of cytotrophoblast cells
TCTP	Translationally controlled tumor protein	Could be involved in cellular calcium uptake activity and binding capacity

iv. S100A8 S100A8 is a 10-kDa CaBP (Lackmann *et al.*, 1992) that usually forms a complex with the S100A9 protein. It is a secreted protein and acts as a potent pure chemoattractant for mouse and human neutrophils and macrophages (Lackmann *et al.*, 1992, 1993). Human S100A8 is the most closely related member of the human S100 family to mouse S100A8, although the level of homology is <60%, and the human protein lacks chemotactic activity (Lackmann *et al.*, 1993). The presence of S100A8 was studied in human placenta using a biotinylated DNA probe for *in situ* hybridization. During the first and second trimesters of pregnancy high levels and synchronous expression were detected in cytotrophoblast cells, placental-tissue macrophages, fibroblast-like cells, endothelial cells, and monocytic lineages in fetal capillaries. Its highest expression was observed in cytotrophoblast cells and stromal-cell populations at around 8–11 weeks. The term placentas had low levels of S100A8, mainly in the myelomonocytic lineages of fetal blood vessels. The peripheral monocytes in the maternal space also expressed this protein at high levels, mainly during the first and second trimesters (Sato *et al.*, 1999). It was suggested that the presence of this protein in cytotrophoblast cells, placental-tissue macrophages, and fibroblasts could play an important role in the production of placental hormones and in the immunoregulation of fetal acceptance (Sato *et al.*, 1999). In addition, target disruption of the S100A8 gene in mice causes rapid and synchronous resorption at day 9.5 of development in 100% of null embryos (Passey *et al.*, 1999). It was shown that null embryos are infiltrated with maternal cells before overt signs of resorption. A nonredundant function of this member of the S100 gene family was also shown, implying a role in the prevention of maternal rejection of the implanting embryo (Passey *et al.*, 1999).

v. S100A9 This protein of about 12 kDa is mainly known for its cytokin proinflammatory action, like a pituitary hormone, and for being a negative regulator of corticosteroid action on the immune response. However, many research groups have suspected that it is implicated in the reproductive process (Suzuki *et al.*, 1996a,b; Wada *et al.*, 1997) and pregnancy (Zeng *et al.*, 1993). Arcuri *et al.* (1999) have shown that this protein is expressed in villous chorionic cytotrophoblasts of human placenta from 6 to 10 weeks of pregnancy. They also suggested that the action of this protein on macrophages present at the maternal-fetal interface could play a critical role in the implantation and development of the embryo during the first trimester of pregnancy.

vi. S100A10 S100A10 is a dimeric protein composed of two 11-kDa subunits. It represents a distinct member of the S100 family, because mutations in its two EF-hand domains modify its binding capacity for calcium, rendering it insensitive to calcium (Gerke and Weber, 1985). Typically, in most cell types, S100A10 is found to bind to its annexin A2 ligand as the heterotetrameric [(S100A10)2(annexin A2)2] complex, AIIt. In addition to

an intracellular distribution, S100A10 is present on the extracellular surface of many cells (Kwon *et al.*, 2005). Recently, van de Graaf *et al.* (2003) showed that this complex is the first auxiliary protein of TRPV5 and TRPV6, favoring a calcium influx pathway into a variety of epithelial cells. They also showed that this complex is associated specifically with the conserved sequence VATTV located in the C-terminal tail of TRPV5 and TRPV6 (van de Graaf *et al.*, 2003), where the first threonine plays a crucial role in its binding capacity to S100A10. Furthermore, they showed that the complex is colocalized with the calcium channels in renal tubules expressing TRPV5 and in duodenal cells expressing TRPV6. They concluded that the S100A10–annexin 2 complex plays a crucial role in routing of TRPV5 and TRPV6 to the plasma membrane. Another study obtained similar results and demonstrated that the Allt complex is associated with the cytoskeleton (Kaczan-Bourgois *et al.*, 1996). Moreover, they showed that the mRNA expression levels of S100A10 and annexin 2 increase progressively during pregnancy, suggesting that this complex may play a role in the differentiation process of the placenta and in the function of the mature microvillous structure (Kaczan-Bourgois *et al.*, 1996). Thus, this protein seems to be of vital importance for many processes in the placenta.

vii. S100A11 S100A11 has been extensively studied since it is observed in relation to different cancer types. It has a molecular mass of about 13 kDa, is often found as a dimer (Todoroki *et al.*, 1991), and interacts with annexin 1 in a calcium-dependent manner (Mailliard *et al.*, 1996). S100A11 was found to have a localization distinct from other S100 proteins examined, is mostly localized in the nucleus, with slight variations among different glioblastoma cell types (Inada *et al.*, 1999), and under the influence of calcium may translocate to the cell plasma membrane (Mandinova *et al.*, 1998). It is highly expressed in human placenta, but its expression level is reduced in many other human and fetal tissues (Inada *et al.*, 1999). However, its function in placenta is still unknown.

viii. S100B S100B is found as a homodimer and possesses a molecular mass of about 10 kDa (Isobe and Okuyama, 1978). It has four binding sites for zinc and two for calcium (Baudier *et al.*, 1986). *In vitro*, this protein binds zinc with a high affinity of $0.01-1.0\ \mu M$, while it binds calcium with 1000 less affinity (Baudier and Cole, 1988; Baudier *et al.*, 1986). In placenta, it is localized in villous and intermediate trophoblasts and during pregnancy the intensity of its protein immunostaining increases progressively. S100B is also localized in amnion, decidual cells of fetal membranes, and endothelial cells of umbilical vessels at all gestational ages (Gazzolo *et al.*, 2002; Marinoni *et al.*, 2002). Moreover, in human placenta, its expression level and localization are not influenced by complications of pregnancy, such as IUGR (Gazzolo *et al.*, 2002). In all cell types, the immunostaining of S100B is localized in the cytoplasm, where it

acts as a calcium-modulated protein, interacting with different targets in a calmodulin-like fashion. In particular, it could react with many enzymatic pathways, such as adenylyl cyclase, PLC, and PKC, known to be involved in many cellular processes (Klee and Vanaman, 1982).

ix. S100P This protein, initially found in placenta, has a molecular mass of about 10 kDa (Becker *et al.*, 1992). This placental protein of 95 amino acid residues shares about 50% sequence homology with brain S100 proteins alpha and beta (Becker *et al.*, 1992) and 38% homology with S100A6 (calcyclin) (Emoto *et al.*, 1992). It interacts with its target proteins following a calcium-dependent conformational modification involving hydrophobic residues of the C-terminal extension (Becker *et al.*, 1992). Although its presence was initially demonstrated in placenta (Becker *et al.*, 1992; Emoto *et al.*, 1992; Jin *et al.*, 2003), actually no specific role was assigned to this protein in placenta. Interestingly, it has been shown that the S100P protein is regulated by androgens and may play a role in the etiology of prostate cancer (Averboukh *et al.*, 1996).

x. The Calbindin D9k (CaBP-9k or CALB3) CaBP-9k is the best known and studied protein of the S100 family (Marenholz *et al.*, 2004). It was originally named intestinal calbindin, but was recently renamed CALB3 based on a new nomenclature (Marenholz *et al.*, 2004). Unlike other EF-hand proteins, this protein can be a monomeric protein of 78 amino acids with a molecular mass of 9 kDa, which has the capacity to bind two calcium ions by the protein molecule. This structural difference is also reflected in calbindin functions as calcium buffer versus the signaling activities frequently associated with the other S100 proteins (Marenholz *et al.*, 2004). The human CaBP-9k shows 88.6% homology with porcine and bovine CaBP-9k, followed by 78.5% homology with rat and 75.9% homology with mouse (Jeung *et al.*, 1992b). Until now, it was detected in a large variety of tissues, including intestine (Davie, 1981), alveolar epithelial cells of rat lung (Dupret *et al.*, 1992), pig (Jeung *et al.*, 1992a) and mouse (Li and Christakos, 1991) kidneys, bone cartilage (Balmain *et al.*, 1986), uterus (Delorme *et al.*, 1983; Warembourg *et al.*, 1987), and placenta (Muller *et al.*, 2000a). Functionally, CaBP-9k expressed in most tissues is regulated at both the transcriptional and posttranscriptional level by 1,25-$(OH)_2D_3$ (Darwish and DeLuca, 1992; Roche *et al.*, 1986; Song *et al.*, 2003; Wasserman and Fullmer, 1989). In many species, the induction of CaBP-9k coincides with exponential fetal growth and the maximum period of calcium accumulation in fetal bone. Results obtained in ewe show increasing mRNA levels in endometrium and trophoblast epithelium according to fetal needs for calcium (Morgan *et al.*, 1997). Moreover, Reiswig *et al.* (1995) showed that the expression of CaBP-9k was localized to the maternal caruncular epithelium, fetal chorionic epithelium, and trophoblastic binucleated cells of the bovine placenta. Within the maternal epithelium the intensity of staining increases from the second

trimester to term pregnancy, indicating a higher intracellular concentration of CaBP-9k in the epithelium at term. They hypothesized that this increase at the end of gestation is in response to the increasing demand for calcium to supply the mineralization of the fetal skeleton. Also, CaBP-9k may play a role in enhancing calcium transport across the placenta in cattle (Reiswig *et al.*, 1995). In human placenta, a low expression of this protein was observed in cytotrophoblast cells, while a higher concentration was found in syncytiotrophoblast (Belkacemi *et al.*, 2004), and active calcium transport across epithelia was correlated with CaBP-9k or CaBP-28k (Nikitenko *et al.*, 1998). However, the exact role of this protein in human placenta is still unknown, but it may act as a buffering protein. In rodent, it seems that mRNA expression of CaBP9k is under the control of estrogens and progesterone, but not under that of 1,25-$(OH)_2D_3$ (L'Horset *et al.*, 1994). Effectively, L'Horset *et al.* (1994) showed that the expression of CaBP9k is strictly regulated by 17β-estradiol in rat uterus and by progesterone in mouse uterus (An *et al.*, 2003; Cao *et al.*, 2002; Tatsumi *et al.*, 1999). Finally, Kovacs *et al.* (2002) showed that in the PTHrP gene-deleted or PTH/PTHrP receptor-null placentas in which placental calcium transfer is decreased, the expression of CaBP9k was downregulated in the intraplacental yolk sac (the mice homologue of human syncytiotrophoblast), showing the potential regulation of this CaBP by PTHrP.

b. The Calbindin D28k (CaBP-28k or CALB1) CaBP-28k was initially discovered by the Wasserman group at a high concentration in chicken intestine (Wasserman *et al.*, 1966). Since then, it has been found in many mammalian tissues such as kidneys (Wood *et al.*, 1988), brain (Wood *et al.*, 1988), pancreas (Christakos *et al.*, 1989), bone (Christakos *et al.*, 1989; Thomasset *et al.*, 1982), and placenta (Belkacemi *et al.*, 2003). It possesses four binding sites for calcium, contains six EF-hand domains, and is a member of the calmodulin superfamily (Kawasaki *et al.*, 1998). CaBP-28k shares a 58% homology with calretinin (a hexa EF-hand protein associated with the ER) (Berggard *et al.*, 2002); however, in spite of their size and sequence similarities, both are differentially localized in many organs and possess distinct functions. This protein was recently localized in human placenta by our research group (Belkacemi *et al.*, 2003). We demonstrated the presence of CaBP-28k in trophoblast cells, showing an increase of mRNA and protein level in the differentiation of cytotrophoblast cells into syncytiotrophoblast, which is correlated with the increase of calcium uptake in syncytiotrophoblast (Belkacemi *et al.*, 2003, 2004; Moreau *et al.*, 2002a,b). Our results are in accordance with increasing fetal calcium needs during the third trimester of pregnancy. Moreover, we showed for the first time that CaBP-28k is upregulated in the presence of 1,25-$(OH)2D_3$ (Belkacemi *et al.*, 2005). Consequently, it is possible that CaBP-28k participates in the

increasing buffering capacity of this cell type at the end of pregnancy and/or eventually participates in the stimulation of the mechanism(s) involving in syncytiotrophoblast calcium entry (Belkacemi *et al*., 2003). According to Husain and Mughal (1992), it could act as an intracellular transporter to facilitate calcium shuttling through the cytosol of trophoblast cells. Moreover, it may be directly involved in calcium storage into particular organs such as growing fetal bone. Moreover, studies conducted in mice pancreatic islets demonstrated that CaBP-28k acts as a calcium–buffering protein and as an important transitory intracellular calcium modulator (Sooy *et al*., 1999). Finally, both calbindins (CABP-9k and CaBP-28k) are rarely expressed in the same period, in the same tissue, except for mice kidneys (Li and Christakos, 1991) and human placenta (Belkacemi *et al*., 2003, 2004).

c. The Placental 57-kDa Calcium-Binding Protein (CaBP57k) The CaBP-57k is a high-molecular-mass CaBP of 57 kDa identified by the Tuan group. It is expressed in both human and rodent placentas, and is localized solely in trophoblast cells (Lin *et al*., 1997; Tuan, 1982, 1985; Tuan and Kirwin, 1988). This 417 amino acid protein shows significant sequence homology with calreticulin, an ER-associated calcium-binding protein (Hershberger and Tuan, 1998). This protein is expressed as a function of gestation, maximally early in the third trimester (Hershberger and Tuan, 1998). The upregulation of this CaBP during and/or following the differentiation of Rcho-1 cells, a trophoblast cell line derived from a rat choriocarcinoma, into trophoblastic giant cells emphasizes the importance of this protein in trophoblast maturation and transport capacity (Hershberger and Tuan, 1999). Moreover, in relation to trophoblast differentiation, calcium uptake capacity is higher in differentiated trophoblast cells compared to undifferentiated ones. These results are in accordance with the increasing expression level of this protein in pregnancy (Hershberger and Tuan, 1998, 1999). Moreover, the expression of CaBP-57k is upregulated by the mid-molecule fragment of PTHrP [PTHrP(67–84)] (Hershberger and Tuan, 1998). In addition, using immunohistochemistry detection, it was demonstrated that this protein is expressed in trophoblast giant cells and spongiotrophoblasts in latter stages of mice placental development (Tuan and Kirwin, 1988). Consequently, it is possible that this protein plays a cardinal role in placental calcium transport as a buffering protein and/or by favoring trophoblast differentiation (Hershberger and Tuan, 1999).

d. Human Calcium-Binding Protein (HCaBP) This protein has a molecular mass of about 150 kDa and is composed of dimers of 70 kDa. It is an acidic protein containing 27% aspartic and glutamic acids. Its specificity in

human placenta was evaluated by immunodiffusion by the Tuan group (Tuan, 1982), and its expression increased throughout pregnancy. Unfortunately, little is known about this protein in the human placenta.

e. The Oncomodulin Oncomodulin belongs to the protein family encompassing calmodulin, parvalbumin, and S-100 proteins (Baba *et al.*, 1984). Initially, the oncomodulin was found in human and rat placenta (MacManus, 1979) and in various tumors (MacManus *et al.*, 1985). In rat placenta it is composed of 750 nucleotides, and in tumors and placenta, a plot of mRNA versus protein revealed a direct relationship suggesting transcriptional control of oncomodulin abundance (Gillen *et al.*, 1988). Oncomodulin may represent an oncodevelopmental protein (Gillen *et al.*, 1987) since the only normal tissues expressing this protein are embryo tissues during implantation (MacManus *et al.*, 1990), placentas of different species, mainly in invasive cytotrophoblast cells (Brewer and MacManus, 1985, 1987), and tumors (Banville and Boie, 1989). The placental oncomodulin could play an important role in calcium-dependent cytotrophoblast cell motility, a vital phenomenon for trophoblast invasion into the uterine wall (Pauls *et al.*, 1996).

f. The Translationally Controlled Tumor Protein (TCTP) This protein is highly conserved and abundantly expressed in mammals and in a wide range of both animal and plant kingdoms (Bommer and Thiele, 2004). It was initially considered a tumor protein, but it is now clear that it is not an exclusive feature of cancer cells since it is found in several normal cells and tissues (Sanchez *et al.*, 1997). Many studies focus on its role as a histamine-releasing factor (MacDonald *et al.*, 1995), in cell growth (Bohm *et al.*, 1989; Thomas and Thomas, 1986), and as antiapoptotic factors (Li *et al.*, 2001a). In addition, different teams reported calcium-binding activity for this protein (Arcuri *et al.*, 2004; Rao *et al.*, 2002; Xu *et al.*, 1999), and Kim *et al.* (2000) reported the presence of a calcium-binding site for this protein. Recently, Arcuri *et al.* (2005) demonstrated the presence of TCTP in the first trimester and term placenta, specifically localized in villous trophoblasts, with the highest mRNA and protein expression levels reached at term. Moreover, downregulation of this protein using siRNA in HTR-8/SVneo cell line reduced cellular calcium uptake activity and binding capacity (Arcuri *et al.*, 2005).

Thus, in spite of the essential roles of these proteins in placental calcium regulation during pregnancy, many characteristics of these CaBPs remain unknown. Because fetal growth is dependent on maternal calcium supply, it is of the utmost importance to continue to elucidate all the physiological functions of these specialized proteins in calcium handling.

4. Plasma Membrane Calcium ATPase (PMCA)

The regulation of calcium homeostasis plays a key role in cellular functions. The fundamental part of this regulatory system is the ATP-driven calcium pump located in plasma membranes. The activity of this pump is modulated to a great extent by membrane fluidity, which is significantly linked to the lipid and protein environments of the cell membrane. In the human placenta, this enzyme responds to the specific needs of the fetus for calcium. Effectively, during the third trimester of pregnancy, the human placenta transfers about 140 mg/kg/day (Salle *et al.*, 1987), and PMCA seems to be the main protein involved in calcium extrusion from the syncytiotrophoblast.

PMCA is a high-affinity, low-capacity calcium pump present in most eukaryotic cells. Its main function is to remove excess calcium from the cell, and in a resting state, to maintain cytosolic calcium concentration at a nanomolar concentration, preventing cells from a lethal overload of calcium. It is a calmodulin-regulating enzyme. There are four genes in mammals, giving rise to four separate PMCA isoforms, 1 to 4, and due to alternative splicing, more than 20 variants are known. The mammalian PMCAs are encoded by a gene family composed of at least four nonallelic members. The main differences between them are in the N- and C-terminal sections (Stauffer *et al.*, 1993; Strehler and Zacharias, 2001), which are both located intracellularly. This enzyme is composed of 10 putative transmembrane domains and two major cytosolic loops representing the transduction and the catalytic domains, respectively, and an extended C-terminal tail containing the PDZ domain. The first intracellular loop region (between transmembrane domains 2 and 3) that corresponds to the ''transduction domain'' is embedded between a putative G-protein-binding sequence and the site of phospholipid sensitivity, and could differ according to the splice variants. This domain is supposed to be involved in the long-range transmission of conformational changes occurring during the transport cycle. In addition, this region contains the phospholipid-binding (PL) and the autoinhibitory domains. The other large cytosolic region is between transmembrane domains 4 and 5 and corresponds to the ''catalytic domain.'' It includes the ATP-binding site and the aspartate residue that forms the acyl phosphate intermediate during ATP hydrolysis and the calcium transport cycle. The C-terminal domain encompasses the most important regulatory domain of the PMCAs (Carafoli, 1991; Penniston and Enyedi, 1998; Strehler, 1991) and differs substantially. This C-terminal portion contains the phosphorylation sites that can be activated by protein kinase A (PKA) and PKC, and the calmodulin-binding sites (A and B) (Paszty *et al.*, 2002). The affinity of the PMCAs for calcium and calmodulin is affected by alternative splicing. The maximum velocity (V_{max}) of the activated enzyme is also modified by splicing (Elwess *et al.*, 1997; Enyedi *et al.*, 1994; Penniston and Enyedi, 1998;

Preiano *et al.*, 1996). In addition, calmodulin may act as a pseudosubunit for some of the PMCAs (Vorherr *et al.*, 1991), because the calcium–calmodulin affinity of some isoforms of the PMCA is in the low nanomolar range (Caride *et al.*, 1999; Elwess *et al.*, 1997; Hilfiker *et al.*, 1994), at least two to three orders of magnitude below calmodulin concentrations in tissues like the brain (Carafoli, 1987).

In a recent excellent review on the diversity of PMCA according to splice variants, Strehler and Zacharias (2001) suggest that the amino acid sequences of the four PMCAs include regions of variable or lower homology that are mostly associated with specific functions of each specific isoform. Thus each pump could be associated with a unique role in the specific cell or tissue in which it is expressed, and conserved regions between isoforms could be linked to the essential structure necessary to ensure basic functions such as catalytic activity and transport. They also stated that the differences among splice variants include differential protein–protein interactions with scaffolding and/or regulatory proteins (e.g., via differential binding to PDZ motifs), differential binding to and regulation by calcium–calmodulin, as well as differential regulation by kinases (Strehler and Zacharias, 2001). PMCA1 and PMCA4 are ubiquitous, suggesting that they are necessary for housekeeping functions, while PMCA2 and PMCA3 are mostly tissue specific, suggesting that they serve tissue-specific functions (Stauffer *et al.*, 1995). Moreover, Zylinska *et al.* (2002) suggest that the expression of PMCA2 and 3 may be involved in a developmental and differentiation process, and the regulation of PMCA isoforms may be crucial for cellular survival. Many PMCA knockout animal models were done; the majority are viable with minor to major defects, but PMCA1 knockouts cause embryonic lethality (Kozel *et al.*, 1998; Okunade *et al.*, 2004; Penheiter *et al.*, 2001).

The implications for this enzyme in calcium extrusion from syncytiotrophoblast membranes was demonstrated in 1991, using purified plasma membrane vesicles of human placenta. Lafond *et al.* (1991) demonstrated an ATP-dependent calcium transport in this membrane that necessitates the presence of magnesium. It was the first demonstration of the role of PMCA in the transplacental calcium movement from mother to fetus. Thereafter, Howard *et al.* (1992) demonstrated the presence of mRNA from both PMCA1 and PMCA4, the latter being the more abundant form, and similar expression was found in second trimester and term placentas. Strid and Powell (2000) obtained similar results demonstrating the presence of both PMCA1 and PMCA4 proteins in human term placenta, with no modulation of the expression between 32 and 42 weeks of pregnancy. However, the activity of these pumps increased linearly over the same gestational period, suggesting that this increase is critical in supplying the rapidly growing fetus with sufficient amounts of calcium for bone mineralization (Strid and Powell, 2000). This team also demonstrated that PMCA activity is increased in the

presence of IUGR and of insulin-dependent diabetes, without modification in the presence of gestational diabetes. However, these results contradict those obtained by Husain *et al.* (1994) and Hamilton *et al.* (2000) using experimental models of diabetes in the rat. In addition, using a reverse transcriptase-polymerase chain reaction (RT-PCR) technique, a sensitive method for mRNA detection, we demonstrated the presence of all isoforms of PMCA (1 to 4) mRNAs in BeWo cells (Moreau *et al.*, 2003a) and in human syncytiotrophoblast (Moreau *et al.*, 2003b). In human placenta, this enzyme is specifically localized at the BPM surface of the syncytiotrophoblast, allowing this calcium transporter to function properly in maternal-fetal calcium exchange (Fisher *et al.*, 1987). PMCA has been identified as a contributor to cell calcium extrusion from the placenta toward the human fetal circulation (Lafond *et al.*, 1991; Strid *et al.*, 2003). Moreover, placental PMCA and calcium transport are inhibited by the addition of erythrosin B (an inhibitor of PMCA) (Tuan and Bigioni, 1990; Tuan and Kushner, 1987), suggesting an important role for PMCA in calcium homeostasis. Furthermore, erythrosin B time- and dose-dependently increases calcium-cell-associated extrusion, suggesting an important role of PMCA in calcium extrusion from placental cells (Moreau *et al.*, 2002b). In addition, in rat placenta, using Northern blot hybridization, the gene expression of PMCA was increased two- to three-fold during the last period of gestation, while an increase of 72-fold was observed in the calcium transport process, combined with a 135-fold increase in placental CaBP-9k mRNA expression during this period (Glazier *et al.*, 1992). It was suggested that the expression of placental CaBP-9k, but not PMCA, may be rate limiting in placental calcium transport in the rat.

In addition, PMCA activity is greatly modulated by PTHrP, a hormone that is produced almost throughout pregnancy (Senior *et al.*, 1991) and is known to participate in transplacental calcium movement and in the maintenance of the maternal-fetal calcium gradient (Kovacs, 1995; Kovacs *et al.*, 1996). Strid *et al.* (2002) showed that PTHrP(38–94) stimulated ATP-dependent calcium transport (PMCA dependent) at a physiological range (5 pg/ml) and the effect (10–38% increase) was concentration–dependent, while PTHrP(1–34) stimulated only at a supraphysiological concentration. Moreover, it was demonstrated that this increase of calcium uptake was associated with placental PKC activity. Thus, taken together, all these data strongly support the important role of PMCA in placental calcium extrusion and its regulation by PTHrP (mid-molecule).

5. Na^+/Ca^{2+}-Exchanger (NCX)

A second system involved in intracellular calcium extrusion is the NCX. In mammals, NCX is a translocating molecule having a low affinity and a high capacity for calcium, and is assumed to play an important role, in parallel

with the PMCA, in the maintenance of intracellular calcium homeostasis (Blaustein and Lederer, 1999; Philipson and Nicoll, 1993). This structure is responsible for the countertransport of three sodium ions for one calcium ion across the plasma membrane of a variety of cells. Its primary role is to extrude calcium from cells, but in some cases this exchanger could favor calcium influx (Philipson, 1996). The exchanger thereby always tends to maintain an adequate cytoplasmic calcium level, which depends primarily on membrane potential and the sodium gradient. Mammal NCXs are encoded by three genes, NCX1 (Nicoll *et al.*, 1990; Shieh *et al.*, 1992), NCX2 (Li *et al.*, 1994), and NCX3 (Nicoll *et al.*, 1996). These isoforms share about 70% amino acid homology and have similar topology, consisting of 11 putative transmembrane domains and a large central hydrophilic loop between transmembrane domains 5 and 6, also called loop *f* (Nicoll *et al.*, 1990). This loop is supposed to contain the calcium-binding regulatory domain (Matsuoka *et al.*, 1993). In addition, the gene coding for NCX1 is characterized by six exons (A to F) coding for a variable region in the C-terminus section of this large loop. Alternative splicing of these exons generates multiple tissue-specific variants of NCX1 and NCX3 (Quednau *et al.*, 1997; Nakasaki *et al.*, 1993; Schulze *et al.*, 1996). Compared to NCX1, a deletion of 37 amino acids in the intracellular loop is observed in NCX2 and NCX3 (Quednau *et al.*, 1997), and NCX1 possesses two internal repeats called the α and β repeats. Both α-1 and α-2 motifs are between transmembrane domains 2 and 3 and 8 and 9, respectively. These α repeats are involved in the ion-binding/translocation reactions of the exchanger (Nicoll *et al.*, 1996), whereas the β repeats are located in loop *f* and are mostly involved in the calcium-binding capacity of NCX. The binding site is specifically located in the β-1 repeat and resides between β-1 and β-2, does not contain an EF-hand motif, and binds calcium in a cooperative manner (Levitsky *et al.*, 1994; Matsuoka *et al.*, 1993, 1995). Thus the calcium-binding site is distinctly localized from the transport site (Levitsky *et al.*, 1994; Matsuoka *et al.*, 1993, 1995). NCX1 is highly expressed in cardiac tissues and widely distributed in many other tissues, whereas NCX2 and NCX3 are less extensively distributed and mainly found in brain and skeletal muscle (Li *et al.*, 1994; Nicoll *et al.*, 1990, 1996). All NCXs are regulated by intracellular calcium, and no significant difference in their properties is observed. The only difference resides in their respective response induced by depletion of cellular ATP. NCX1 and NCX2 activity was inhibited, while the activity of NCX3 was unaffected. In addition, exchange activities of NCX1 and NCX3 were modestly increased by agents that activate PKA and PKC (Linck *et al.*, 1998). Interestingly, in a recent study, Iwamoto and Shigekawa (1998) showed that NCX3 is 10-fold less sensitive to inhibition by nickel or cobalt, but 3-fold more sensitive to inhibition by the isothiourea derivative KB-R7943 (a reverse mode inhibitor) than NCX1 and NCX2. In addition,

the presence of nickel accelerates sodium-dependent calcium uptake in NCX3, but not in NCX1 or NCX2. It was suggested that some differences exist in the kinetic and pharmacological properties in NCX3 compared to NCX1 and NCX2. Other physiological regulators of NCX include the signaling lipid phosphatidylinositol-4,5-bisphosphate (PIP_2), free radicals, pH, and temperature as well as protein kinases with stimulation of exchange activity by agents acting via PKC (Blaustein and Lederer, 1999). However, proof is still lacking for the physiological significance of these messengers as regulators of NCX *in vivo*. Interestingly, NCX1 is activated by PTHrP in osteoblasts exclusively via a cAMP-dependent pathway (Azarani *et al.*, 1995). In rabbit kidney, TRPV5 is completely colocalized with CaBP-28k, NCX, and PMCA (Hoenderop *et al.*, 2000).

Knockout models were generated to study the function of this exchanger, specifically the NCX1, since few specific inhibitors were available. Homozygous $NCX1^{-/-}$ mice are embryonic lethal at around 9–11 days postconception (Cho *et al.*, 2000; Koushik *et al.*, 2001; Reuter *et al.*, 2002; Wakimoto *et al.*, 2000). At 9 days postconception, homozygous null embryos are of normal size, but at 10 days postconception they are severely retarded in size, although they are still developing despite the lack of a heartbeat, indicating that diffusion of growth factors/nutrients is not sufficient to maintain normal growth of the embryo at later developmental stages. Koushik *et al.* (2001) reported that lethality is most likely due to a complete lack of vascular morphogenesis within the $NCX1^{-/-}$ yolk sac of placenta. Cho *et al.* (2003) confirmed these results, since even using a knockout model expressing the canine NCX1 in a cardiac-specific manner, embryos survived with hearts beating until 10½ days postconception and died thereafter. They also observed that embryos died due to a lack of an organized vasculature in the yolk sacs and that the placental labyrinth layer is reduced in size and is largely avascular. Thus they suggested that defects in the extraembryonic compartments are causal to lethality (Cho *et al.*, 2003).

In addition, the presence and the activity of this exchanger were also studied in human placenta based on its important role in intracellular calcium homeostasis (Cho *et al.*, 2003; Kofuji *et al.*, 1992; Lafond *et al.*, 1991; Moreau *et al.*, 2003a,b; Williams *et al.*, 1991). In placenta, Kofuji *et al.* (1992) demonstrated by Northern blot analysis a major transcript of 7.2 kilobases. Thereafter, Kamath and Smith (1994) showed its localization specifically in BPM of syncytiotrophoblast of human term placenta. However, its role in transplacental calcium exchange is still subject to debate. Using human term perfused placental lobules, Williams *et al.* (1991) suggested that NCX does not contribute significantly to the transplacental movement of calcium from mother to fetus, but is more probably associated with PMCA. In addition, using syncytiotrophoblast purified BPM vesicles from human term placenta, Lafond *et al.* (1991) obtained similar results since they were unable to

demonstrate the role of NCX in the calcium movement through these purified membranes (BPM). More recently, mRNAs of two cloned human NCX isoforms (NCX1 and NCX3) were revealed in syncytiotrophoblast of human placenta. More specifically, both splice variants NCX1.3 and NCX1.4 were amplified. However, calcium flux studies in sodium-free incubation medium also indicated that NCX played a minimal role in cell calcium fluxes (Moreau *et al.*, 2003b). Similar results were obtained in BeWo cells (Moreau *et al.*, 2003a). Thus all this information tends to confirm that NCX could be mostly involved in the establishment of placental vascularization instead of transplacental calcium movement.

IV. Factors Associated With Decreased Calcium Accretion During Pregnancy

Some factors are known to influence *in utero* mineral accretion. Seasons could influence fetal bone metabolism through modification of 1,25-$(OH)_2D_3$ metabolism (Namgung and Tsang, 2003; Namgung *et al.*, 1992, 1993, 1994, 1996, 1998; Weiler *et al.*, 2005). In Northern countries it is thought that vitamin D levels are decreased in autumn and winter due to reduced sunny periods (Webb *et al.*, 1988). A severe deficiency in vitamin D could impair the growing skeleton by causing reduced mineralization of bone tissue (leading to osteomalacia) and of the growth plate (which manifests as rickets). In addition, profound 1,25-$(OH)_2D_3$ deficiency during pregnancy can result in neonatal rickets (Russell and Hill, 1974). Also, recently, following a longitudinal study in the United Kingdom, a research team suggested that maternal lifestyle, fat stores, and seasonality of early pregnancy could influence maternal skeletal status (Javaid *et al.*, 2005). Their findings support a role for vitamin D supplementation of women pregnant during winter, especially those with low calcium intake who are pregnant for the first time. Also, in a study conducted in Canada, Weiler *et al.* (2005) demonstrated that 46% of the mothers and 18% of the newborns suffered 25-hydroxyvitamin D_3 deficiency. These deficient infants were larger and longer at birth, but they had a lower bone mass relative to their body weight, suggesting that vitamin D levels during pregnancy could play an important role in fetal calcium accretion. Thus, adequate 1,25-$(OH)_2D_3$ levels during pregnancy seem to be crucial to provide the calcium needed for fetal bone mineral accretion that mainly occurs in the last trimester.

Interestingly, Kovacs *et al.* (2005) obtained very interesting results that are in conflict with those obtained in humans. Using a vitamin D receptor (VDR) gene knockout mice model, they studied the effects of maternal and fetal absence of VDR in fetal-placental calcium transfer and fetal mineral

homeostasis, and their results demonstrated that VDR is not required by fetal mice to regulate placental calcium transfer, circulating mineral levels of calcium or magnesium, and skeletal mineralization. In addition, the absence of maternal VDR resulted in effects on fetal growth that seem to be mostly related to maternal calcium intake, but did not specifically affect fetal mineral homeostasis (Kovacs *et al.*, 2005). Thus, these results in mice contradict those obtained in humans and demonstrated that the role of 1,25-$(OH)_2D_3$ during human pregnancy is still unclear and remains elusive.

Another factor that could be related to abnormal fetal calcium metabolism is PE. PE is a disorder characterized by maternal hypertension, proteinuria, and edema, as well as fetal growth retardation. Although the etiological details are still being debated, a consensus exists that its initial stage includes deficient placentation in the first half of pregnancy. Despite these possible mechanisms, the pathogenesis of PE is still the subject of much debate. However, in this hypertensive disorder during pregnancy, metabolic abnormalities regarding calcium metabolism were observed, including a decrease in serum 1,25-$(OH)_2D_3$ concentration by about 25% (in maternal serum and cord blood) (Saudan *et al.*, 1998), an increase in serum PTH concentration by 50% (Seely *et al.*, 1992), and a decrease in urinary calcium excretion by about 50% (Seely *et al.*, 1992). Hypocalciuria was reported in women with PE in some studies (Saudan *et al.*, 1998; Seely *et al.*, 1992) but not in others (Masse *et al.*, 1993). Epidemiological studies also showed potential effects of dietary calcium intake, and several clinical studies have demonstrated associated alterations in maternal calcium metabolism (Ohara, 2001; Ritchie and King, 2000). Moreover, maternal hormone levels involved in calcium homeostasis are significantly different in PE compared to healthy pregnancies. Decreased circulating 1,25-$(OH)_2D_3$ concentrations were reported in several small (less than 20) clinical studies. Also, in pregnancy-induced hypertension (with and without proteinuria), 1,25-$(OH)_2D_3$ levels did not increase during pregnancy and were comparable to those in nonpregnant women (Frolich *et al.*, 1992; Seely *et al.*, 1992). Moreover, maternal low 1,25-$(OH)_2D_3$ levels were more pronounced in PE than in pregnant women with chronic hypertension or healthy pregnant women (August *et al.*, 1992). In this small study, PTH was slightly increased, serum ionized calcium remained unchanged, and urinary calcium was abnormally low in PE pregnancies in comparison to other conditions. It was recently suggested that decreased production of 1,25-$(OH)_2D_3$ by syncytiotrophoblast could contribute to the alteration in maternal calcium metabolism observed in PE (Diaz *et al.*, 2002). These data are consistent with recent evidence about the role of 1,25-$(OH)_2D_3$ in the direct regulation of the renin-angiotensin system (Li *et al.*, 2004), suggesting its role as an antihypertensive agent. During normal pregnancies, the renin-angiotensin system is activated, with increased levels of plasma renin and angiotensin II, whereas maternal blood pressure is maintained normally.

In addition, maternal alterations in calcium metabolism could be related to an inadequate production of PTHrP in PE (Halhali *et al.*, 2001), an important hormone produced by the fetal-placental unit. Moreover, oxidative stress observed in PE induces an important reduction of the PMCA of the syncytiotrophoblast, the enzyme responsible for calcium extrusion from this cell type (Carrera *et al.*, 2003; Casart *et al.*, 2001). Thus, the alteration of maternal calcium metabolism could result in fetal undernourishment, causing impaired fetal development. In addition, PE is strongly associated with IUGR and postnatal neurodevelopmental disturbances (Many *et al.*, 2003). Therefore, it is possible that transplacental calcium movement may be one of the mechanisms responsible for developmental disturbances observed in children from PE pregnancies.

Infants from diabetic mothers also occasionally present with hypocalcemia at birth. It seems that bone resorption is increased in a fetus from an insulin-dependent diabetic mother (Demarini *et al.*, 1995). The infant bone mass content is inversely correlated with poor control of maternal glycemia, specifically during the first trimester of pregnancy (Mimouni *et al.*, 1988). Using experimental models of diabetes in the rat, Husain *et al.* (1994) studied the placental unidirectional maternofetal flux of calcium and magnesium in diabetic rats. They observed that fetal calcium and magnesium accretion is reduced in the presence of untreated maternal diabetes mellitus, conjointly with a reduction of the expression of placental CaBP-9k by about 11–12 times. They suggested that untreated maternal diabetes mellitus could affect the expression of placental transport components involved in the maternal-fetal transfer of these cations. Concerning the gene expression of placental CaBP-9k in diabetic rats, similar results were observed by Hamilton *et al.* (2000), but interestingly, contrary to other studies, they suggested that PTHrP and estradiol may be involved in the regulation of the placental expression of CaBP-9k, instead of 1,25-$(OH)_2D_3$. Many other factors, such as maternal smoking (Colak *et al.*, 2002; Jones *et al.*, 1999; Seller and Bnait, 1995), alcohol consumption (Keiver *et al.*, 1996, 1997), and caffeine use (Nakamoto *et al.*, 1989), could impair fetal bone development.

V. Concluding Remarks

Calcium is, among all ions, one of great importance since it is known to be implicated in many physiological processes. This chapter is an overview of most placental proteins involved in calcium homeostasis during pregnancy. Many hormones and growth factors are involved in placental and fetal development. The human syncytiotrophoblast, the specialized cell in the placenta involved in calcium regulation and transport, is also greatly influenced by these factors

in the uteroplacental environment. Its exceptional polarized structure allows it to be differentially regulated by maternal or fetal stimuli, contributing to adequate fetal growth and development. However, the complex calcium movement in the syncytiotrophoblast necessitates many structures that specifically have a functional role in this process. It is hypothesized that active transcellular calcium transport proceeds through a well-controlled sequence of events consisting of apical calcium entry via TRPV5 and TRPV6, present only in BBM, its cytosolic diffusion through its binding to CaBPs, and its basolateral extrusion mainly through PMCA, and to a lesser extent by NCX. In addition, it was demonstrated that transplacental calcium movement is under hormonal regulation, and since 1991, we and others have demonstrated that the syncytiotrophoblast possesses all the above cited elements involved in calcium transport and a variety of signaling pathways needed to

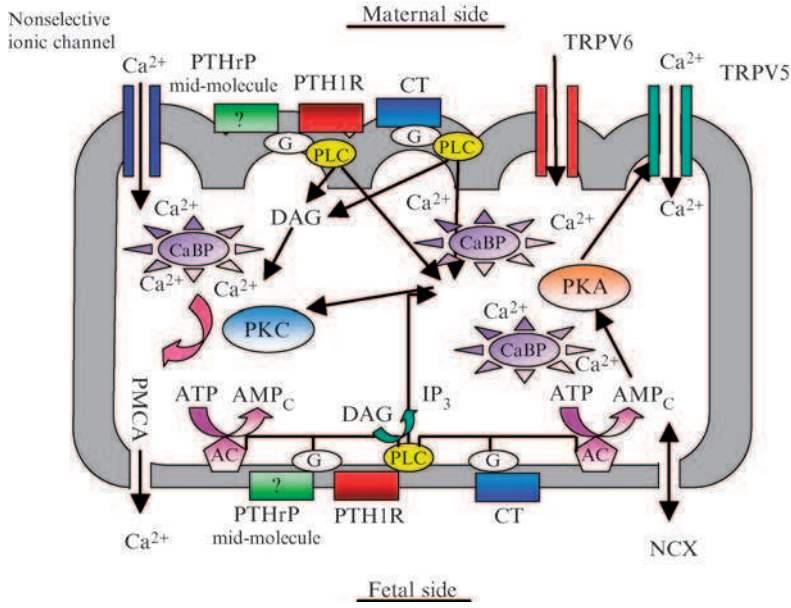

FIG. 1 Schematic representation of a syncytiotrophoblast of human placenta and its associated proteins implicated in cellular calcium homeostasis. AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; ATP, adenosine triphosphate; Ca^{2+}, calcium; CaBP, calcium-binding proteins; CT, calcitonin receptor; DAG, diacylglycerol; G, G protein; IP_3, inositol-1,4,5-triphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PTH1R, parathyroid hormone/parathyroid hormone-related peptide receptor; PTHrP mid-molecule, putative receptor that binds the mid-molecular fragment of PTHrP; TRPV, transient receptor potential, vanilloid family.

respond to the hormonal regulation of transplacental calcium movement (Fig. 1). However, specific events are still undetermined. Thus, many studies are still necessary to understand all the complex mechanisms found in this specialized unit in terms of calcium movement.

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New Insights into the Cell Biology of the Marginal Zone of the Spleen

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In the marginal zone of the spleen the bloodstream passes through an open system of reticular cells and fibers in which various myeloid and lymphoid cells are located. Macrophages in this region are well equipped to recognize pathogens and filter the blood by virtue of unique combinations of pattern recognition receptors. They interact with a specific set of B cells that can be found only in the marginal zone and that are able to react rapidly to bacterial antigens in particular. This combination of strategically located cells is an important factor in our defense against blood-borne pathogens. New data on the development of the marginal zone itself and the marginal zone B cells are reviewed and discussed in light of the function of the spleen in host defense.

KEY WORDS: Macrophages, B cells, SIGNR1, Lymphotoxin, TNF-α, Notch signaling, Embryonic development.

I. Introduction

The marginal zone of the spleen is strategically positioned between the lymphoid compartment of the spleen, the white pulp, and the more innate, scavenging red pulp compartment. Most of the arterial blood that enters the spleen runs through the marginal zone where sets of cells, such as macrophages expressing unique combinations of pattern recognition receptors as well as marginal zone B cells that can be readily activated, are located to intercept and react to pathogens in the blood.

Although the important function of the spleen has been appreciated for a long time, the anatomical basis for its function has only gradually emerged. Less than 25 years ago Bishop and Lansing (1982) started an overview of

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normal and pathological anatomy of the human spleen stating that the spleen is "an organ of unique anatomic and functional importance, and yet the morphologic correlates of its various functions remain somewhat mysterious." Our own review, which appeared a decade later (Kraal, 1992), was probably the first that focused on the marginal zone only and described the constituents and function of this area as they were known at that time. It is striking how much data and new insights have been generated since then, and from how many different angles of interest the spleen and the marginal zone have been studied. Especially from the field of developmental immunology, but also from that of studies on pattern recognition receptors and B cell immunology, many exciting new data on the function of the spleen and the interactions between the various cells and molecules have been generated and have helped to solve at least some of the mysteries. Here we will describe these findings correlated with the function of the spleen, and the intriguing marginal zone in particular.

The spleen is composed of a branching splenic artery that eventually ends in venous sinuses. The arterial branches, called central arterioles, are surrounded by a layer of lymphoid tissue as a continuous sheath, the white pulp. It consists of T cell areas and B cell follicles, more or less resembling the organization found in lymph nodes, except for the apparent absence of high endothelial venules. Although smaller branches of the central arteriole run through the white pulp, most of the arterial blood (Schmidt *et al*., 1993) ends in a sinusoid system in the area surrounding the T and B cell zones, thereby forming an anatomical border between the white and red pulp, the marginal zone (Kraal, 1992). It is here that the blood opens up in sinusoid spaces formed by lining cells continuous with the endothelium of the arterioles. Especially in rodents, in which the spleen has been investigated most thoroughly, the marginal zone is a conspicuous area consisting of a network of sinus lining cells and reticular fibroblasts through which the blood freely percolates on its way to the red pulp. Macrophage populations and marginal zone B cells are firmly attached to this network, thus allowing a continuous scan of the blood for pathogens and debris. In humans the marginal zone can be divided into an inner and outer area with a small rim of T cell zone in between and surrounded by a more diffuse perifollicular zone where macrophages can be found that surround endings of blood vessels (Mebius and Kraal, 2005; Steiniger *et al*., 2001). The blood runs from the marginal zone through the red pulp cords into the venous sinuses, whereby the structure of the venous cords and sinuses, forming the spleen's red pulp, leads to a functional slow bloodstream, enabling the spleen to exert its function as a filter of the blood. The passage from the cords into the sinuses is an important step for the selection of effete red blood cells and their removal by the macrophages of the red pulp. Interestingly, the splenic vein is connected to the hepatic portal vein, so that all the blood leaving the spleen is then filtered by the portal system of the liver.

II. Cells of the Marginal Zone

Within the reticular framework of the marginal zone, resident cell types can be found that form more or less permanent populations that are not present in other lymphoid organs (Fig. 1). These include the marginal zone macrophages, the marginal metallophilic macrophages, and the marginal zone B cells. In addition, many cell types present in the blood are located in the marginal zone as passing cells, including lymphocytes and granulocytes. In addition, a rather large number of dendritic cells can be found here. They are thought to reside temporarily in the marginal zone and to migrate into the white pulp after stimulation and antigen uptake. Also, lymphocytes may dwell here for longer periods during the process of transmigration into the white pulp. The characteristics of the resident cells will first be described, after which an attempt will be made to correlate the findings on the various cells in this area to the function of the marginal zone in innate and adaptive immunity.

A. Macrophages

Armed with a unique set of pattern recognition receptors, the marginal zone macrophages occupy their strategic position in the marginal zone. In fact, it is impressive to observe the efficiency and speed by which these cells can clear the blood from an experimentally administrated antigen for which they have the appropriate receptors, such as FITC-Dextran. Similar to tissue macrophages, they display receptors involved in recognition and phagocytosis of opsonized particles, such as Fc and complement receptors, but their outstanding capacity for binding and uptake of nonopsonized particles is based upon the expression of surface receptors such as the C-type lectin SIGNR1 (Geijtenbeek *et al.*, 2002a; Kang *et al.*, 2003, 2004), and MARCO, a type I scavenger receptor (Elomaa *et al.*, 1995; van der Laan *et al.*, 1999) (Fig. 1A and B).

1. Structure and Function of SIGNR1

That marginal zone macrophages are capable of recognizing polysaccharide antigens and play a role in T cell-independent antibody responses has been well established in earlier studies (Amlot and Hayes, 1985; Amlot *et al.*, 1985; Humphrey and Grennan, 1981; Kraal *et al.*, 1989b). With the discovery of DC-SIGN as a human dendritic cell-specific C-type lectin (van Kooyk and Geijtenbeek, 2003) and subsequent isolation of murine homologues, the SIGNR1 molecule was identified as an important C-type lectin present on

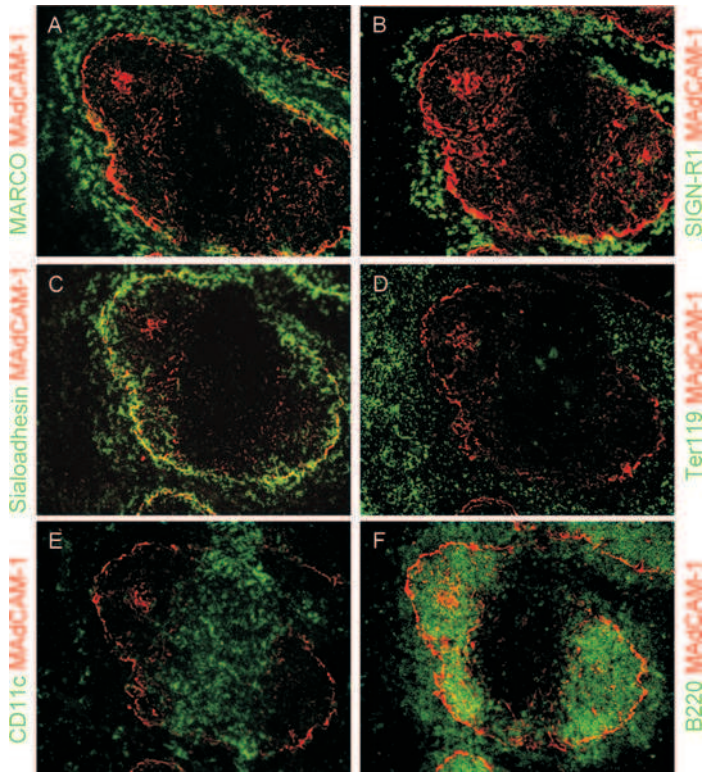

FIG. 1 The cellular constituents of the marginal zone. Serial sections of a mouse spleen have been stained with various monoclonal antibodies to reveal the position of the different cell populations in the marginal zone. In all sections the expression of MAdCAM-1 (red) on the cells of the marginal sinus is used to clearly indicate the demarcation between white pulp and marginal zone. In addition, MAdCAM-1 stains cells, probably follicular dendritic cells in the B cell follicles. In (A) and (B) the marginal zone macrophage population is clearly visible based on its expression of MARCO [(A) green] and SIGNR1 [(B) green]. In (C) the compact ring of sialoadhesin-positive marginal metallophilic macrophages can be seen. In (D) the staining for Ter119 (green) on erythrocyte membranes demonstrates the strict separation of freely passing blood in the marginal zone and the red pulp and the absence of erythrocytes in the white pulp, clearly illustrating the selective barrier that exists for leukocytes to enter the white pulp from the marginal zone. In (E) the localization of dendritic cells, based on the expression of CD11c, in the T cell zone of the white pulp, in a bridging channel [visible in the top of (E)], and in the marginal zone is seen. Finally, in (F) the presence of B cells, most of them belonging to the distinct population of marginal zone B cells, can be seen outside the MAdCAM-1-positive sinus. In addition, within the white pulp two B cell follicles (green), separated by the T cell zone, can be seen. Magnification: $\times 10$.

marginal zone macrophages and responsible for recognition of carbohydrate antigens (Geijtenbeek *et al*., 2002a; Park *et al*., 2001). The C-type lectin domain of mouse SIGNR1 has 74% similarity with that of human DC-SIGN, and the amino acid residues important for both Ca^{2+} and ligand binding are conserved in the murine homologue (Geijtenbeek *et al*., 2002b). The ligand-binding specificities of SIGNR1 are therefore quite similar to the ones described for DC-SIGN, based on the presence of the EPN sequence motif in the carbohydrate recognizing domain (CRD). The EPN motif determines their affinity, in particular to mannose-containing carbohydrates, but additional amino acids in the CRD will determine the specific structures that are recognized (Appelmelk *et al*., 2003; Feinberg *et al*., 2001; Galustian *et al*., 2004), and many of the pathogens that are recognized by DC-SIGN can also interact with SIGNR1 (see Table I). Detailed analysis of the carbohydrate specificity of SIGNR1 showed interaction with mannose-, fucose-, and *N*-acetylglucosamine-terminating oligosaccharides, as demonstrated by their ability to react with high mannose *N*-glycans and proteins containing *N*-linked glycans such as invertase and soybean agglutinin. That SIGNR1 also reacts with fucose was inferred from binding studies with Lewis antigens (Galustian *et al*., 2004).

The SIGNR1 molecule is sufficient for both binding and internalization of ligands, after which the complex is localized in lysosomes, as shown by

TABLE I
Overview of Pathogens and Molecules that Bind to SIGNR1[a]

	Pathogen or molecule	References
	ICAM-2	(Geijtenbeek *et al.*, 2002a)
Yeast	Zymosan	(Takahara *et al.*, 2004; Taylor *et al.*, 2004)
	Candida albicans	(Nagaoka *et al.*, 2005)
Bacteria	*Mycobacterium tuberculosis* (ManLAM)	(Koppel *et al.*, 2004)
	Streptococcus pneumoniae	(Lanoue *et al.*, 2004)
	Escherichia coli	(Nagaoka *et al.*, 2005)
	Salmonella typhimurium	(Nagaoka *et al.*, 2005)
Virus	Coronavirus (S protein)	(Marzi *et al.*, 2004)
	Marburg virus	(Marzi *et al.*, 2004)
	Ebola virus (glycoprotein)	(Marzi *et al.*, 2004)
	HIV (gp120)	(Geijtenbeek *et al.*, 2002a)

[a]In parentheses is the ligand or subcellular part of the pathogen recognized by SIGNR1.

transfected cells (Geijtenbeek *et al.*, 2002a). The cytoplasmic region of the murine SIGNR1 lacks a dileucine motif that is responsible for the internalization of human DC-SIGN, but it contains a triacid cluster (DDD) in its cytoplasmic region (Koppel *et al.*, 2004; Park *et al.*, 2001). This motif functions as an internalization motif in DEC-205, an endocytic receptor on dendritic cells, where it is responsible for targeting to lysosomes for antigen processing (Mahnke *et al.*, 2000). It is therefore assumed that the lysosomal targeting of internalized ligand–SIGNR1 complexes is due to this triacidic cluster. Indications that the SIGNR1 receptor can also interact with other receptors begin to emerge. Cooperation with Dectin-1, the β-glucan receptor, leads to efficient uptake of zymosan and production of tumor necrosis factor (TNF)-α (Taylor *et al.*, 2004), although this is probably not as relevant for the situation in the marginal zone where dectin-1 is hardly expressed (Reid *et al.*, 2004). For marginal zone macrophages the observed interaction of SIGNR1 with the Toll-like receptor (TLR) 4 seems to be more important (Nagaoka *et al.*, 2005). In studies on the ligand specificity of SIGNR1 it was observed that lipopolysaccharide (LPS) from *Escherichia coli* bound to SIGNR1 via oligosaccharides in the nonreductive end of the LPS core region. In transfectant cells with both SIGNR1 and TLR4, LPS binding to SIGNR1 led to oligomerization of TLR4 and degradation of IκB-α and it was found that SIGNR1 associated with the TLR4−MD2 complex leading to cytokine production (Nagaoka *et al.*, 2005). It is obvious that this type of interaction greatly enhances the efficiency by which the marginal zone macrophages can eliminate pathogens.

In addition to interactions with apparent pathogens, SIGNR1 also interacts with murine ICAM-2, which is widely expressed on lymphocytes, and could therefore function as the leukocyte ligand mediating contact between SIGNR1$^+$ marginal zone macrophages and leukocytes in the marginal zone (Geijtenbeek *et al.*, 2002a). Close contacts between B cells and the marginal zone macrophages have been described, which suggests that the marginal zone macrophages may play a role in the migration and retention of B cells in the marginal zone.

In addition, as described in B cell-deficient mice and in mice where B cells were induced to disappear, B cells are essential for the early differentiation of the macrophage populations in the marginal zone but also for their maintenance during adult life (Crowley *et al.*, 1999; Karlsson *et al.*, 2003; Nolte *et al.*, 2004).

2. Role of MARCO on Marginal Zone Macrophages

As for SIGNR1, the expression of the Macrophage Associated Receptor with COllagenous structure (MARCO) is not restricted to marginal zone macrophages, but it is the combination of these receptors that makes

these cells special. Furthermore, the expression of MARCO seems to be constitutive on marginal zone macrophages, whereas it can be rapidly induced on many other macrophage populations, such as alveolar macrophages and macrophages in the liver (Arredouani *et al.*, 2004; Elomaa *et al.*, 1995; van der Laan *et al.*, 1999). This enables the body to initiate a swift and functional upregulation of pattern recognition receptors under conditions of heavy pathogen load. For MARCO a broad range of pathogenic ligands has been described, whereby MARCO and SIGNR1 are often complementary in their specificity. Bacteria such as *Escherichia coli* and *Staphylococcus aureus* readily bind to MARCO, whereas yeast cells (zymosan) do not (Elomaa *et al.*, 1995). Ficoll, which is avidly captured by the marginal zone macrophages, does not bind to MARCO, whereas acetylated low-density lipoprotein (LDL) does (Kraal *et al.*, 2000). When MARCO is expressed in other cell types *in vitro* it induces changes in cell shape, and profound changes in cytoskeleton rearrangements, which may be related to the function of the molecule in the cellular changes needed to accommodate phagocytosis and engulfment of larger particles (Granucci *et al.*, 2003; Pikkarainen *et al.*, 1999).

MARCO belongs to the group of class A scavenger receptors (SR-A) that was originally defined as binding to LDLs and was studied extensively in relation to the development of artherosclerosis (Suzuki *et al.*, 1997). Its structure resembles the SR-A1 molecule, consisting of a trimer of disulfide-bonded proteins with a collagenous structure. MARCO has short intracellular and transmembrane domains, as well as a large extracellular domain composed of a spacer domain, a long collagenous domain, and a C-terminal fifth domain, forming a scavenger receptor cysteine-rich domain (SRCR) (Elomaa *et al.*, 1995).

SRCRs are ancient and highly conserved protein modules of ~100–110 amino acids, of either soluble or membrane-bound receptors expressed by hematopoietic and nonhematopoietic cells (Resnick *et al.*, 1994; Sarrias *et al.*, 2004). Based on the numbers of cysteine residues two groups are distinguished, with group A containing six cysteine residues, encoded by two exons, and group B usually containing eight cysteines and encoded by a single exon. Group A members, to which SR-A1 and MARCO belong, are multidomain mosaic proteins with single SRCR domains associated with other functional domains, such as enzymatic (protease) domains or collagenous regions. Group B members are composed of tandem repeats of SRCR domains, thought to be involved in oligomerization but never associated with protease domains. Representatives of either group are found in different animal species, from low invertebrates (sponges) to high vertebrates (mammals), and it is thought that based on the high degree of structural and phylogenetic conservation of SRCR domains, they have basic functions in innate immune defense (Lehrer, 2004; Sarrias *et al.*, 2004).

Using human and mouse MARCO variants with deletions or single amino acid substitutions the bacteria-binding properties of MARCO were localized to the fifth SRCR domain, with an RXR motif as an essential element for high-affinity bacterial binding (Brannstrom *et al.*, 2002). Interestingly, for SR-A1 the ligand-binding function has been localized to the collagenous domain, and so far not to the SRCR domain (Acton *et al.*, 1993; Doi *et al.*, 1993).

B. Metallophilic Macrophages

In contrast to the position of the marginal zone macrophages that are located in a seemingly random way throughout the width of the marginal zone, the marginal metallophilic macrophages form a distinct line of cells at the border of the marginal zone at the white pulp side of the marginal sinus (Fig. 1A–C). As such their position closely resembles the rim of macrophages lying just underneath the subcapsular sinus of the lymph node (Nolte *et al.*, 2000). Both sites form a transitional area, where cells and molecules can enter the white pulp or the lymph node parenchyma, suggesting an important role for the marginal metallophilic macrophage cells in selection and scavenging.

Originally, before the development of specific antibodies, the cells at this site had been characterized as macrophages by the presence of acid phosphatase, but differed from other macrophage populations by their high content of nonspecific esterase (NSE) (Eikelenboom, 1978). A role for this enzyme in cleaving fatty acids as well as for the detoxification of LPS has been described, as well as a direct association of NSE and removal of apoptotic T cells by macrophages in the thymus (Feng *et al.*, 2002). This suggests that the marginal metallophilic macrophages are involved in scavenging pathogens and apoptotic cells based on the function of NSE in membrane destabilization. In line with this is the abundant expression of Siglec-1 (sialoadhesin) on these cells (Fig. 1C), a receptor associated with binding to oligosaccharide self ligands on many cells and extracellular matrix components (Crocker *et al.*, 1995, 1997). The family of siglecs (sialic acid-binding Ig-like lectins) can be divided into two subsets, the highly related and large group of CD33-related siglecs, and a second group composed of sialoadhesin, CD22, and myelin-associated glycoprotein (MAG). Although the latter group is more distantly related, it is represented by well-conserved orthologues in all mammalian species examined so far (Crocker, 2005). Except for MAG, all siglecs are expressed on cells of the immune system and consist of an N-terminal V-set Ig domain that mediates sialic acid binding, and a number of C2-set Ig-like domains. Furthermore, several of them contain immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tails, suggestive

of a role in immune regulation, but the precise role of the various siglecs, with the exception of CD22, is still not clear. Sialoadhesin, which in addition to its expression on marginal metallophilic macrophages is also found on macrophage subsets at inflammatory sites and in tumors, differs from other siglecs by the fact that it is constitutively active. Most others siglecs are thought to be masked through *cis* interactions with sialylated ligands on the same plasma membrane and have to be activated, e.g., by sialidases to become functional. That this is not the case for sialoadhesin could be due to the fact that with its 17 domains it stands out from the crowd of other molecules in the plasma membrane and is therefore less likely to interact in *cis* (Crocker *et al.*, 1994; Munday *et al.*, 1999).

The role of sialoadhesin in recognition of apoptotic cells was inferred from the expression of its ligands on many cells, in particular granulocytes, but remains circumstantial (Crocker *et al.*, 1995). Based on the position of marginal metallophilic macrophages at the entrance site of the marginal zone and white pulp, a role for sialoadhesin in lymphocyte migration has been suggested. Activation of lymphocytes leads to changes in the glycosylation patterns of molecules such as CD43, CD45, and PSGL-1, all of which are ligands for sialoadhesin based on expression of clustered sialic acids. Their interaction may facilitate the retention of lymphocytes in the transit zone and subsequent entrance into the white pulp (Crocker *et al.*, 1997). In fact, elimination of macrophages from the marginal zone with the use of toxic liposomes led to reduced entrance of lymphocytes into the white pulp, although it cannot be completely ruled out that this was related to general changes in the hemodynamics in the marginal zone (Kraal *et al.*, 1989a). Also for blood-borne tumor cells with altered glycosylation, binding and removal through sialoadhesin have been suggested (Nath *et al.*, 1999).

Although it was always assumed that the N-terminal V-set Ig domain of sialoadhesin was the sole ligand-binding site, it was recently found that sialoadhesin can also interact with ligands that bind to other parts of the molecule (Kumamoto *et al.*, 2004). In a study on the migration of dermal macrophages into lymph nodes it was found that these cells interacted with the sialoadhesin expressed on the subcapsular macrophages in the node through M galactose C-type lectins (MGL1). This interaction was independent of sialic acid but involved *N*-glycans on the stalk of the sialoadhesin molecule (Kumamoto *et al.*, 2004). In light of the aforementioned similarities between these subcapsular and the marginal metallophilic macrophages, similar interactions can be expected to occur in the marginal zone and it will be very interesting to see what cell types are involved.

Although sialoadhesin is not a phagocytic receptor, it may be involved in phagocytosis through interaction with other receptors. Uptake of pathogens for which recognition through sialic acid by sialoadhesin was crucial was reported for *Neisseria meningitides* (Jones *et al.*, 2003), and for arterivirus in

the case of porcine alveolar macrophages (Delputte and Nauwynck, 2004). Together it is clear that both the strategic position of the marginal metallophilic macrophages and the expression of a receptor with some striking characteristics point to essential functions of these cells at the interface of the marginal zone and white pulp. However, full appreciation of their role needs further investigation.

C. B Cells

Marginal zone B cells (MZ B cells) represent a distinct naive B cell lineage (Fig. 1F), different from mature follicular B cells and the B-1 cell lineage, predominantly nonrecirculating and specialized to respond rapidly to blood-borne pathogens (Gray *et al.*, 1982; Martin *et al.*, 2001; Oliver *et al.*, 1997). Upon encounter with bacteria they respond swiftly by differentiation toward plasma cells that produce IgM as well as acquiring the capacity to function as antigen-presenting cells (Martin *et al.*, 2001; Oliver *et al.*, 1999). MZ B cells are particularly well equipped to deliver a response to T-independent antigens. With their rapid response to bacterial antigen and their strategic location in the marginal zone, where blood flows into open sinuses between the red and white pulp, they deliver a first line of defense against blood-borne pathogens and therefore help to fill the gap between the fast but nonspecific innate immune response and the adaptive, T cell-dependent antibody response that needs considerable time to reach its peak (Lopes-Carvalho and Kearney, 2004). In addition, they deliver immune complexes to follicular dendritic cells by migrating into the B cell follicles (Gray *et al.*, 1984; Heinen *et al.*, 1986).

1. Phenotype and Lineage Commitment of Marginal Zone B Cells

MZ B cells have a unique phenotype by expression of high levels of IgM, CD21, and CD1d, and low levels of IgD, CD23, and B220, whereas follicular B cells are characterized by high levels of IgD and CD23, but intermediate to low levels of IgM, CD1d, and CD21 (Martin and Kearney, 2002). In addition, CD9 has been identified as a marker to delineate MZ B cells from follicular B cells (Martin and Kearney, 2002).

To what extent are MZ B cells different from follicular B cells and how is this determined? Together with the B-1 B cells, MZ B cells are encompassed in the group of innate lymphocytes, of which the antigen receptor repertoire is germ-line encoded with limited diversity (Bendelac *et al.*, 2001), and they are not part of a constantly migrating lymphocyte pool, but instead reside at certain anatomical sites. The B-1 B cells form an early lineage that already develops in neonatal life and shows a restricted repertoire of its V genes

biased to recognize T-independent antigens. B-1 B cells are mainly found in the peritoneal cavity and along mucosal surfaces (Bendelac *et al*., 2001). MZ B cells develop later in life and are also characterized by a restricted repertoire and a fixed position. It is now well established that in addition to negative selection of B cells with the B cell receptor (BCR) reacting strongly with self (Rolink and Melchers, 1996), B cells are also subjected to positive selection during their development based on interactions of their BCR, whereby the affinity of the receptor, accessory signaling molecules, and the nature of the antigen itself play a role (Lopes-Carvalho and Kearney, 2004). It is becoming clear that the signaling through the BCR is an important cell-fate decision step in the differentiation of MZ B cells (Pillai *et al*., 2005), whereby interaction with self-ligands plays an important role in their recruitment and selection (Dammers and Kroese, 2005; Wen *et al*., 2005). Positive selection, based on the very nature of the antigen, was very nicely demonstrated by Martin and Kearney, using mice transgenic for different Ig heavy chains. They showed clearly that the ability to become an MZ B cell, based on both phenotypic characteristics and localization, was dependent on the composition of the Ig molecule (Martin and Kearney, 2000). This means that the distinct subsets of B cells, B-1 cells, MZ B cells, and follicular B cells, not only occupy different domains of the immune system, but do so with different specificities, evolved to deal with antigens that are most likely encountered at their sites.

2. Precursor Relationships between MZ B Cells and Follicular B Cells

MZ B cells are quite long-living cells, compared to follicular B cells, as demonstrated in conditional knockout Rag-1 mice. Whereas in these mice, upon deletion of *Rag* follicular B cells gradually disappear, the numbers of MZ B cells are stable. This is suggestive of a self-renewing capacity, either through replication of MZ B cells themselves or from recirculating precursors (Hao and Rajewsky, 2001; Pillai *et al*., 2005).

Newly formed B cells, transitional type 1 (T1), emerge from the bone marrow and predominantly depend on BCR stimulation for their survival. These cells develop into T2 cells, which need additional survival signals such as BAFF (B cell-activating factor of the TNF family). These T2 cells will give rise to both follicular and marginal zone B cells, whereby intermediate precursor stages are discerned based on expression of various markers (Fig. 2). The existence of a separate $CD23^{+}$ $CD21/35^{high}$ precursor cell for MZ B cells was suggested (Loder *et al*., 1999). Cells with this phenotype are found only in spleen and not in bone marrow, blood, or lymph nodes, and are absent in mice that lack MZ B cells such as Aiolos null mice and $Notch2^{-/-}$ mice. Because of their absence in Aiolos null mice, they are

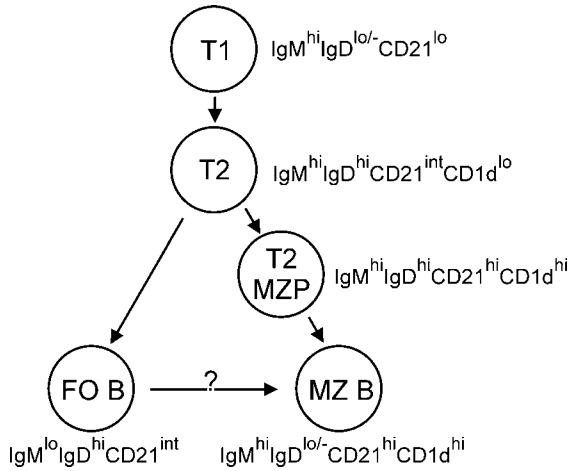

FIG. 2 Developmental pathways of marginal zone B cells and follicular B cells. Both B cell populations are derived from transitional type 2 (T2) B cells. The existence of a distinct marginal zone precursor B cell (T2 MZP) has been described but debated, as is the differentiation of marginal zone B cells from follicular B cells.

thought to be different from precursors for follicular B cells, since the development of the latter is normal in these mice (Cariappa *et al*., 2001; Loder *et al*., 1999; Saito *et al*., 2003). Other investigators support the idea that these $CD23^{+}$ $CD21/35^{high}$ cells are transitional cells that develop from mature follicular B cells (Allman *et al*., 2004; Srivastava *et al*., 2005) (Fig. 2). This is based on observations using lymphopenic mice in which follicular B cells were transferred and gave rise to marginal zone B cells.

Irrespective of the precise precursor relationship, it is clear that MZ B cells use differentiation pathways that are distinct from follicular B cells. In models proposed to explain the two pathways, a major difference emerges: the difference in signaling strength of the BCR needed to develop the two cell types. MZ B cell development requires weak BCR signaling, whereas intermediate signaling is required for follicular B cells. Important data to support this came from studies in Aiolos null mice. As a zinc-finger protein of the Ikaros family, Aiolos is mainly expressed in B cells, and functions to repress target genes. When Aiolos is absent, the strength of BCR signaling increases as seen by enhanced B cell proliferation upon BCR crosslinking, leading to expanded numbers of follicular B cells, but MZ B cells do not develop (Wang *et al*., 1998). This was related to the fact that Aiolos negatively influences Bruton's tyrosine kinase (Btk) activity (Cariappa *et al*., 2001). Btk is a member of the Tec family of protein tyrosine kinases (PTKs) and plays a vital role in many cellular processes. Mutations in the Btk gene cause

the primary immunodeficiency disease X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (Xid) in mice (Lindvall *et al.*, 2005). In Xid mice and Btk null mice, follicular B cells are strongly reduced in numbers, but MZ B cells are generated, although in smaller numbers (Kraal *et al.*, 1988a; Loder *et al.*, 1999). In the absence of both Aiolos and Btk, Cariappa *et al.* (2001) showed that MZ B cells do develop, indicating that Btk is a negative regulator of MZ B cell development, supportive of the idea that MZ B cells require low signal strength through their BCR to differentiate. In contrast, indications that strong BCR signaling, especially from low-dose self-antigens, is essential for MZ B cell maturation have recently been given using a monoclonal BCR mouse (Wen *et al.*, 2005). It is becoming clear that the decision steps for lineage commitment depend on many factors, partly BCR related, and are strongly influenced by environmental factors. Also signaling in B cells via the NF-κB pathway, and in p50 component in particular, is required for the proper development of MZ B cells (Cariappa *et al.*, 2000), but this pathway does not seem to interact directly with Btk activation or to influence the signaling strength of the BCR.

3. Notch Signaling and MZ B Cell Development

In addition to apparent differences in the threshold of BCR signaling, MZ B cells are critically dependent on Notch2 signaling for their survival and probably also for their maintenance. Notch signaling is important for many cell-fate decision steps during ontogenetic development, but also during adult life. Furthermore, it has been suggested that there is an inverse relationship of BCR and Notch signaling, whereby strong BCR signals could downregulate the Notch pathway by induction of inhibitors or downregulation of activating factors (Pillai *et al.*, 2004). Notch2 belongs to the group of four notch receptors described in mammals, which are composed of two noncovalently linked fragments in the cell membrane. Ligation induces the release and translocation into the nucleus of the intracellular domain of Notch (Notch IC), where it interacts with transcriptional repressors such as RBP-J (repressor recombination binding protein-J) to convert it into a transcriptional activator. Recent studies with mice targeted in several genes of the Notch pathways have revealed the role of this signaling route in MZ B cell development. It was first demonstrated that RBP-J was crucial for the proper development of MZ B cells (Tanigaki *et al.*, 2002), followed by the observations that Notch2 was specifically expressed in MZ B cells, and not in follicular B cells, coinciding with an absence of MZ B cells in $Notch2^{-/-}$ mice (Saito *et al.*, 2003). In addition, transfer of fetal liver cells from $MINT^{-/-}$ mice into $RAG^{-/-}$ mice led to the preferential development of MZ B cells (Kuroda *et al.*, 2003). MINT (Msx2-interacting nuclear target protein) negatively regulates activation through Notch by interfering with the interaction of Notch IC and RBP-J.

MINT is highly expressed in follicular B cells, consistent with suppression of Notch-regulated genes (Kuroda *et al.*, 2003).

4. Additional Transcription Factors

Recently a role for the helix-loop-helix protein E2A in MZ B cell development was described, balanced by its antagonist Id3 (Quong *et al.*, 2004). The E2A gene encodes for two proteins, E12 and E47, which are formed by alternative splicing, and these E proteins are involved in the early commitment and survival of lymphoid precursors into the B cell lineage (Lazorchak *et al.*, 2005). $E2A^{+/-}$ mice show an increase in levels of MZ B cells, whereas follicular B cell numbers decrease. In Id3 null mice more follicular B cells are generated (Quong *et al.*, 2004). A relationship with Notch signaling was proposed by the authors as it has been demonstrated that the Notch pathway negatively regulates E47 activity (Nie *et al.*, 2003). By controlling the levels of E47, Notch may regulate the cell-fate decision that leads to MZ B cells. Id3 levels, on the other hand, are controlled by Pyk-2 kinase activity, corresponding to the fact that in the absence of this kinase no MZ B cells can be formed (Guinamard *et al.*, 2000). A complex picture of signaling pathways that are necessary for the development of MZ B cells versus follicular B cells slowly starts to emerge, but the unraveling of the precise interactions of these routes awaits further studies (Fig. 3).

5. Extracellular Signals Involved in MZ B Cell Generation

In addition to these opposing interactions at the level of gene transcription, it is necessary to know more about the activating steps at the plasma membrane that lead to these intracellular signaling pathways. Signaling through Notch

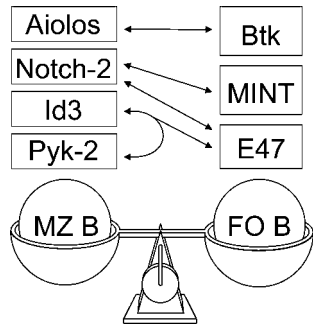

FIG. 3 Cell fate decisions between marginal zone B cells (MZ B) and follicular B cells (FO B). The points at which the decision for lineage commitment is made are dependent on the balance between various signaling cascades that either cooperate or counteract. See the text for details on these interactions.

as a receptor complex on the plasma membrane requires interactions with ligands on cells or extracellular matrix, different from cognate interaction with antigen and BCR. Delta-like-1 (Dl-1) is involved as a molecular ligand, since genetic ablation of Dl-1 also leads to significant loss of MZ B cells. Furthermore, Dl-1 was found to be expressed by dendritic cells in the spleen (Hozumi *et al*., 2004). Dendritic cells also produce growth and survival factors such as BAFF and APRIL (a proliferation–inducing ligand) (Balazs *et al*., 2002), and the signaling cascades through these factors may interfere with the above-mentioned transcription routes and lead to preferential induction or maintenance of MZ B cells.

Roles for other molecules on the surface of B cells related to BCR signaling such as CD19, CD21, and CD22 have been amply documented (Lopes-Carvalho and Kearney, 2004). The CD19 and CD21 molecules seem to act in concert and help to lower the threshold for BCR triggering. It is not clear what the ligand for CD19 is, but the molecule is important for the development of MZ B cells as shown in gene-targeted mice (Rickert *et al*., 1995), whereas the absence of the complement receptor CD21 leads to increased numbers of MZ B cells (Cariappa *et al*., 2001). Interestingly, the expression levels of CD21 on B cells seem to be related to the Notch pathway, since in Notch2$^{-/-}$ mice B cells exhibit reduced levels of CD21, while in E2$^{+/-}$ B cells the levels of CD21 are increased, again allowing a link between Notch2 signaling and the activity of E proteins (Quong *et al*., 2004; Saito *et al*., 2003).

CD22 is also a negative regulator of BCR signaling, based on the presence of an ITIM in its cytoplasmic tail and the reduced MZ B cell compartment in CD22–deficient mice (Samardzic *et al*., 2002). CD22 is a siglec molecule, with its N-terminal domain binding to glycans containing sialic acids. Recently it was found that CD22 is predominantly reacting *in cis* with sialic acids on other CD22 molecules, forming homomultimeric complexes, thereby masking its reactivity (Han *et al*., 2005). Interestingly, MZ B cells express an unmasked form of CD22, suggestive of an interaction with external stimuli (Danzer *et al*., 2003).

Dendritic cells have been mentioned as putative candidates with which the MZ B cells can interact during their development, but also the marginal zone macrophages are likely candidates to provide survival or differentiation signals. By *in vivo* infusion of an antibody against the MARCO scavenger receptor on marginal zone macrophages it was found that MZ B cells started to migrate out of the MZ and into the follicles (Karlsson *et al*., 2003). Recently we analyzed MARCO null mice and mice mutated in both MARCO and SR-A-I and found an impairment of the T-independent type 2 (TI-2) response in these mice, although no apparent differences in the presence of MZ B cells were found (Chen *et al*., 2005). The dependence of MZ B cell formation on NF-κB-p50 could point to interactions with microbial antigens encountered in the marginal zone and their signaling through

TLR via the NF-κB route. Together it is clear that the formation of MZ B cells is a complex process involving delicate balances in signaling pathways of both BCR and accessory molecules, through interactions with antigen, but also ligands produced by local cells in the marginal zone.

6. Retention Mechanisms for Marginal Zone B Cells

One of the most obvious outcomes of the complex differentiation schemes that lead to marginal zone B cells is the fact that they occupy a different niche and do not recirculate, as compared to follicular B cells. It is not clear whether the development of marginal zone B cells initially starts in the marginal zone or whether their retention is a result of this differentiation. At any rate, some of the molecular bases of why they remain localized are becoming more clear. An important breakthrough was derived from studies using the drug FTY720, which was found to have a major impact on lymphocyte egress from lymphoid organs (Cyster, 2005). Upon injection the drug becomes readily phosphorylated, thereby mimicking sphingosine-1-phosphate (S1P), and can engage four of the five known S1P receptors. S1P is a lysophospholipid, produced by many cells, including macrophages, and is found in high concentrations in blood compared to lymph. This concentration gradient from lymph to blood may be a driving force for lymphocytes to exit from lymph node upon expression of S1P receptors. Being G-protein coupled receptors they transduce signals upon binding to the lysophospholipids by association with G proteins, leading to a variety of downstream events, including survival signals as well as cell motility. The receptor $S1P_1$ is the most important S1P receptor associated with lymphocyte egress, as inferred from transfer studies with cells from $S1P_1$-deficient mice. $S1P_1$ on lymphocytes is downregulated and inactivated by the FTY720 drug, leading to the inability of cells to exit lymphoid organs (Matloubian *et al.*, 2004). Marginal zone B cells express high levels of $S1P_1$, which turned out to be important for the retention of the cells. Not only did treatment of mice with FTY720 lead to a rapid displacement of MZ B cells from marginal zones into the white pulp follicles, but in $S1P_1$-deficient mice marginal zones were absent (Cinamon *et al.*, 2004). Interestingly, normal numbers of B cells with the MZ B phenotype were found in their spleen, although the mice lacked a clearly localized population of MZ B cells, suggesting that the development of these cells may precede the lodging into their anatomical localization (Cinamon *et al.*, 2004).

In contrast to the active emigration of lymphocytes from lymph nodes upon $S1P_1$ engagement, MZ B cells use $S1P_1$ to retain their position. This apparent discrepancy may be explained by higher concentrations of S1P in the blood, to which the cells in the marginal zone are continuously exposed, leading to signaling cascades that favor retention over migration. Effects of

S1P on cytoskeleton rearrangements and adhesion have been described in other systems (Rosen and Goetzl, 2005), and a role for integrins and additional chemokines in MZ B cell retention has been given (Lu and Cyster, 2002). In this respect the selective expression of CD9 on MZ B cells deserves attention. CD9 belongs to the family of tetraspanin molecules that associates with signaling receptors on the plasma membrane of leukocytes, thereby forming so-called microdomains, which are thought to facilitate the signaling processes on leukocyte membranes (Wright *et al.*, 2004). CD9, in particular, is associated with integrin molecules (Shaw *et al.*, 1995), and its more or less restricted expression on marginal zone B cells, as well as on B-1 B cells and plasma cells, both of which are nonrecirculating cells, may imply a role in the adhesion and sessile nature of these cell types (Fig. 4).

In addition, $S1P_1$ may be involved in survival of the marginal zone B cells, because it is well established that the intracellular signaling cascades initiated by S1P can result in antiapoptotic survival signals (Rosen and Goetzl, 2005), and MZ B cells as a population are long–living cells (Hao and Rajewsky, 2001; Pillai *et al.*, 2004). This involvement of $S1P_1$ is not absolute, and other signaling pathways will also account for their survival, because MZ B cells are still present in $S1P_1$-deficient mice (Cinamon *et al.*, 2004). Activation with LPS or with cognate interaction overrides S1P-mediated retention and leads to immigration of the MZ B cells into the follicles of the spleen (Groeneveld *et al.*, 1985). This immigration is dependent on the chemokine CXCL13.

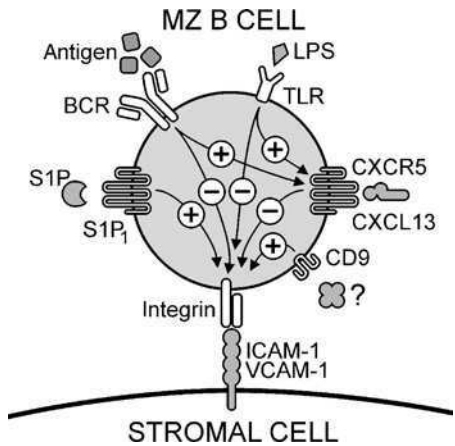

FIG. 4 Molecular interactions involved in the retention of marginal zone B cells. S1P is crucial for integrin activation and retention of MZ B cells through adhesion to stromal cells, and this signal is possibly strengthened through CD9. The signal can be negatively influenced by activation via the antigen receptor and via Toll-like receptors, leading to enhanced sensitivity of CXCR5 for CXCL13, whereupon the MZ B cell can migrate into the white pulp.

However, when this chemokine is absent, $S1P_1$ is no longer required to keep the B cells in the marginal zone, showing a hierarchy of responsiveness (Cinamon *et al*., 2004). Expression of $S1P_1$ on MZB cells was shown to be required to overcome the chemotactic response toward CXCL13, which is expressed in the B cell follicles. By triggering of $S1P_1$, B cells either fail to respond to CXCL13, or are induced to express adhesion molecules or other receptors required for retention in the marginal zone (Fig. 4). As such, expression of the integrins LFA-1 ($\alpha L\beta_2$) and $\alpha_4\beta_1$ has been implicated as adhesion molecules required for retention of MZB cells in this compartment (Lu and Cyster, 2002). Data from gene-targeted mice displaying defects in integrin and chemokine signaling support these findings. $DOCK2^{-/-}$ mice, which were shown to have a selective defect in chemokine-induced integrin activation (Nombela-Arrieta *et al*., 2004), have no MZ B cells. This could be attributed to the inability of lymphocytes to activate Rac, required for actin polymerization, which is in turn necessary for migration (Fukui *et al*., 2001). A strong reduction of MZ B cells was found in the absence of Lsc, which acts downstream of the chemokine receptors in polymerization of actin (Girkontaite *et al*., 2001). In all these mice, the inability to correctly signal through chemokine or lipid receptors can explain the defect in MZB cell localization.

However, there are still molecule(s) other than S1P involved in this lodging and retention, since in the absence of $S1P_1$ as well as CXCR5, the receptor for CXCL13, MZB cells are still retained within this compartment (Cinamon *et al*., 2004). Treatment with pertussis toxin leads to the disappearance of the B cells from the marginal zone. Since this toxin acts on $G\alpha$ protein signaling, additional chemokines could be involved to retain MZ B cells (Guinamard *et al*., 2000). However, pertussis toxin treatment will also lead to the disappearance of macrophages from the marginal zone, since marginal zone macrophages require the chemokines CCL21 and, to a lesser extent, CCL19 to localize to the marginal zone (Ato *et al*., 2004). Expression of the scavenger receptor MARCO by marginal zone macrophages was recently described as essential for localization of B cells to the marginal zone, because infusion of antibodies against MARCO led to migration of the B cells into the follicles, suggesting a disruption in the interaction of MZ B cells and macrophages (Karlsson *et al*., 2003) (Fig. 5).

III. Marginal Zone as a Transit Area for Lymphocytes

Although extensive branching of the central arteriole can be found in the white pulp, it is generally assumed that there are no formal endings of the arteriolar bloodstream in the white pulp, and there are only scant indications

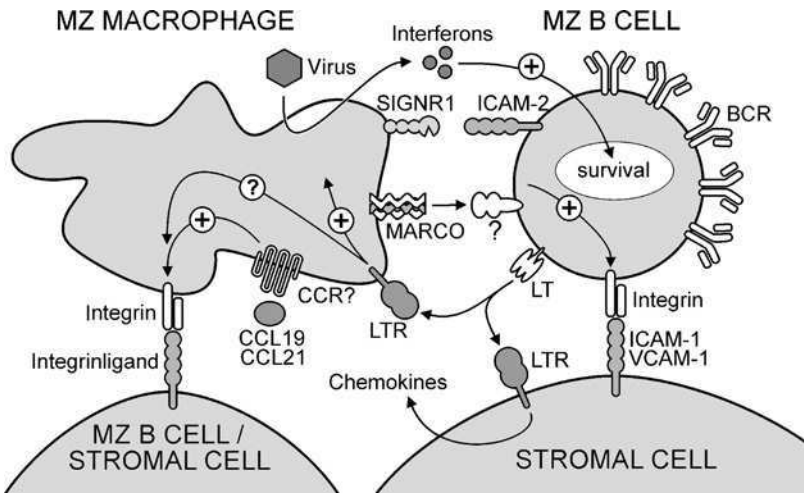

FIG. 5 The cellular interactions between cells in the marginal zone. Marginal zone macrophages (MZM) and MZ B cells influence each other through several pathways. Viral-induced production of interferons by MZM acts antiapoptotically and enhances the survival of MZ B cells. Lymphotoxin–lymphotoxin receptor interaction (LT-LTR) is thought to be necessary for the survival of MZM, possibly through positive effects on integrin-mediated retention. MARCO, acting through unknown mechanisms, influences the retention of MZ B cells. MZM and MZ B cells additionally interact via SIGNR1 and ICAM-2, whereas LT on B cells may be involved in triggering stromal cells to produce chemokines involved in the retention of MZ B cells and macrophages.

for specialized parts in blood vessels that allow cells to transverse from blood to white pulp, comparable to high endothelial venules in lymph nodes (Grayson *et al.*, 2003). This means that most, if not all, of the lymphoid and myeloid cells found in the white pulp enter and leave this region through the marginal zone. The fact that no red blood cells and almost no granulocytes can be found in the white pulp points to an active selection process, and suggests a role for chemokines (Fig. 1D). This was indeed demonstrated using pertussis toxin, which blocks chemokine receptor-associated Gα1 protein signaling and led to a blockade of the entry of lymphocytes into the white pulp (Cyster and Goodnow, 1995). The chemokines involved in the localization of the T- and B-lymphocytes into their respective white pulp compartments were similar to those in lymph node homing: CCR7–CCL19/CCL21 interactions are necessary for the localization of T cells in the white pulp T cell zone, and cooperation between CCR7 and CXCR5 and their ligands is required for B cell entry into the follicles (Muller *et al.*, 2003). Whether chemokine receptor triggering is also essential for the initial activation of lymphocytes in the marginal zone is not formally proven.

The selectivity and chemokine involvement point to mechanisms resembling the entry modes described for the entry of lymphocytes into lymph nodes through high endothelial venules, where the key adhesion molecules are well established (Kraal and Mebius, 1997). Initial rolling to ligands for L-selectin or integrins on high endothelial venules is followed by chemokine-triggered activation and firm adhesion of the cell onto the endothelial wall by interactions of integrins and integrin receptors. Important integrins that play a role in the adhesion and diapedesis are LFA-1(CD11a/CD18), $\alpha_4\beta_1$, and $\alpha_4\beta_7$. In the spleen, however, L-selectin was not found to be important, as inferred from experiments in which trypsin was used to remove this important rolling ligand from lymphocytes, nor did LFA-1 seem to play a major role based on studies with LFA-$1^{-/-}$ mice (Nolte *et al.*, 2002). In later studies, using blocking antibodies in transfer experiments, LFA-1 interaction did seem to be important, especially in combination with $\alpha_4\beta_1$ (Lo *et al.*, 2003). As counterreceptors, both ICAM-1 and VCAM-1 were required, but, because a complete blockade of the immigration could never be found using mixtures of antibodies against LFA-1 and $\alpha_4\beta_1$ and their counterreceptors, other ligands, such as fibronectin, may be involved (Lo *et al.*, 2003).

An obvious molecular candidate that is supposed to play an important role in the immigration process of cells from marginal zone into white pulp is MAdCAM-1 (see Fig. 1). Originally described as an integrin ligand on high endothelial venules in lymphoid organs at mucosal sites, such as mesenteric lymph nodes and Peyer's patches, it is expressed on sinus-lining cells of the marginal zone, right at the border of the white pulp (Kraal *et al.*, 1995). Several attempts to link this suggestive expression at such a strategic position to a functional role in cell migration using classic antibody interference approaches have so far not led to any results (Kraal *et al.*, 1995; Lo *et al.*, 2003; Nolte *et al.*, 2002). Nevertheless, indirect evidence for a role of MAdCAM-1 comes from two sources (Girkontaite *et al.*, 2004; Pabst *et al.*, 2000). First, targeted disruption of NKX2.3, a transcription factor important for tissue differentiation, led to the finding that this factor was essential for the expression of MAdCAM-1 in the spleen, and that this expression was not only necessary for the proper structure of the splenic marginal zone, but was also required for cell migration based on the diminished size of the spleen (Pabst *et al.*, 2000). However, the NKX gene also affected the position of marginal metallophilic macrophages, and may therefore have more profound effects than can be ascribed to the lack of MAdCAM-1 only. The second indication for a role of MAdCAM-1 comes from a study on the $S1P_3$ receptor (Girkontaite *et al.*, 2004). We previously showed that S1P is an important ligand in the retention of MZ B cells by interactions with its receptors $S1P_1$ and $S1P_3$ (Cinamon *et al.*, 2004).

In addition, it has been shown that S1P is an important factor in controlling the organization of endothelial cells through $S1P_3$. Using mice

deficient in $S1P_3$, a disruption of the normal organization of the marginal zone was found, and the effects on lymphocyte localization could be attributed to the initial effects of the lack of S1P signaling on the endothelial cells expressing MAdCAM-1 (Girkontaite *et al.*, 2004). Again, it cannot be ruled out that additional effects on the overall structure of the spleen are more important, and that the effects on MAdCAM-1 are secondary and not indicative of its precise function in the spleen.

In addition to lymphocytes, the marginal zone is also an important area for the entry and transit of dendritic cells (DC) (Fig. 1C). It is assumed that blood-borne DC reside in this zone for longer times and that activation upon pathogen encounter (Leisewitz *et al.*, 2004) or the uptake of apoptotic cells (Morelli *et al.*, 2003) will trigger them to actively migrate into the white pulp T cell zone to present processed antigens. The expression of the chemokine receptor CCR7 seems to be crucial for this migration process (Gunn *et al.*, 1999).

The cells that are responsible for the local production of chemokines in the marginal zone are either stromal cells or endothelial cells, and the induction of chemokine synthesis is probably dependent on lymphotoxin signaling. This is based upon the fact that in mice deficient for components of this signaling pathway, such as LTα, LTβ, and LTβ-receptor (LTβ-R) KO, an intact marginal zone is absent (Martin and Kearney, 2002). The expression of lymphotoxin $\alpha_1\beta_2$ on marginal zone B cells as well as on immigrating follicular B cells could be needed to induce LTβ-R expressing stromal and/or endothelial cells to upregulate the required chemokines. This means that the continuous interaction between stromal cells and resident cells is necessary for an optimal configuration and function of the marginal zone (Fig. 5).

IV. Functions of the Spleen in Host Defense

The major factors in the marginal zone that are instrumental in the removal and destruction of pathogens or the initiation of adaptive immune responses against them are the macrophage subtypes, the marginal zone B cells, and the dendritic cells. New insights in the interactions between the various pattern recognition receptors on these cells involved in the uptake of particles have made it clear that phagocytosis inevitably leads to some sort of activation, and whether this activation has a more pro- or anti–inflammatory nature is dependent on whether the phagocytized material is apoptotic cells, bacteria, viruses, or parasites (Stuart and Ezekowitz, 2005). In some extreme cases, especially after parasitic infections, activation can lead to major changes in the overall organization of the marginal zone that impede the clearance of the infection (Engwerda *et al.*, 2002; Weiss, 1990). At any rate, it is clear that

these activation signals can stimulate other cells leading to an effective cooperation between the various cells in the marginal zone in their effort to control homeostasis.

A good example is the role of SIGNR1 on marginal zone macrophages in the control of pulmonary *Streptococcus* infection (Koppel *et al*., 2005). Mice deficient in this important receptor fail to clear pneumococcal infections (Koppel *et al*., 2005; Lanoue *et al*., 2004); it was suggested that this was mainly because capture and concentration of the bacteria in the spleen were insufficient to activate the marginal zone B cells to produce protective IgM antibodies (Koppel *et al*., 2005). A similar dependence on the marginal zone macrophages to control infections by adaptive immune responses has been described for viral infections (Oehen *et al*., 2002). Although uptake and clearance could be important factors to initially reduce the viral load, marginal zone macrophages can also contribute to viral protection as important producers of interferons (Eloranta and Alm, 1999). In addition, interferons give an anti-apototic signal to B cells (Fig. 5) and enhance BCR signaling (Braun *et al*., 2002).

A. Marginal Zone and T-Independent Immune Responses

The spleen is regarded as the major lymphoid organ capable of mounting immune responses against multivalent TI-2 antigens. This is related to the efficient capturing of these antigens, many of them bacterial capsular polysaccharides, by the macrophages in the marginal zone and the role of MZ B cells in producing antibodies against them. Splenectomy results in increased susceptibility against encapsulated bacteria, and vaccination against these pathogens before the age of two is difficult, probably because the marginal zone is not yet well developed (Cowan *et al*., 1978; Likhite, 1976). The important role of marginal zone B cells became clear from experiments in various mice with gene deficiencies correlating a defect in MZ B cell development with a concomitant inability to handle gram-negative bacteria or their TI-2 antigens (Cariappa *et al*., 2000; Guinamard *et al*., 2000; Tanigaki *et al*., 2002). Their rapid maturation into plasmablasts upon antigen encounter is partly due to their BCR, which can respond more strongly, and probably also to the presence of TLRs on their surface that will help in the recognition of the bacterial antigens, leading to additional intracellular signaling and maturation of the cell. Also CD21, the complement receptor, which is highly expressed on MZ B cells, may aid in concentrating complement-coated polysaccharides (Guinamard *et al*., 2000; Martin *et al*., 2001). Although the MZ B cells are independent of T help for the production of IgM antibodies in the initial responses, they do interact and need dendritic cells for their stimulation. Dendritic cells in the marginal zone provide factors such as

BAFF and APRIL that will sustain the MZ B cell population and are involved in the generation of T-independent responses. This interaction is thought to replace the CD40 ligand–CD40 interaction between Th and B cells during B cell activation (Balazs *et al*., 2002; Litinskiy *et al*., 2002).

B. Marginal Zone and T-Dependent Immune Responses

In addition to the presence of marginal zone B cells with their distinct features as outlined in the previous sections, especially in humans and rats, there is ample evidence for the existence of B memory cells in the marginal zone. These cells have been generated as a result of classical T-dependent responses, involving germinal center reactions, and showing somatic hypermutation and high affinity of their BCRs (Dunn-Walters *et al*., 1995; Liu *et al*., 1991; Shih *et al*., 2002; Tierens *et al*., 1999). They may react swiftly to antigen, and as such the marginal zone can form a reservoir of memory B cells.

MZ B cells also play a role in T-dependent responses as a result of their ability to function as antigen-presenting cells. Their basal levels of the costimulatory molecules CD80 and CD86 can be upregulated by stimulation via their antigen receptor, CD40 ligation, or LPS, and it has been shown using mice in which a transgenic anti-HEL BCR was introduced that MZ B cells can, in fact, readily prime naive CD4 T cells, whereas follicular B cells cannot (Attanavanich and Kearney, 2004).

A third level at which the marginal zone is important for the generation of T-dependent immune responses is activation of dendritic cells. It is assumed that the dendritic cells reside in the marginal zone as sentinels, which start to migrate into the white pulp upon appropriate activation. It is not known whether the dendritic cells are efficient enough to scavenge material from the blood on their own, or benefit from the concentrating effect of the large marginal zone macrophage population, or from the observed direct interactions with marginal zone B cells.

C. Autoimmunity and Tolerance

In view of the selection of MZ B cells on the basis of recognition of self-antigens, there is a potential risk for the development of autoreactive B cell clones. But in general, MZ B cells do not produce natural antibody and they do not engage in T cell-dependent responses with high affinity maturation, whereas autoimmune, pathogenic antibodies are almost always highly somatically mutated (Lopes-Carvalho and Kearney, 2004). Nevertheless, in several autoimmune-prone mice models, indications that MZ B cells could be involved in the expansion of the autoimmune disease have been given.

In the autoimmune NZB mouse strain, enhanced numbers of MZ B cells are found, showing an activated phenotype (Wither *et al*., 2000), and in a lupus-prone model, early expansion of MZ B cells is found, which is responsible for the production of anti-DNA antibodies (Schuster *et al*., 2002). Also, studies in which autoreactive transgenic BCRs were introduced showed that negative selection of these transgenes occurred but that the marginal zone B cells, in particular, were autoreactive (Goodnow, 1996). Mice overexpressing BAFF, the factor critical for the survival and maturation of MZ B cells, spontaneously develop an autoimmune SLE-like syndrome with increased numbers of MZ B cells (Mackay *et al*., 1999). Although these and several other studies (Viau and Zouali, 2005) point to a role for MZ B cells in autoimmunity, a direct mechanistic link between MZ B cells and the initiation of autoimmunity is not clear.

Autoimmune disorders can be seen as the perturbance of tolerance to self, which is a highly regulated intrinsic characteristic of B and T cell development. In addition, immunologic tolerance can be induced against exogenous antigens as a result of an active immune response. The best studied examples are immune responses that are generated along mucosal surfaces against protein antigens, involving the generation of mucosal regulatory cells (Faria and Weiner, 2005; Samsom, 2004). The majority of these tolerogenic responses do not lead to any involvement of the spleen (Samsom *et al*., 2005; Unger *et al*., 2003), with the exception of immune responses that are generated in the eye (Camelo *et al*., 2005; Stein-Streilein and Streilein, 2002). Antigen introduced into the anterior chamber of the eye is carried to the marginal zone by a subset of macrophages, expressing the F4/80 antigen, and leads to the induction of CD8 T cells that can suppress the immune response against the antigen. This anterior chamber-associated immune deviation (ACAID) model is clearly dependent on the marginal zone of the spleen, whereby the F4/80 molecule plays a crucial role (Lin *et al*., 2005). Whether this involvement of the marginal zone is typical for eye-associated tolerance or whether the induction of CD8 suppressor cells is a general function of the marginal zone is an interesting question that needs further investigation.

V. Embryonic Development of the Marginal Zone of the Spleen

A. Development of the Spleen

During embryonic development of the mouse, the first evidence of splenic development is the condensation and proliferation of mesenchymal tissues in the dorsal part of the mesogastrium seen at dE12.5 (embryonic day 12.5 or

12.5 days postcoitus) (Green, 1967; Roberts *et al.*, 1994). In the following days the spleen increases in size because of expanding centers of myelopoiesis and erythropoiesis (Metcalf and Moore, 1971; Sasaki and Matsumura, 1988). The first macrophages that carry the F4/80 antigen, characteristic of the macrophages in the red pulp, can be detected from dE15, while a subset of Siglec-1 (sialoadhesin)-positive macrophages appears between dE18 and birth (Morris *et al.*, 1991, 1992). At this time there is no apparent organization of the macrophage populations. Only in the late phase of splenic development can lymphocytes be found, and the first mature IgM^+ B cells are present at dE17, although their precursors can be isolated from the spleen as early as dE13.5 (Godin *et al.*, 1999; Velardi and Cooper, 1984). In rodents the first mature T cells leave the thymus in large numbers only after birth, leading to the subsequent development of separate splenic compartments from that time on (Friedberg and Weissman, 1974; Metcalf and Moore, 1971; Rugh, 1968).

During the development of the spleen, as early as dE13.5, a population of $CD4^+CD3^-$ cells can be found in the splenic anlage. These lineage-restricted progenitors have been well characterized in early lymph nodes and have the potential to differentiate to antigen-presenting cells, NK cells, and follicular cells, but not to T or B cells (Mebius *et al.*, 1996, 1997). Importantly, they are crucial for the development of several lymphoid organs such as nasal-associated lymphoid tissue (NALT), Peyer's patches (PP), and lymph nodes (Eberl *et al.*, 2004; Finke *et al.*, 2002; Fukuyama *et al.*, 2002) by giving instructive signals to stromal cells in the organ anlagen that lead to further differentiation (Mebius *et al.*, 1997). This has been well documented using mice deficient in genes of the TNF or NF-κB superfamilies. Interestingly, so far none of these defects, which in some cases can lead to complete absence of any lymph node, affects the initial formation of the spleen, although developmental aberrations are found (see below). This means that the formation of the spleen is regulated by early developmental genes and differs from the development of other secondary lymphoid organs.

Again, with the help of genetically altered mice some of the molecular interactions that are intrinsic to the formation of the spleen have been identified. A mesodermal-derived cell layer, the splanchnic mesodermal plate (SMP), which is part of asymmetric left–right morphogenic development, can be viewed as an organizing center for the development of the spleen. When the SMP is not formed, as in mice lacking the dominant hemimelia (Dh) gene or the homeobox transcription factor Bapx1, no spleen is formed (Green, 1967; Hecksher-Sorensen *et al.*, 2004; Lettice *et al.*, 1999; Tribioli and Lufkin, 1999). In mice that are deficient in the homeobox gene Hox11 the anlage of the spleen begins normally at dE11.5, but is rapidly followed by resorption due to apoptosis (Dear *et al.*, 1995) or persists as an unorganized rudiment (Kanzler and Dear, 2001). In aggregation chimeras

generated between Hox11$^{-/-}$ and normal mice the defect could not be rescued, which indicates that this defect is intrinsic to this cell population and is not due to the inability of the splenic anlage to attract and retain lymphocyte precursors (Kanzler and Dear, 2001). Also, deficiency in the basic helix-loop-helix transcription factor Capsulin (Lu *et al.*, 2000), and the Wilm's tumor gene product Wt1 (Herzer *et al.*, 1999), results in the complete absence of the spleen. Some of these genes may act in concert, whereby Bapx1 and Dh control the development of the SMP. Wt1 and Hox11 are probably regulated independently of each other (Herzer *et al.*, 1999). Future studies will be necessary to uncover the precise molecular interactions that are essential for the development of the spleen.

B. Development of the Marginal Zone

In both rats and mice the marginal zone is still not distinguishable at day of birth and gradually develops in the course of the next 2 weeks (Takeya and Takahashi, 1992). Macrophages that will later enter into the marginal zone can be found dispersed throughout the entire spleen during the first neonatal days, while the actual formation of the marginal zone starts around day 5 in mice. From this day on these macrophages start to localize at the interface of the red and white pulp and establish a marginal zone, which is then also populated by marginal zone B cells (Kraal *et al.*, 1988b; Morris *et al.*, 1992; Takeya and Takahashi, 1992). Around day 10 the marginal zone attains its full mature appearance, and at this stage marginal zone bridging channels can also be recognized, which represent protrusions of the white pulp area across the marginal zone into the red pulp (Mitchell, 1973; Takeya and Takahashi, 1992).

C. Molecules Involved in the Organization of the Marginal Zone

Although little is known about the molecular interactions that regulate the formation of the separate splenic compartments, development of genetically altered mice has revealed molecules and pathways that are important in this process. A large number of knockout mice have been generated in the past decade that appeared to have minor or major defects in splenic organization. In mice that lack a single member of the TNF or NF-κB superfamily, severe defects in the formation of lymphoid tissue, such as the absence of (certain) lymph nodes and/or Peyer's patches, are seen (Mebius, 2003). In all these mice a spleen is formed; however, anatomical abnormalities, with respect to

the formation of distinct B and T cell areas as well as the marginal zone, are observed in the spleens of these mice.

1. Lymphotoxin and Its Receptors

Lymphotoxins (LT) are members of a complex communication system between lymphocytes and surrounding stromal cells. With its close homology to TNF, it is encompassed in the TNF superfamily, and although TNF and LT often work together in signaling networks, sharing receptors, it has become clear that LT has quite a distinct role, especially in the development of the immune system. Two distinct structural forms of LT can be discerned, LTα and LTβ, which form trimeric molecules. As a membrane-bound form it can be found as a heterotrimer, either $LT\alpha_1\beta_2$ or $LT\alpha_2\beta_1$, and in in secreted form as a homotrimer, $LT\alpha_3$ (Ruddle, 1992; Ware, 2005). Homotrimeric forms of both TNF-α and LTα can bind and activate each of the two defined TNF receptors, TNF-RI (p55) and TNF-RII (p75), while the LT heterotrimers, but not $LT\alpha_3$, interact through the LTβ-R (Fu and Chaplin, 1999; Ware *et al*., 1995).

Genetic disruption of either the LTα gene or the LTβ gene results in complete disruption of the splenic microarchitecture: follicles, their follicular dendritic cells (FDC), and germinal centers are lacking, as well as normal constituents of the marginal zone, such as macrophages, B cells, and sinus lining cells (Alimzhanov *et al*., 1997; Banks *et al*., 1995; De Togni *et al*., 1994; Matsumoto *et al*., 1996). In LTα-deficient mice, T and B cells have no separate areas and are completely intermingled, while LTβ-deficient mice show B cells localized in a ring around a central T cell area (Koni *et al*., 1997). Compared to LTβ-deficient mice, LTβ-receptor-deficient mice showed an even more severe phenotype, suggesting the involvement of additional ligands. It was found that LIGHT, a closely related trimeric ligand, was responsible (Wang *et al*., 2002). The introduction of LIGHT as a transgene in LTα-deficient mice did lead to restoration of most of the defects in the spleen, except for the organization of the marginal zone, emphasizing the importance of LTα in the development of this region. The importance of LT for the architecture of the spleen was also evident from studies that used a soluble LTβ-R-Ig fusion protein to block normal LTαβ/LTβ-R interactions. Injection of this fusion protein as a decoy receptor during embryonic life severely interferes with the formation of the marginal zone and the T and B cell compartments of the white pulp, comparable to the phenotype of LTβ-deficient mice (Ettinger *et al*., 1996; Rennert *et al*., 1996). Interestingly, experiments in which adult animals with a functional marginal zone were treated with the LTβ-R-Ig fusion protein showed a disturbed marginal zone and loss of B cell follicles. This clearly indicates that signaling through LTβ-R is not only important for adequate development of normal

splenic organization, but also for its maintenance in adult life (Mackay *et al.*, 1997).

2. TNF-α and Its Receptors

Although LT signaling appears to be crucial for proper postnatal development and proper compartmentalization of the spleen, signaling through TNF-α and its receptors is also important in this process: in mice that are deficient in either TNF-α or TNF-RI the marginal zones are present, but they are clearly less well developed than in wild-type mice. Reduced numbers of marginal zone and metallophilic macrophages are found, while MAdCAM-1 expression is completely absent (Matsumoto *et al.*, 1996; Neumann *et al.*, 1996b; Pasparakis *et al.*, 2000). In addition these mice lack splenic follicles and FDCs, although T and B cells are segregated as seen in LTβ-deficient mice (Körner *et al.*, 1997; Le Hir *et al.*, 1996; Pasparakis *et al.*, 1996). In contrast, spleens of TNF-RII-deficient mice appear normal, indicating that this receptor is either not important in splenic formation or that its function can be taken over by TNF-RI (Matsumoto *et al.*, 1996; Neumann *et al.*, 1996a; Pasparakis *et al.*, 2000).

Soluble TNF-RI-Ig can reduce the expression of MAdCAM-1 (not other marginal zone markers) and disrupt the splenic T/B cell compartments, but only when this receptor decoy is administered during ontogeny (Rennert *et al.*, 1996, 1997). In contrast to the findings with LTβ-R, injections of soluble TNF-RI-Ig in adult mice did not lead to any effects, thus indicating that signaling through this receptor is important only for splenic development, but not for its maintenance (Mackay *et al.*, 1997). Importantly, bone marrow transplantation experiments have revealed that expression of TNF-RI and LTβ-R, required for proper development of the splenic microarchitecture, is necessary on radioresistant stromal cells, while LTα, LTβ, and TNF-α are derived from cells of hematopoietic origin, which are most likely B cells (Endres *et al.*, 1999; Matsumoto *et al.*, 1996, 1997; Mebius *et al.*, 1998; Tkachuk *et al.*, 1998). This could explain why the marginal zone in B cell-deficient mice is not developed, and when B cells are experimentally depleted in adult life, this leads to a disappearance of marginal zone macrophages and the expression of MAdCAM-1 (Nolte *et al.*, 2004). LTβ-R or TNF-R triggering leads downstream to the activation of NF-κB. This ubiquitously expressed transcription factor family is involved in numerous cellular responses and serves as a critical regulator of the inducible expression of many genes (de Winther *et al.*, 2005). In mammalian systems, this family is composed of subunits p50, p52, RelA (p65), c-Rel, and RelB, which can all form dimeric complexes, depending on the cell type and activation state (Baeuerle and Henkel, 1994; Ghosh *et al.*, 1998; Mercurio and Manning, 1999). These dimers exist in the cytoplasm in an inactive form, due to

interaction with inhibitory proteins termed IκBs. Upon activating signals, these IκBs are phosphorylated and consequently degraded, which results in nuclear translocation of the NF-κB dimeric complex and transcription of its target genes (de Winther *et al*., 2005; Hayden and Ghosh, 2004).

Several phenotypes that affect the marginal zone by deficiencies in the NF-κB route exhibit defects that are similar to those seen with LTβ-R-deficient mice, being parts of the same activation and signaling route. This is the case for mice deficient in NIK, the natural mutant *aly/aly* mice (Shinkura *et al*., 1999), which in addition to lacking lymph nodes and Peyer's patches, have no marginal zone (Karrer *et al*., 1997; Koike *et al*., 1996; Miyawaki *et al*., 1994; Yamada *et al*., 2000). Also, relB expression is absolutely required for the development of the splenic marginal zone (Weih *et al*., 2001). RelB acts downstream of LTβ-R, and perhaps TNF-R, and is most likely expressed by endothelial and/or stromal cells present in the marginal zone (Weih and Caamano, 2003).

VI. Concluding Remarks

In summarizing the multitude of data that have been generated on the function and development of the splenic marginal zone, the most obvious conclusion involves the complex interplay that takes place in this region between cell types from completely different lineages. Compared to our knowledge a decade ago, it is clear that a general picture of what the marginal zone was and how it functioned existed, but little was known about these intricate cellular interactions. It is clear that the marginal zone is a highly dynamic region in which the continuous interaction of the various resident and transmigrating cells is necessary to maintain its active state. In particular, the interaction between macrophages, MZ B cells, and dendritic cells is crucial for the integrity of this region. Through growth factors and hormones and cell–cell contacts, a picture of delicately balanced interactions is emerging, and any perturbance of this balance can have direct consequences for the function of the marginal zone.

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Cell Biology of T Cell Activation and Differentiation

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T cells are major components of the adaptive immune system. They can differentiate into two different populations of effector cells—type one and type two—and may also become tolerant. T cells respond to immune challenges by interacting with antigen-presenting cells of the innate immune system. These latter cells can identify the nature of any immune challenge and initiate adaptive immune responses. Dendritic cells are the most important antigen-presenting cells in the body. The T cell recognizes both peptides associated with MHC molecules on the antigen-presenting cells and also other molecules in a complex structure known as an immunological synapse. The nature of the antigen, the cytokine environment, and other molecules on the dendritic cell surface instruct the T cells as to the response required. A better understanding of the biology of T cell responses offers the prospect of more effective therapeutic interventions.

KEY WORDS: T cell activation, Th1, Th2, Dendritic cells, T cell differentiation, Leukocyte migration, Immunologic synapses.

I. Introduction

A. Mammalian Immune Response

The mammalian immune system is extremely efficient in defending the host against microbial pathogens. The system is composed of two well-defined parts—the innate and the adaptive immune systems (Germain, 2004; Germain and Jenkins, 2004).

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The innate immune system is the first line of defense that comes into action as soon as invaders are detected. The cells involved in this response include macrophages, neutrophils, natural killer (NK) cells, and dendritic cells (DC). The macrophages and neutrophils engulf and even destroy the invaders; they also release cytokines that help to destroy the pathogen in other cells, by inducing a series of distinct processes. Cytokines are small proteins produced by many specific cell types. They are important in the activation, proliferation, differentiation, and chemotaxis of immune cells—that is, they orchestrate the whole immune response. ''Cytokines'' include growth factors, colony-stimulating factors, interleukins, monokines, interferons, and chemokines (Ozaki and Leonard, 2002; Zlotnik and Yoshie, 2000). NK cells recognize and destroy transformed cells (for example, tumor cells) and virally infected cells. DCs are extremely important in the induction of B and T cells, which constitute the second line of immune defense; this is called adaptive immunity. DCs also link the innate immune response to the adaptive immune response.

When the invader escapes or survives the innate immune system, the adaptive response is activated. This response is mediated by B and T cells. In contrast to the innate immune response, the adaptive immune response has an immunological memory and receptors that are highly specific for the invaders (Germain, 2004). B cells recognize antigens through B cell receptors, which are IgM molecules located in the cell membrane. Once the B cells are activated, they proliferate and differentiate into plasma cells, which are the actual antibody-producing cells. The secreted antibodies then neutralize the pathogens in the tissues and fluids of the host. B cells recognize antigens directly. In contrast, the T cell receptor recognizes only short peptides. These are formed by proteolysis of foreign proteins and are presented to the T cell in association with MHC class I or class II molecules, which are present on the cell membrane of antigen-presenting cells (APC). The most important APCs are the DC, which are able to prime naive T cells. B cells and macrophages are also important APCs involved in secondary T cell responses (Trombetta and Mellman, 2005).

Almost all somatic cells in the organism have MHC class I molecules. These molecules allow infected or transformed cells to be recognized and destroyed by previously activated cytotoxic T (Tc) cells. MHC class II molecules are, however, found only on the cell surface of so-called ''professional'' APCs. These molecules present their antigenic peptides to helper T (Th) cells. These ''professional'' APCs (the DCs, macrophages, and B cells) have both classes of MHC molecules, necessary for priming both kinds of T cell (Trombetta and Mellman, 2005).

Tc cells recognize peptides with an average length of 8–10 amino acids bound to the peptide recognition site of the MHC class I molecules. These peptides are generated by the action of the proteasome; this process is called

endogenous antigen processing and presentation. In contrast, Th cells mainly recognize peptides with an average length of 14–25 amino acids derived from extracellular proteins; these are associated with MHC class II molecules. This is called the exogenous pathway of antigen processing and presentation (Yewdell and Bennink, 1990).

B. Th1/Th2 Concept

Development of adaptive immunity starts with the differentiation of naive T lymphocytes into cells capable of exerting an immune response, that is, into effector cells. Early studies in the 1970s showed that there are two types of response—one associated with inflammation or proinflammatory processes, and the other associated with high levels of antibodies or humoral events (Marrack and Kappler, 1975; Tada *et al.*, 1978). In the 1980s Mosmann and colleagues (Cherwinski *et al.*, 1987; Mosmann *et al.*, 1986) demonstrated that Th cells could be divided into two different groups based on the type of cytokines they released. When murine Th clones were stimulated *in vitro* with the T cell mitogen concanavalin A or with an antigen, one group of clones secreted mainly interleukin (IL)-2 and interferon (IFN)-γ but not IL-4 or IL-5; these were called Th1 cells. The other group of clones secreted mainly IL-4 or IL-5, but not IL-2 and IFN-γ; these were called Th2 cells. This dichotomy of the immune response is also observed *in vivo*. Th1 cells promote proinflammatory responses, also called cellular responses, and Th2 cells promote the antibody responses, also called humoral responses. A similar pattern occurs in humans (Del Prete *et al.*, 1991); as will be discussed later, however, the differences between the Th1 and Th2 responses in humans have been less well characterized.

Over the years the Th1/Th2 concept has evolved as more cytokines and other factors have been described (Mosmann and Sad, 1996). We now know that the representative cytokines for Th1 cells are IFN-γ and lymphotoxin; these not only promote inflammatory events [for example, induction of delayed-type hypersensitivity (DTH), macrophage activation] but also help B cells to produce immunoglobulin (Ig)G_{2a} or IgG_3 (in mice and humans, respectively) antibodies. The representative cytokines for Th2 cells are IL-4, IL-5, IL-6, and IL-13. These together induce those antibody responses (particularly involving IgG_1 and IgE) that promote (1) degranulation of mast cells, (2) eosinophil proliferation and function, and (3) immediate-type-hypersensitivity reactions.

Recently the Th1/Th2 concept has changed further since it has become clear that the patterns of cytokines characteristic of both T cell types are also found in other cell types. It is therefore more appropriate to use the terms type one and type two immune responses (Mosmann, 2000). Thus, in

an immune response, the cytokine pattern in a given time will depend on an intricate network of interactions, in which cross-regulation by the different cytokines plays an important role. IFN-γ blocks the proliferation and differentiation of Th2 cells, while IL-4 blocks the proliferation and differentiation of Th1 (Fiorentino *et al.*, 1989; Furumoto and Irahara, 2002; Mosmann, 2000; Mosmann and Coffman, 1989). This reciprocal control allows the immune system to respond in a controlled fashion by transiently regulating Th1 and Th2 responses. This makes it difficult for us to observe a very distinct response after certain challenges (Allen and Maizels, 1997). In acute infections both Th1 and Th2 responses are simultaneously induced, while in more chronic conditions separate responses can be more clearly observed.

One of the most intriguing questions is "How do Th cells decide to become Th1 or Th2 cells?" There have been several explanations involving a number of factors, but we still do not know exactly how such factors might work *in vivo*. Clearly one of the relevant factors is the cytokine environment during the initial response. For instance, if IL-12 is present, a type one response would be favored; on the other hand, if IL-4 is present, a type two response would follow. It also seems that there may be discrete microenvironments simultaneously capable of inducing both kinds of responses (Gor *et al.*, 2003). Other important factors include the kind and amount of antigen and the phenotype of the APC (Hosken *et al.*, 1995).

II. Cell Biology of Dendritic Cells

A. Antigen Processing and Presentation

Antigen presentation to T cells is carried out by "professional" APCs. The best characterized are dendritic cells, macrophages, and B cells, although in some cases endothelial cells also perform the role of "professional" APCs.

The DCs, present in most peripheral tissues, are the most efficient APCs in the body. They differentiate from bone marrow precursors, which have penetrated peripheral tissues after extravasation from the blood. After differentiation they maintain immune surveillance in the tissue. On contacting antigens and/or danger signals coming from pathogens or stressed tissues, they are activated and migrate to the regional lymph node, where they present antigen to both Tc and Th cells (Randolph, 2001). DCs have basal levels of MHC class I and class II molecules as well as the costimulatory molecules CD80 and CD86 (ligands of CD28 on T cells), which increase dramatically during activation. All these molecules provide

stimulatory signals to T cells; this is why these cells are efficient in inducing primary responses. In the immature stage, DCs have a greater capacity for endocytosis than any other cell in the body; this enables them to ingest antigens very efficiently (West *et al.*, 2004). While the DCs migrate to the lymph node, they mature while processing and presenting antigen (Trombetta *et al.*, 2003). DCs can also stimulate the differentiation of both type one and type two Th cells by poorly understood mechanisms. It is clear, however, that the characteristics and concentration of the antigen, surface molecules, and cytokine environment are important elements determining the balance between type one and type two cells (Lanzavecchia and Sallusto, 2001b).

It has recently become clear that macrophages are less efficient than DCs and B cells in inducing primary T cell responses, despite their substantial capacity for phagocytosis (which enables them actively to defend the host against invading pathogens). The reason for this lower efficiency is that macrophages have very low levels of class II and costimulatory molecules on their surfaces. Perhaps the main role of macrophages as APCs is at the site of the infection, where they may present antigen to activated effector cells, and to receive from T cells signals that increase their microbicidal capacity (Delemarre *et al.*, 1990).

B cells have very high levels of class II and costimulatory molecules on their surfaces. This makes them second to the DCs in efficiency as APCs. Unlike DCs and macrophages, B cells have very low general endocytotic activity. Antigen uptake is selective because it depends on the presence of an antigen-specific receptor, which increases the internalization of the antigen up to 1000-fold (Watts, 1997). The capacity of B cells to present antigen to Th cells ensures that Th cells provide the B cells with the necessary signals for proliferation and differentiation into antibody-producing plasma cells.

There are two different routes for antigen presentation—the endogenous and the exogenous pathways. In the endogenous pathway, the cytosolic proteasome complex breaks down proteins, including viral and tumor antigens, to yield small peptides 8–10 residues in length. These peptides are transported into the endoplasmic reticulum by a nucleoside triphosphate-dependent process involving a heterodimeric complex of two proteins called TAP1 and TAP2 (TAP, transport associated with antigen processing). Those peptides, which are specifically recognized (that is, for which there is a high affinity), bind in the groove formed by the α_1 and α_2 domains of the MHC class I molecules. At this point each class I molecule is associated with an invariant β_2-microglobulin. The peptide, now in a very stable heterotrimeric structure, is carried to the cell surface where it is recognized by Tc cells (Shastri *et al.*, 2002). Through this process Tc cells are able to sense and destroy virus-infected and/or tumor cells.

Longer peptides (14–25 residues) associated with MHC class II are derived from extracellular antigens; these are taken up by endocytosis and carried to the endosomal/lysosomal system, where they are processed. The mature endosome has an acidic pH and contains a number of proteases that contribute to the unfolding and partial degradation of the antigenic proteins. The endosome containing these peptides fuses with another endosome-like compartment, the MHC class II compartment (MIIC). There the peptides meet the class II–Ii complex from the trans-Golgi network (Hiltbold and Roche, 2002).

The separate α and β chains of the class II molecules associate with each other in the lumen of the endoplasmic reticulum to form dimers. Immediately three of these dimers associate with a further three molecules of the invariant chain protein (Ii), forming a nine-member heterononamer; this has the amino terminal region of Ii bound in the peptide-binding groove of the class II molecules (Watts, 2001). After binding to the MHC class II molecule, the Ii chain is degraded, leaving only a small peptide called CLIP (class II associated Ii peptide) bound in the peptide-binding groove (Lennon-Dumenil *et al.*, 2002). The function of CLIP may be to prevent the nonspecific binding of peptides, or even regions of unfolded proteins present in the endoplasmic reticulum (ER), by allowing only the peptides with high affinity for the peptide-binding groove to compete with itself and bind in the groove. This process is facilitated by the class II-like molecule DM, which interacts directly with the class II molecules to promote peptide exchange (Denzin and Cresswell, 1995). Once the peptide is bound to the class II molecule, the complex migrates to the cell membrane where it is recognized by Th cells.

DCs have the ability to present exogenous antigens to MHC class I-restricted Tc cells, an event called antigen cross-presentation. Exogenous proteins (not those synthesized by DCs) are an important source of peptide ligands for MHC class I molecules, which then activate Tc cells. Cross-presentation is an important route for the induction of Tc cells specific for tumor antigens and pathogens that do not infect DCs. Although the mechanism of cross-presentation is not fully understood, recent work has shown that antigens contained in the phagosomes of DCs have to be transported to the cytosol, where they are processed by the proteasome with the resulting peptides entering the ER through TAP, exactly as described above (Trombetta and Mellman, 2005). The mechanism involved in the transport from the phagosome to the cytosol is obscure, but nonspecific leakage and specific active transport of low-molecular-weight protein products (3–20 kDa) are the most likely mechanisms (Guermonprez *et al.*, 2003; Rodriguez *et al.*, 1999). TAP-dependent transport of the peptides processed in the cytosol may be either in the ER or in the heterogeneous phagosome-ER compartment (Guermonprez and Amigorena, 2004). It is

clear, however, that DCs have a unique pathway for presentation of exogenous antigens to Tc cells.

B. Subpopulations of Dendritic Cells

DCs can be subdivided into different subpopulations, but, with a few exceptions, these are all characterized by the presence of CD11c (Shortman and Liu, 2002). CD11c is an integrin (α_k chain) located on the DC membrane. Recently, as new subpopulations have been described, the classification of DCs has become more complex. It is possible, however, that not all of these new populations are truly DC subsets or DCs from the same subset. In mice there are at least three subpopulations of DCs: $CD8\alpha^+$ or lymphoids, $CD8\alpha^-$ or myeloids, and $B220^+$ or plasmocytoids. Manz *et al.* (2001) have shown that both the lymphoid and myeloid populations can differentiate into $CD8\alpha^+$ and $CD8\alpha^-$ cells and indicated that $CD8\alpha$ may be more a differentiation, rather than a subset, marker. In humans, DCs do not have $CD8\alpha$ as a marker.

The distribution of the surface markers CD4 and CD11b (the integrin α_M chain of Mac-1) and the interdigitating CD205 (the multilectin domain molecule DEC205) has allowed at least five different DC subsets to be identified in mice (Shortman and Liu, 2002). Spleen contains three such DCs: $CD4^-CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^-$. In lymph nodes, $CD8^+$ DCs are concentrated in the T cell areas while the $CD8^-$ DCs are in the marginal zones. After pathogen stimulation, however, $CD8^+$ DCs can migrate to, and are found in, the T cell areas.

DCs also play an important role in the immune response in mucosa. Of all the mucosal sites, the DCs from the gastrointestinal tissue are the best characterized with Peyer's patches having at least four different subsets (Bilsborough and Viney, 2004). What is important is that the various DC phenotypes exhibit differences in function, as we will describe below.

C. Dendritic Cell Activation

DCs are activated and mature after they sense "danger" signals in the environment. These "danger" signals can be endogenous or exogenous. One source is damaged cells releasing proinflammatory cytokines, or the release of such cytokines by surrounding cells. Other "danger" signals are uric acid and heat shock proteins (Gallucci and Matzinger, 2001; Pulendran, 2004; Shi *et al.*, 2003). Significant sources of exogenous "danger" signals are the pathogens themselves, through their characteristic recognition molecules. The pathogen-associated molecular patterns (PAMPs) present in the

microorganisms are recognized by pattern-recognition receptors (PRR) in DCs and this recognition leads to cellular activation. These receptors are in two groups: (1) endocytic receptors, present on the cell membrane and mainly involved in the uptake of microbes, and (2) signaling receptors involved in the activation of DCs (Medzhitov *et al.*, 1997). Toll-like receptors (TLRs), also involved in signaling, are type I transmembrane proteins. They have a cytoplasmic region that resembles that of the IL-1 receptor and an extracellular domain with several leucine-rich repeats. There are at least 11 different TLRs in mice and humans that recognize various PAMPs (Finberg and Kurt-Jones, 2004; Takeda and Akira, 2005) (Fig. 1). Recognition of these molecules by the TLRs induces the synthesis of proinflammatory cytokines, which helps to generate an antiviral state in the cell in which protein synthesis is dramatically decreased (Fig. 1) (Akira and Takeda,

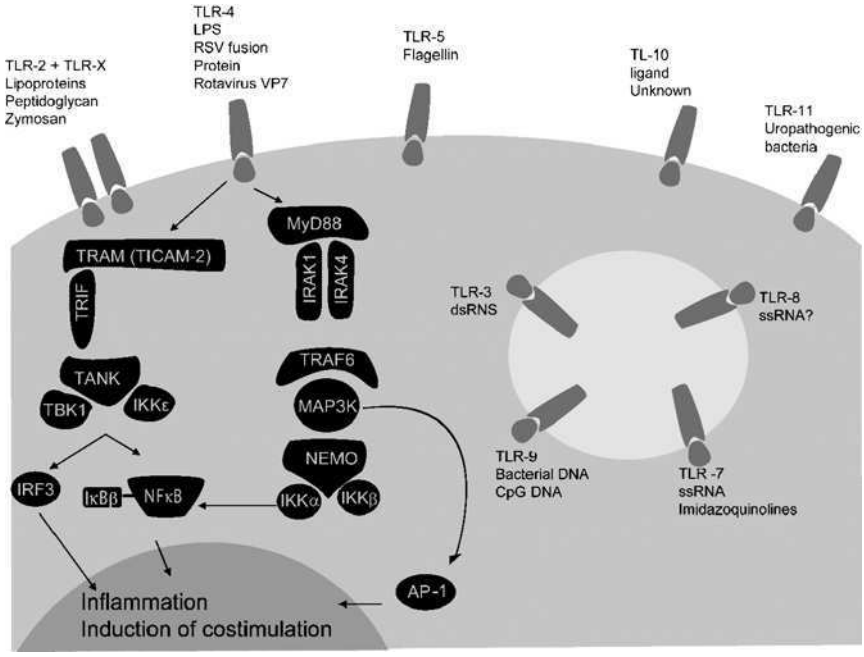

FIG. 1 TLRs present in DCs, their ligands, and signal transduction pathways. Summary of the TLRs present in different subsets of mouse and human DCs. TLR-2 associates with TLR-1 or TLR-6 to form functional heterodimers. TLR-4 requires MD-2 and CD14 to be stimulated by LPS. TLR-3, 7, 8, and 9 are located in endosomes. TLR-9 seems to be absent in human DCs. TLR-11 has been found only in human cells. All TLRs can signal through MyD88 and generate the transcriptional factors NF-κB and AP-1. Only TLR-4 and TLR-3 share a signaling pathway that is MyD88 independent and induce the expression of the transcriptional factor IRF-3 and therefore the production of interferons α and β.

2004). Another type of microbial recognition involves C-type lectin receptors (CLRs), DC-SIGN, and CLR Dectin-1, which mediate the uptake of antigens (Cambi *et al.*, 2005; Rogers *et al.*, 2005). All these receptors and signals help the DC to sense its environment and decode the nature of the danger. In this way, when antigen presentation takes place, all this information is conveyed to the T cell in the form of cytokine signals and through the presence of costimulatory molecules.

The nature of the microenvironment and the stimuli that activate the DC during antigen presentation are important determinants for the instructions given by DCs to T cells for their differentiation into type one, type two, or regulatory T cells. For instance, IL-10 is known to favor differentiation into type two and T regulatory responses while IL-12 stimulates differentiation into type one. Little is known, however, about the precise nature of the intracellular signaling pathways that result in the secretion of any one cytokine. In general, a stimulus through one of the TLRs will result in a type one response, although this is not always the case (Dillon *et al.*, 2004). This suggests that, in response to external stimuli, the DCs can self-regulate the appropriate type of response.

Shortman and Liu (2002) suggested that the different subsets of DCs were programmed to direct the functional polarization of Th cells. This idea has, however, been challenged (Iwasaki and Kelsall, 1999; Maldonado-Lopez and Moser, 2001; Pulendran, 2005). Although plasmacytoid DCs have a very limited capacity for antigen processing and presentation, they respond strongly to viral and microbial challenge by producing INF-α and -β (Shortman and Liu, 2002); these cells seems to be a DC subset skewed to the induction of type one responses. When the cells are activated by the CD40 ligand, however, they undergo type two differentiation (Rissoan *et al.*, 1999).

The different DC subsets may thus be rather plastic in their ability to control the differentiation of T cells. So the antigen concentration, rather than the DC commitment, is responsible for the induction of the different subsets of T cells (Boonstra *et al.*, 2003). At low antigen exposure, DC cells induce type two responses and, at high antigen exposure, they induce type one responses.

D. Dendritic Cell Migration

Migration of DCs from peripheral tissues to lymphatic vessels is a crucial step for antigen presentation to T cells in lymph nodes. Under both normal (homeostatic) and inflammatory conditions, DCs require the expression of the CC-chemokine receptor 7 (CCR7) (Martin-Fontecha *et al.*, 2003; Ohl *et al.*, 2004). The upregulation of CCR7 in inflammatory

conditions correlates with the upregulation of DC maturation markers; under normal conditions, however, CCR7 is upregulated without the DC maturation. Here, uptake of apoptotic cells seems to be sufficient to induce CCR7 (Verbovetski *et al.*, 2002). Once CCR7 is upregulated, DCs follow a gradient of the CCR7 ligands, chemokine ligand 19 (CCL19) and CCL21.

Endothelial cells synthesize two isoforms of CCL21—CCL21-Leu (with leucine at position 65), made in the lymphatic vessels, and CCL21-Ser (with the leucine replaced by serine), made in the subcapsular-sinus lymphatic vessels and lymph nodes but not in afferent lymphatic vessels. CCL19 is expressed by stromal cells of the lymph node T cell areas and by DCs themselves, but not by the lymphatic vessels. Contrary to the classical view that chemokines migrate toward an anatomical site other than the site of their synthesis, the autocrine CCL19 produced by DCs may direct its migration to the lymphatic vessels, possibly following the flow of the interstitial fluid that enters the vessels. Under the influence of CCL21-Leu, DCs then cross the endothelial cells, and travel to the subcapsular sinus, through lymphatic vessels expressing CCL21-Ser, finally reaching the T cell area of the lymph nodes (Randolph *et al.*, 2005).

III. Subpopulations of T Cells

T cells are defined as those lymphocytes that contain T cell receptors (TCR), complexes composed of two variable chains responsible for antigen recognition and CD3 subunits and ζ chains, both of which initiate signaling cascades. The variable chains (V) of most lymphocytes are the product of random rearrangements of the α and β genes; these give rise to the large repertoire of receptors characteristic of T cells. Some T lymphocytes contain, as variable chains, the products of the γ and δ genes, and are therefore called $\gamma\delta$ T cells. When not otherwise specified, conventional $\alpha\beta$ T cells (which recognize peptides associated with self-MHC molecules presented by APCs) will be referred to as T cells. The $\gamma\delta$ T cells and those $\alpha\beta$ cells known as NKT cells recognize limited and distinct groups of antigens and are separate from the machinery of classical MHC presentation. NKT cells and $\gamma\delta$ cells recognize molecular patterns and react immediately upon encountering antigen; they are therefore considered to be a part of the innate immune system (Dieter and Wesch, 2003; Godfrey and Kronenberg, 2004; Ramsey *et al.*, 2002).

Potentially the γ and δ genes recombine with the same variability as the α and β genes, although in reality they have a more restricted repertoire. This repertoire is further restricted, especially in mature individuals, after only a

few clones have been selected for survival (Kabelitz *et al*., 2005). In human adults, only six Vγ genes are expressed, five of which are very similar to each other and have been grouped as Vγ1−Vγ5; the sixth is named Vγ9. Only three Vδ elements are detected in human adults (Vδ1, 2, and 3). The dominant γδ cell in adult blood is Vγ9Vδ2. This is the product of the expansion, during childhood, of the clones containing this rearrangement (Kabelitz *et al*., 2005). They recognize the malaria parasite as well as bacterial pyrophosphate and alkylamine moieties (Altincicek *et al*., 2001); these cells respond rapidly to such antigens by producing proinflammatory cytokines. Cells of the Vδ1 subset are found in epithelial tissues and recognize mainly MHC class I-related molecules (MICA and MICB), which are induced by stress in neighboring cells (Groh *et al*., 1998). A major response of intraepithelial γδ cells is the production of fibroblast, connective tissue, and keratinocyte growth factors, all involved in wound healing (Kabelitz *et al*., 2005). Vδ1 γδ cells also recognize lipid antigens presented by the MHC-like molecule CD1 (Spada *et al*., 2000).

NKT cells, also activated by antigens presented by CD1, are characterized by the expression of specific invariant α chains. These cells, which owe their name to the presence of surface protein markers characteristic of NK cells, recognize glycolipid antigens. They respond to challenge by secreting either type one or type two cytokines (Godfrey and Kronenberg, 2004). They are also involved in peripheral tolerance by expressing the immunosuppressive cytokine IL-10 (Sharif *et al.*, 2002). The role of NKT cells in defense and tolerance depends on their anatomical location, the strength of the stimulus, and communication with other cells of the immune system. In this way, immune cells work as teams and the immune function of a particular population is governed by the community of cells.

Classical T lymphocytes present either CD4 or CD8 as well as the TCR on their surfaces (CD4 and CD8 cells). CD4 and CD8 are important for the recognition of the MHC presenting the antigen to T cells. CD4 associates with MHC II, while CD8 binds to MHC I. This recognition restricts the range of the antigens presented to CD4 or CD8 cells, because MHC II presents peptides of 14–25 residues, while MHC I presents peptides of 8–10 residues. The origin of the antigens presented is also different, with MHC I presenting endogenous peptides and MHC II presenting endocytosed antigens; there are, however, connections between the two antigen-processing pathways (see Section II.B). MHC II expression is restricted to APC, while MHC I is present in most nucleated cells, allowing activated CD8 cells to recognize and kill any nucleated cell displaying a target antigen on its surface.

The major role of CD4 cells (also called helper cells, Th) is to coordinate the immune response by direct communication with B lymphocytes and macrophages through immunological synapses, and by the production

of cytokines. CD8 cells (also called cytotoxic cells, Tc) respond to stimulation through secreting cytokines and by increasing their cytotoxic capacity. Stimulation of CD8 cells leads to the synthesis of granzymes and perforin and also the expression of the Fas ligand, all of which induce apoptosis in infected cells through cytotoxic synapses (Barry and Bleackley, 2002).

Lymphocytes are mainly produced in the thymus from a lymphoid precursor cell that migrates from the bone marrow. When T cells enter the circulation, they are naive cells, incapable of immune function. After activation, they become either effector cells, responsible for immune responses, or memory cells (fast-responding, long-lived cells that differentiate after a first encounter with an antigen and are ready to respond to a second challenge by the same antigen) (Lanzavecchia and Sallusto, 2001a). Lymphocytes are plastic cells and their phenotype and function depend on their origin and immunological history. There are four subpopulations of CD4 effector cells that differ according to the kinetics of IL-4 and IFN-γ transcription or protein secretion upon stimulation (Seder and Ahmed, 2003). There are several more populations of CD8 effector cells that arise in response to viral challenge (van Lier *et al.*, 2003).

The Lanzavecchia group (Sallusto *et al.*, 1999) demonstrated that memory lymphocytes fall into two categories—central memory cells, T_{CM}, residing in lymph nodes, and peripheral or effector memory cells, T_{EM}, in the periphery. These populations are distinguished by the presence or absence of L-selectin (CD62L) and CCR7, a chemokine receptor responsible for locating T cells in the lymph nodes. Cells are further characterized by the presence or absence of other markers (Sallusto *et al.*, 2004; Seder and Ahmed, 2003). For example, the type one differentiation pattern is characterized by the presence of the chemokine receptor CCR4, while type two cells contain another chemokine receptor CXCR3. Originally it was thought that differentiation of these subsets was sequential from naive cells to T_{CM} to T_{EM}, and it was proposed that T_{CM} were not biased toward any pattern of cytokine production—in contrast to T_{EM}, which were already committed to the type one or type two pattern. Subsequently it was shown that in CD8 cells, T_{EM} was conversely the precursor of T_{CM} (Seder and Ahmed, 2003).

In the case of CD4 cells, differentiation is more complex. Naive cells can give rise to both T_{EM} and T_{CM} directly; these can in turn interconvert, with cells being both committed and uncommitted (Song *et al.*, 2005). The state of commitment depends on the availability of appropriate signals, including cytokines. The nature of the antigen encountered by the cells is also a very important determinant of their phenotypic memory. The extent of this will vary according to the strength of the stimulation, stress signals from damaged tissues, and to cell modifications occasioned by infection with pathogenic organisms, particularly viruses.

IV. Immunological Synapses

A. Description of Immunological Synapses

Communication between antigen-presenting cells and T cells occurs through immunological synapses, so called because of similarities with classical synapses. Contacts between these cells elicit specific patterns of aggregation of surface molecules—supramolecular activation clusters. The TCR, CD4 (or CD8 in cytotoxic cells), and CD28 cluster in the center of the contact between cells. The adhesion molecules CD2 and LFA1 cluster to form a doughnut-like ring around the central aggregation, while the larger molecules CD44 and CD43 are specifically located outside the contact area (Fig. 2) (Monks *et al.*, 1998). The formation of immunological synapses is central for lymphocyte maturation and function of the immune response.

Before entering the circulation, immature T cells are confronted by self-antigens in the thymus; during positive selection, cells that are able weakly to recognize self-MHC-peptides are rescued from apoptosis and enter the circulation, while cells that react strongly against self-MHC-peptides are removed, thereby preventing autoreactive mature T cells from appearing in the periphery (negative selection). This process ensures that only functional and self-MHC-restricted T cells leave the thymus. Mature lymphocytes encounter APCs during the immune response. In the antigen recognition phase, DCs make contact with naive T cells; those lymphocytes with antigen receptors specific for the particular peptide displayed by the DC become activated. Later on, in the effector phase of the immune response, synapses between CD4 T cells and B cells are determinants for the process of isotype switching of immunoglobulins.

Communication of CD4 T cells with macrophages is important for activation of their microbicidal activities; CD8 cells also use immune synapses to kill infected cells. In addition, cytotoxic synapses are also part of the mechanism by which regulatory $CD4^+CD25^+$ T cells eliminate activated clones and maintain peripheral tolerance (Grossman *et al.*, 2004).

In cell-to-cell communication, the outcome from the signals accumulated by all the molecules in contact and the cytokines in the medium determines the cellular response of the two cells in contact (Mosmann and Livingstone, 2004). In this way, lymphocytes also send signals to DCs, which induce the expression of cytokines of the original T cell to other subsets of T cells. Memory T cells from a donor mouse were able to propagate their immune response, in term of the cytokines secreted, to naive T cells in a recipient animal, using DCs as messengers (Alpan *et al.*, 2004). Suppressor $CD8^+CD28^-$ lymphocytes also use DCs to generate tolerance in naive lymphocytes, by inducing these cells to express suppressor molecules, such as immunoglobulin-like transcript 3 (ILT3)

FIG. 2 Representation of an immunologic synapse. The T cell is shown in blue and the APC in pink. During immunological synapses both cells reorganize their interacting molecules from all over the cell surface to the contact area, to help maintain the long-lasting interaction necessary for the full activation of the T cells. At the center of the interaction the α and β chains of the TCR (blue) interact with the peptide (red) bound into the groove of the MHC (yellow). CD4, shown here as a green molecule (or CD8 for cytotoxic T cells), recognizes a different area of the MHC. Still at the center of the synapses, a molecule of CD28 is interacting with its ligand in the APC, a CD80 or CD86 costimulatory molecule. Just one molecule of each is shown for clarity of illustration, but all the TCR and CD28 molecules in the T cell are centered in the synapses. Around the central clustering, there is a ring of CD2 and LFA1 adhesion molecules, recognizing their ligands CD58 and ICAM1 in the APC to help maintain a strong adhesion between the cells. The mesh in each of the cells represents cortical actin cytoskeleton and the thick filaments represent microtubules, some of which carry MHC-peptide complexes to the immunological synapses. Signaling molecules are shown as chains associated with the cytoplasmic face of each

and ILT1 (involved in human heart transplant acceptance; Chang *et al.*, 2002) on their surfaces. The same group reports that endothelial cells can also induce tolerance (Manavalan *et al.*, 2004). Tolerization of DCs by suppressor and regulatory T cells in cancer is a major problem, supposing that we want to eliminate tumors by priming T cells with tumor antigens or to potentiate the cytotoxic potential of tumor-specific clones (Banchereau and Palucka, 2005; Finn, 2003).

New methods for imaging cells and organs, fluorescent probes, and molecular constructs have contributed to an impressive advance in the study of immunological synapses. These structures were first described using systems *in vitro*. Long-lasting interactions were observed, and the minimum times of interaction required to elicit a T cell response were described (Huppa *et al.*, 2003; Iezzi *et al.*, 1998; Lanzavecchia *et al.*, 1999; Monks *et al.*, 1998; Underhill *et al.*, 1999). Later simulation of extracellular matrix in which cells could "crawl" allowed brief interactions between cells and the signaling events affecting lymphocyte activity to be described (Gunzer *et al.*, 2000). In lymph node slices, the interactions between APCs and CD4 or CD8 cells highlight the importance of long-lasting contacts for T cell activation (Ingulli *et al.*, 1997, 2002; Norbury *et al.*, 2002; Schaefer *et al.*, 2001). We can now observe cellular interactions *in vivo* when the intact lymph node is observed by two-photon microscopy (Bousso and Robey, 2003). Recently the migration of DCs loaded with antigen from the periphery to the lymph nodes and the subsequent movement of these cells inside the node as they reach maturity have been described. The initial crawling movements are followed by the settling of the cells. Movement becomes confined to cytoplasmic extensions of dendrites, providing an extensive area in which lymphocytes come in contact with DCs to explore the wide range of antigens (Catron *et al.*, 2004; Germain and Jenkins, 2004; Itano and Jenkins, 2003). Video microscopy has allowed computer simulation of the whole process, leading to a better understanding of one of the key processes of antigen recognition (Catron *et al.*, 2004).

Three stages have been described in the priming of naive CD4 T cells (Mempel *et al.*, 2004). In the first stage, T cells scan the range of antigens on the DCs in the lymph nodes; contacts between cells are therefore brief and abundant, inducing a first, but limited, antigen-independent activation of T cells (Revy *et al.*, 2001). Coreceptor molecules including intercellular adhesion molecule (ICAM) 3 are probably involved in these recognition

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receptor, forming the signal transduction machinery or signalosome. Not shown are lipid rafts, other molecules that are present in the immunological synapses (CD45 in Th cells, chemokine receptors, cytokine receptors, other integrins), and peripheral molecules from the extracellular matrix.

interactions (van Gisbergen *et al.*, 2005). The second stage, dependent on the specific recognition of antigens by the TCR, involves a strong and long-lasting interaction between both cells. The high sensitivity and specificity of this interaction is intriguing, because a very small amount of specific peptide is enough to establish a T cell response. Third, the cells separate and there are again brief interactions before T cells migrate to interact with B cells and macrophages, and finally leave the lymph node. The duration of synaptic interaction is different in CD4 and CD8 T cells and depends on their maturation stage. Naive CD8 T cells do not make long-lasting interactions, as do effector and memory CD8 cells (Bousso and Robey, 2003; O'Keefe *et al.*, 2004). Dustin (2004) has proposed that the duration of the interactions may depend on "stop" signals from the TCR that compete with "go traffic" signals from chemokines and their newly synthesized receptors.

The aggregation of molecules that increases adhesion and signaling between cells depends on several factors:

1. interactions between transmembrane proteins;
2. clustering of lipid rafts;
3. the presence of molecules from the extracellular matrix;
4. modification of actin filaments to adapt the cell shape of the lymphocyte to the surface of the APC and to allow the formation of a scaffold for cell signaling molecules; and
5. movement of molecules and vesicles loaded with MHC-peptide in the dendritic cell by the microtubules to specific locations in the cell membrane.

B. Interactions between Transmembrane Proteins

Interactions between the peptide-MHC complex and the TCR confer the specificity needed for activation of lymphocytes. The affinity of this interaction is very weak and dendritic cells present only a small proportion of the antigen recognized by T cells, most of the peptides presented being self-antigens (Huppa and Davis, 2003). Typically, the affinity of this interaction has a K_d of 10^{-5}–10^{-7} M, quite low considering the concentration of peptides. The amount of peptide needed to activate a T cell depends on the particular circumstances in which the synapses are formed. Only three molecules are needed for activating an immature lymphocyte for negative selection, whereas 300 molecules are needed to activate a naive lymphocyte and 50 to activate an effector cell (Dustin, 2002).

Other interactions are needed to maintain synapses in place and allow the long-lasting interactions needed for full activation of T cells. The adhesion molecules CD2 and LFA1 play a major role in maintaining synapses, but in

naive T cells, these molecules are in an inactive conformation with very low affinity for their ligands. The first interactions of the large molecule CD43 with the MHC I molecule are important for increasing the affinity of CD2 for its ligand (Stockl *et al.*, 1996). The integrin LFA1 develops increased affinity for its ligand in response to initial ("inside-out") signals from chemokines and the TCR (Dustin *et al.*, 2004). Once adhesion molecules are activated, the signals from the TCR, coreceptor, and adhesion molecules interacting with their ligands lead to stabilization of the immunological synapses. The immunological synapses are versatile structures that can be reestablished to enable the polarization of T cells to the APCs that present the peptide with the greatest affinity for the TCR (Depoil *et al.*, 2005).

Interactions between individual proteins in complexes and preassociations between molecules help the establishment of the scaffold of proteins mediating the particular signaling cascade, or "signalosome." Proteins are stabilized within the membrane by hydrophobic and other weak interactions. For example, the CD3-TCR complex has a striking feature in its transmembrane domain because of charged residues that interact between the components to form the complex between the three signaling dimers, CD3$\delta\epsilon$, CD3$\gamma\epsilon$, and $\zeta\zeta$ with the TCR$\alpha\beta$ chains responsible for antigen recognition. An elegant study has shown that the organization of the complex is built around important ionic interactions involving lysine and arginine in the TCR$\alpha\beta$ with aspartate or glutamate residues in the CD3 and $\zeta\zeta$ dimers (Call *et al.*, 2002).

C. Role of Lipid Rafts

Lipid rafts, distinctive membrane areas rich in cholesterol and sphingolipids, have been implicated in the formation and maintenance of the immunological synapses. Two physical states are possible within the lipid bilayer at physiological temperatures, a liquid-disordered phase and a liquid-ordered phase, which is characteristic of lipid rafts. In the latter, liquidity results from the presence of cholesterol, which, despite the presence of long highly saturated fatty acid chains, generates order as well as lowering the fusion point of the membrane. Raft-associated proteins have long transmembrane domains or are anchored to the membrane by association with GPI or posttranslational modifications such as myristoylation or palmitoylation (Quest *et al.*, 2004). The concentration or separation of different membrane proteins in the plane of the membrane is fundamental for rafts to function as platforms for signal transduction (Simons and Toomre, 2000).

Studies of the function of lipid rafts have taken advantage of the insolubility of the rafts during extraction with mild detergent. The principle is that the detergent will more easily solubilize the liquid-disordered bilayer, with

the lipid rafts remaining insoluble. There are a number of proteins that are constitutively associated with lipid rafts or that become transiently associated with these structures upon cellular stimulation. In the most common protocol, membrane studies involve sucrose density gradient ultracentrifugation of extracts from cells lysed with Triton X-100. The problem with this method is that extraction is at low temperature that, by itself, causes aggregation of lipids. The association of the detergent with the membranes further increases this aggregation. Studies of lipid rafts were refined by the use of weaker detergents such as CHAPS, lubrol WX, Brij96, and Brij98 (Chamberlain, 2004). Brij98 allows the extraction of lipid rafts at physiological temperatures (Pike, 2004). Several proteins thought to be raft associated proved to be outside rafts when another detergent was used—proteins in the same microdomain can display different levels of detergent solubility (Chamberlain, 2004). This suggests that there are several types of rafts with different protein compositions and arrangements of sphingolipid and cholesterol; these may also have GPI-linked proteins or gangliosides.

The involvement of lipid rafts in immunological synapses has been questioned by new evidence from studies using fluorescence resonance energy transfer (FRET), which allows visualization of the associations between molecules (<10 nm apart) in living cells. Glebov and Nichols (2004) were unable to detect any clustering of the traditional raft markers, GPI-linked proteins and GM1, in lymphocyte cell lines in the regions in contact with anti-CD3 beads. With the development of the single-particle tracking technique, we can follow the behavior of individual molecules moving in the cell membrane. Such studies support the existence of lipid rafts in the lymphocyte membrane (Pralle *et al.*, 2000). Methods for quantifying diffusion parameters, such as florescence correlation spectroscopy, have confirmed the existence of raft components by monitoring the diffusion coefficient of green fluorescent protein (GFP)-tagged proteins that partition in the lipid rafts and by showing differential trapping relative to other membrane proteins (He *et al.*, 2005).

The current model of involvement of lipid rafts in the establishment of the supramolecular activation cluster involves the TCR/CD3 complex being translocated to the raft microdomains after ligation (Horejsi, 2003). He *et al.* (2005) proposed a new model of TCR signal transduction in raft microdomains in which TCR is located in a microdomain rich in cholesterol and sphingolipids (not necessarily with GPI or GMI ganglioside) that can be isolated by Brij98 but is disrupted by cold Triton X-100. This structure is dynamic, allowing rapid molecular movement in and out of the rafts. This model better explains why experiments that target the central molecules of the supramolecular activation cluster outside rafts diminish cell activation. Many interactions between proteins are of weak affinity and will not be sustained in a disordered membrane environment, but are possible within lipid rafts, and are essential organizers or specific rafts' subsets.

Compelling evidence suggests that different sorts of rafts exist side by side in the same cells (Fielding and Fielding, 2003). Several classes of proteins can alter the morphology or function of raft membrane domains in response to environmental signals—such as caveolin (implicated in movements of special raft structures involved in endocytosis, named caveolae, by linking of microfibers and microtubules to membrane receptors), the reggie/flotillins, LAT, MAL/BENE, stomatins, and VIP36 (Quest *et al.*, 2004). With this view it is important to keep in mind that classical raft-associated proteins, such as GPI-containing proteins or GMI, may or may not colocalize with proteins that occur in specialized types of rafts.

The Glimpcher group (Maldonado *et al.*, 2004) has shown that an association between the IFN-γ receptor and the TCR during T cell activation leads to the differentiation of lymphocytes to a type one pattern. They proposed that the close contact between the TCR and IFN-γ receptor could allow cross-talk between the signaling proteins preassociated with each of these receptors in specialized lipid rafts. The inhibitory signals coming from IL-4 prevent the two receptors from associating and result in a type two differentiation. The IFN-γ receptor is associated with rafts rich in caveolin, glucose-regulated protein 58, a thiol-dependent isomerase, and chaperones that associate with the JAK-STAT signaling machinery in a complex called a "statosome" (Sehgal, 2003). Altogether these results suggest that rafts function as pre-assembled cassettes of signaling proteins that may come together in response to signals from the environment, thereby affecting the response of the cell.

D. Role of the Extracellular Matrix

The extracellular matrix plays important roles in cell survival, delivery of cell signals, development, migration, and cell shape. It is also important in the aggregation of molecules during lymphocyte priming. Proteoglycans establish a network that traps soluble signals on its surface. When soluble ligands are presented to T cells attached to proteoglycans, they induce the clustering of receptors on the lymphocyte membrane. Chemokines usually attach to proteoglycans, which also help to establish the concentration gradient that guides the movement of the cells.

Agrin, a characteristic proteoglycan of the extracellular matrix, is directly involved in the establishment of immunological synapses. Khan *et al.* (2001) showed that minutes after activation, lymphocytes alter the epitopes recognized by two different anti-agrin monoclonal antibodies (mAbs), changing them from "agrin$_{res}$" to a smaller form of the molecule named "agrin$_{act}$," which promotes synapse formation. Dustin (2002) proposed that the change in the molecular mass of agrin might be the result of the removal of heparan

sulfate from agrin during activation. This idea is supported by $agrin_{act}$ being produced by heparanase *in vitro*, and because activated T cells produce heparanase (Fridman *et al.*, 1987; Parish *et al.*, 2001). An important role of agrin in the clustering of acetylcholine receptors in the neuromuscular junction has long been known (Hall and Sanes, 1993).

Glycosylation patterns of cell-surface proteins affect the distribution of the TCR during antigen presentation and hence establish a threshold for T cell activation. Naive lymphocytes are highly glycosylated on their cell surface and have a higher threshold of activation than effector or memory cells. A knockout study of mice lacking *N*-acetylglucosaminyltransferase has shown that the glycosylation pattern of the cell surface limits raft clustering in resting T cells (Demetriou *et al.*, 2001). A high level of glycosylation promotes the binding of galectin 3 to glycoproteins and inhibits TCR capping. As predicted, the knockout mice exhibited increased TCR clustering and lymphocyte activation. The phenotype of these knockout mice is mimicked by treatment with a sugar inhibitor of galectin 3 binding. This suggests that galectin 3 binding to carbohydrates on the cell surface would limit the movement of molecules in the cell membrane, preventing the formation of the supramolecular activation cluster.

Integrin-mediated adhesion to the extracellular matrix or to other cells induces signaling cascades leading to rearrangement of the actin cytoskeleton. This mechanism is important in cell migration and also in the changes of cell shape that allow better contact between cells during antigen presentation. Integrins also induce downstream signals that lead to survival, proliferation, and induction of effector functions in lymphocytes (del Pozo *et al.*, 2004).

E. Actin Cytoskeleton Rearrangements

The first visible sign of cell activation is the polarization of the rounded lymphocyte into an elongated flexible cell. Integrin signals trigger signaling cascades that induce the formation of focal contacts and actin polymerization in particular structures: filopodia, lamellipodia, and pseudopodia. Activation of specific forms of the motor protein myosin in contractile actin fibers contributes to the elongation of the cells and the definition of a front (leading) and a rear end (uropod) (del Pozo *et al.*, 1998; Gaus *et al.*, 2005; Jacobelli *et al.*, 2004; Tooley *et al.*, 2005).

The association of the cortical actin cytoskeleton with the cell membrane maintains cell shape. This association depends on proteins of the ezrin-radixin-moesin (ERM) family that link the actin cytoskeleton to cytoplasmic tails of abundant surface proteins. ERM proteins link the cortical actin filament network with the plasma membrane. Their adaptor function

depends on the phosphorylation of a threonine motif that is common to all these proteins. In a resting cell, ERM proteins are phosphorylated and the cortical cytoskeleton is closely associated with the entire plasma membrane with abundant transmembrane proteins like CD43 and CD45. Initial signaling cascades trigger the activation of Rac1 (a protein of the Rho family of GTPases) that leads to dephosphorylation and detachment of ERM proteins from the membrane receptors, making the cells less rigid. Altogether these changes serve to maximize the contact area between APCs and lymphocytes (Dustin and Cooper, 2000; Faure *et al.*, 2004).

Initial signals are mediated by chemokines, cytokines, and contacts between proteins protruding from the surface of the cell. The sparse signals of a few TCR complexes with MHC-peptides are also important in initiating cell activation. In addition to inducing actin polymerization and flexibility in T cells, the primary signals also generate "inside-out signaling," which increases the affinity of integrins for their ligands in the APC (Dustin *et al.*, 2004).

Integrins have three stages of activation. When integrins are inactive, they are in a closed conformational state and are unable to bind to their ligands. Activation signals, coming from selectins or chemokines, result in conformational changes which increase affinity for their ligands. Another important change is an increase in integrin mobility, promoted by the association of integrin tails with the polymerizing cytoskeleton. This involves the membrane association of the protein adaptors RapL and Talin, which link integrin tails to polymerizing actin. The net result of increased motility with increased ligand affinity is the clustering of integrins on the cell in contact with the antigen-presenting cell (Dustin *et al.*, 2004).

The proximity of cells due to interactions between adhesion molecules makes it easier for the TCR to interact with the MHC-peptide. Coreceptors, also redistributed in the contact area during cell polarization, make contact with their ligands, and, acting together, the interacting proteins, the cortical cytoskeleton, proteins from the extracellular matrix, and the lipid rafts stabilize the immunological synapses.

F. Special Delivery of Vesicles

The immunological synapse has two main roles: (1) to identify antigens and hence elicit an adaptive immune response, and (2) to direct lytic molecules or cytokines to the contact area during the effector phase of lymphocytes. The tubulin cytoskeleton is important in both processes. During the recognition phase, microfilaments containing MHC-peptides are directed toward the membrane in a rapid response to lipopolysaccharide (LPS) stimulation (Boes *et al.*, 2002; Chow *et al.*, 2002). This response dramatically increases

the amount of antigen presentation on the surface or the APC. Apart from this general response, antigen-loaded MHC molecules are specifically directed to the contact area of the established immunological synapse by microtubules loaded with vesicles from the lysosomal compartment of the APC (Boes *et al.*, 2002).

During the effector phase of the immune response, cytotoxic lymphocytes deliver lysozyme, perforin, and granzymes to infected cells presenting the target cytotoxic T lymphocyte (CTL) antigen. This mechanism serves specifically to concentrate these dangerous substances at the surface of the cells that are to be eliminated. The directed delivery of vesicles to the contact area is not restricted to the process of cytotoxic lysis, as it is also observed for the directed delivery of cytokines during contact between cells (Alpan *et al.*, 2004).

V. T Cell Activation

When T cells encounter mature APCs loaded with their cognate antigen, they become activated. Cell activation is a generic term that groups signaling events leading to positive cell responses; these include cell survival and proliferation, cytoskeletal rearrangements, changes in gene expression, and increased metabolic activity. Cells can also respond in other ways—for example, by ignoring the antigen or by becoming unresponsive or apoptotic (Fig. 3). Positive responses are actually accompanied by later negative signals that help to maintain homeostasis (Heissmeyer *et al.*, 2004; Riley and June, 2005).

Cell activation starts with the phosphorylation of immunoreceptor tyrosine activation motifs (ITAMs) located in the cytoplasmic tails of the CD3 and ζ chains of the TCR and of some coreceptor molecules. This phosphorylation results in the clustering of enzymes and adaptor molecules in the proximity of the plasma membrane receptors, forming the signalosome, a "scaffold" of signaling molecules (Fig. 3) (Alarcon *et al.*, 2003). The proteins involved in the TCR-dependent signaling mechanism include tyrosine kinases, serine-threonine kinases, small GTPases [and their associated regulators, the guanine exchange factors (GEFs) and the guanylate activating proteins (GAPs)], phosphatases, adaptor proteins, and transcription factors (Baldari *et al.*, 2005; Jordan *et al.*, 2003; Koretzky, 2003; Veillette, 2004a,b). It is beyond the scope of this chapter to encompass all the work published in this field; we have therefore confined ourselves to a brief account of the major pathways and those recent advances that have led to a better understanding of the overall process.

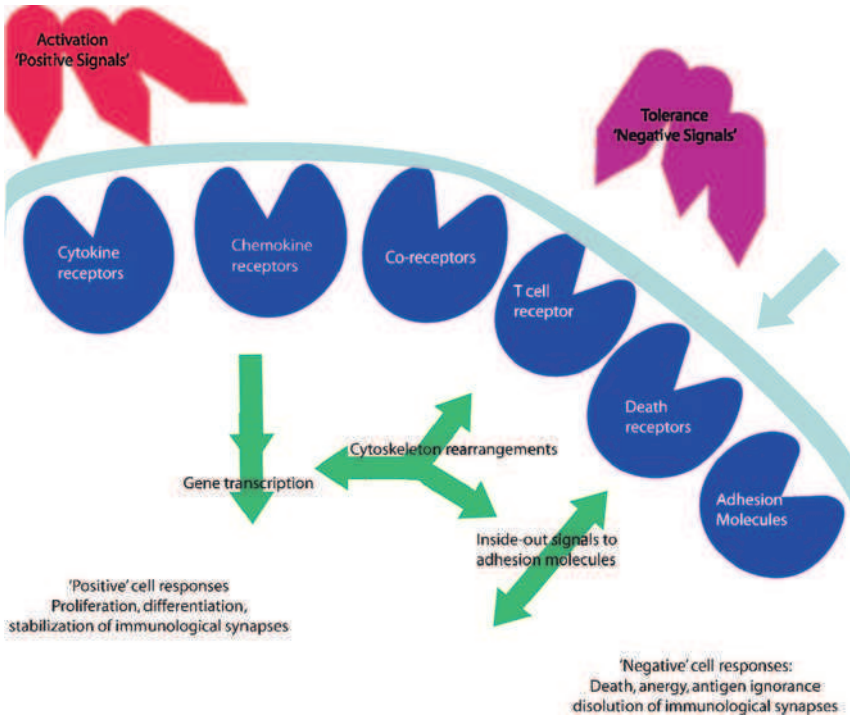

FIG. 3 Summary of T cell responses. T cells receive a plethora of positive and negative signals from their environment. These signaling molecules are recognized by cell-surface receptors that activate or inhibit cell processes (gene transcription, cytoskeleton rearrangements, modification of cell traffic, inside-out signals to adhesion molecules) that result in positive or negative cell responses.

There are four families of nonreceptor tyrosine kinases involved in T cell activation—Src, Syk/SAP-70, Tek, and Csk.

In T cell activation, the most important Src kinases are lck and fyn. These phosphorylate the receptor ITAMs. These phosphorylated ITAMs are docking sites for the tyrosine kinases SAP-70 and Syk, both of which contain SH2 domains. In turn, these latter enzymes phosphorylate adaptor proteins that cause clustering of other signaling molecules (Fig. 4). Of these adaptor proteins, LAT is phosphorylated early by SAP-70 and acts as a link to downstream signal transduction events by recruiting PLCγ—also phosphorylated by SAP-70—and Itk (and other members of the Tek family of tyrosine kinases) to the plasma membrane (Sommers *et al.*, 2004). Itk further phosphorylates phospholipase (PL) Cγ, which initiates two other signaling pathways—one PLCγ product, diacylglycerol, recruits protein kinase Cs

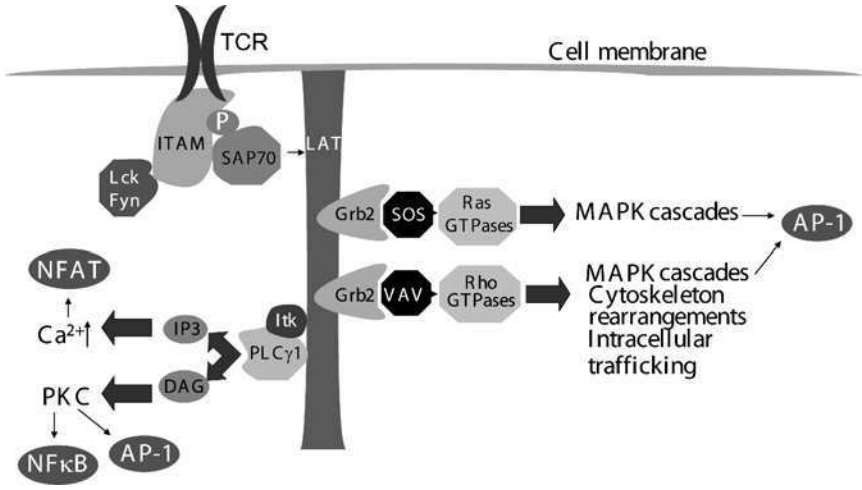

FIG. 4 Signaling pathways initiated by the TCR. T cell activation starts by tyrosine phosphorylation of ITAMs present in the cytoplasmic tails of ζ and CD3 chains of the TCR (or other coreceptors). SAP70 tyrosine kinase binds these phosphorylated ITAMs and in turn recruits the scaffold protein LAT. PLCγ, adaptor protein Grb2, and tyrosine kinase Itk bind to LAT. Itk and SAP70 phosphorylate PLCγ to activate its catalytic activity, allowing the production of diacylglycerol (DAG) and inositol 3,4,5-triphosphate (IP_3) from phosphatidylinositol 4,5-biphosphate. DAG activates PKC, which leads to the activation of NF-κB and AP-1 transcription factors, and IP_3 opens calcium channels in the endoplasmic reticulum resulting in an increase in cytoplasmic Ca^{2+}, finally resulting in the translocation of NFAT transcription factors. LAT-associated Grb2 binds the GTPase activators vav and sos, which in turn activate the Rho and Ras GTPases, resulting in MAPK cascades and the activation of the AP-1 transcription factor. In summary, the TCR signalosome initiates four signaling cascades (arrows), which result in the activation of three transcription factors and other cellular responses not shown (cytoskeletal rearrangements and changes in intracellular traffic).

(PKCs) to the plasma membrane while the other, inositol 2,4,5-triphosphate, opens intracellular Ca^{2+} channels, leading to a movement of Ca^{2+} from the endoplasmic reticulum to the cytosol (Koretzky, 2003; Krogsgaard and Davis, 2005).

The increase in cytosolic [Ca^{2+}] activates Ca^{2+}-calmodulin-dependent kinases (CamK) and the phosphatase calcineurin. The effect of the cytosolic [Ca^{2+}] depends on the time over which the raised concentration persists. A high, but very transient, release of Ca^{2+} leads to the activation of mitogen-activating kinases (MAPK), while a sustained peak of Ca^{2+} results in activation of calcineurin (Berridge, 1997). Calcineurin dephosphorylates NFAT (nuclear factor of activated transcription factors). This dephosphorylation unmasks the nuclear localization signal, allowing NFAT to translocate to the nucleus, where it induces cytokine gene expression—including that of IL-2, which promotes proliferation of T cells (Hogan *et al.*, 2003).

The most important serine-threonine kinases involved in TCR signaling are the MAPKs, PKCs, and PKB (also known as Akt) (Bauer and Baier, 2002; Matsuda and Koyasu, 2002). These enzyme families are the prime constituents of the specific signal-transduction pathways. Each of them is composed of several members; it is the specific members of each family that determine the cell response. For any given response, the specific proteins associate with each other according to the nature of their binding and other regulatory domains. There are very many associations. For example, although SH2 domains recognize specific sequences of amino acids including phosphorylated tyrosine, there are 116 different SH2 domains, all varying from each other in their signature sequences (Machida and Mayer, 2005). Regulatory domains of proteins also provide specificity (Pawson and Nash, 2003), and, to add further complexity, each kinase family can be activated by different mechanisms.

There are three major MAPKs—p38 MAPK, extracellular signal-regulated kinase (ERK), and c/Jun NH_2/terminal kinase (JUNK) (Fig. 5). These enzymes control phosphorylation of transcription factors and chromatin

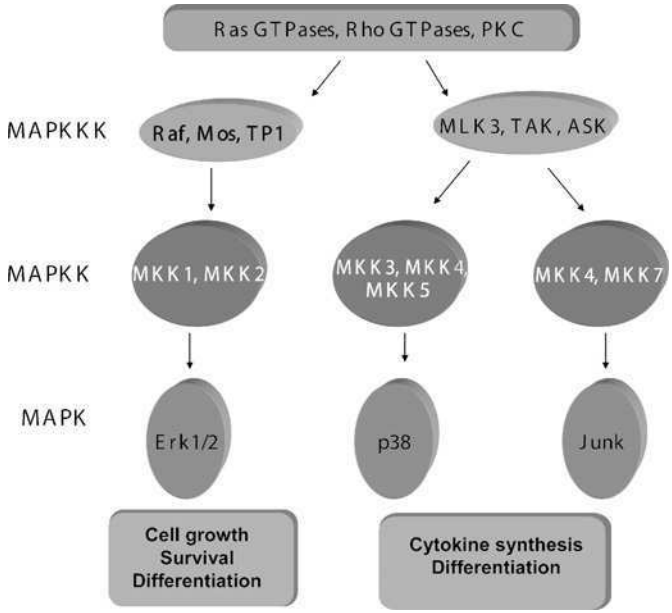

FIG. 5 MAP kinases cascades. Ras and Rho GTPases and PKC signals initiate MAP kinase cascades by activating MAPKKK. These enzymes in turn activate MAPKK, which are MAPK activators. This activation cascade is carried out by phosphorylation chains. Three main MAPK are activated: ERK, which promotes cell growth, differentiation, and survival, and p38 and JUNK, which promote cytokine synthesis and growth.

remodeling, processes leading to various important biological responses. The ERK pathway primarily mediates cell growth, differentiation, and survival, while the p38 and JUNK pathways control the synthesis of cytokines leading to proinflammatory responses, cell differentiation, and, in some cases, proapoptotic and growth inhibitory signals (Matsuda and Koyasu, 2002).

Upstream from the MAPKs are MAPK kinases (MAPKK), which activate MAPKs by phosphorylation of both threonine and tyrosine residues (Fig. 5). The ERK kinases are phosphorylated by the MAPKKs Mkk1 and Mkk2, p38 MAPK by Mkk3, Mkk4, and Mkk6, and JUNK by Mkk4 and Mkk7. These Mkks are in turn controlled by phosphorylation by MAPKK kinases (MAPKKK). The MAPKKK activating the ERK pathway are Raf kinases, Mos and Tpl2, while those activating JNK and p38 include mixed-lineage kinases (Mlks), Mekk kinases, Tak1, Ask1 and 2, and Tlp-2. The extensive MAP kinase networks and the multiplicity of regulators involved, with the tight associated specificities, permit the wide range of cellular responses to the diverse stimuli encountered (Johnson *et al.*, 2005; Uhlik *et al.*, 2004).

The relocation of signaling molecules to the specific area where receptors are clustered is important for their activity. Still upstream of the MAPKKK are small G proteins of the Ras (for Erk pathway) and Rho (for JUNK and p38 pathways) families, whose controlling proteins associate with adaptor proteins in the signalosome. Vav and SOS are among the best known small GTPases activators, associated with adaptor proteins in the early signalosome, that activate Ras and Rho GTPases, respectively (Fig. 5) (Blanchard, 2000; Gallucci and Matzinger, 2001; Tybulewicz, 2005).

PKC activation leads to the induction of MAPK pathways and also to the activation of inhibitory kappa kinases (IKK), a complex of two kinases, IKK1/α and IKK2/β, and the NEMO/IKKγ regulatory subunit (Fig. 6). Phosphorylation of a group of inhibitory proteins (IκB) by IKKα and β marks IκB for ubiquitinylation and degradation, allowing NF-κB to migrate to the nucleus and activate the expression of cytokines and survival genes. The road from PKC to IKK activation has only recently been described, after the discovery of the adaptor protein CARMA (Lin and Wang, 2004; Schmidt-Supprian *et al.*, 2003). CARMA is a member of the guanylate kinase (MAGUK) proteins, which present a signature motif and play important roles in regulating the interface between membrane components and cytoskeletal proteins. CARMA is constitutively associated with the plasma membrane and is recruited to the immunological synapses after lymphocyte stimulation. CARMA brings together the Bcl10, PKCθ, and IKK complex, resulting in the ubiquitinylation of NEMO/IKKγ, leading to IKK activity. PKCθ is phosphorylated and recruited to the membrane by the 3-phosphoinositide-dependent kinase 1 (PDK1), which is also important for the recruitment of the CARMA complex to lipid rafts (Lee *et al.*, 2005).

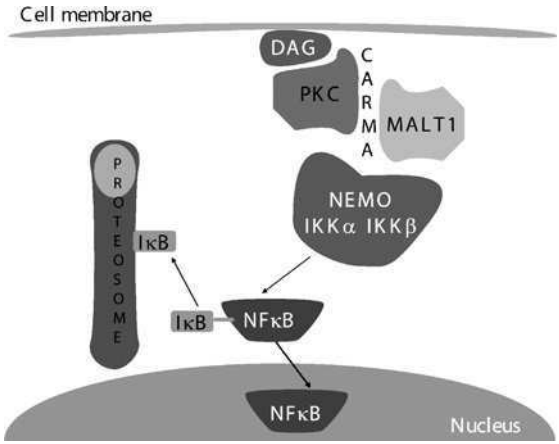

FIG. 6 Activation of NF-κB by PKC-mediated signals. PKC binds DAG in the plasma membrane and associates with the guanylate kinase adaptor protein CARMA, constitutively associated with the plasma membrane, which is recruited to the immunological synapses upon lymphocyte stimulation. CARMA bring together MALT, PKC, and the IKK complex, resulting in the ubiquitinylation of NEMO, leading to IKKα/β activity. Phosphorylation and ubiquitinylation of IκB by activated IKKα/β tags the protein for degradation by the proteasome. At the same time, the NF-κB transcription factor is liberated from its IκB inhibitor and is translocated to the nucleus, where it activates transcription of specific genes.

Naive T cells have a low metabolic rate that is greatly increased on activation. This increase in metabolic activity is a result of external stimuli. One of the surface proteins known to be involved is the coreceptor CD28 (Frauwirth *et al.*, 2002). After the T cell has bound through the TCR, CD28 activates PI_3K with the resultant formation of phosphatidylinositol 3,4,5-triphosphate at the intracellular face of the cell membrane. This constitutes the intracellular docking site for proteins containing a PH domain, the most important of which is PKB.

PKB, also activated by phosphorylation by PI_3K, phosphorylates several targets in T cells, leading to an increase in cell cycle initiation coupled with a decrease in apoptosis (Fruman, 2004). This is accompanied by increased cell growth, together with parallel changes in rates of protein synthesis, general metabolism, and glucose transport (Plas and Thompson, 2005). The extent of cell cycling and of basal metabolism is constrained by inhibitory factors in naive cells. The overall effect of the action of PKB is to stimulate activity by counteracting these negative regulators (Fig. 7) (Song *et al.*, 2004). PKB phosphorylates and thereby tags for degradation the tuberin complex TSC1-TSC2, which inactivates the mammalian target of rapamycin (mTOR). The degradation of its inhibitors results in mTOR activation and

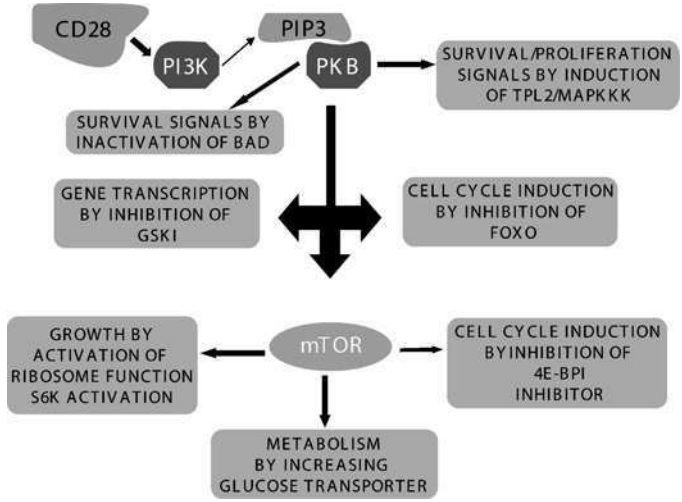

FIG. 7 Signaling pathway for energy, life, and proliferation. CD28 activates an energy and survival pathway through the activation of PI_3K. The resultant formation of phosphatidylinositol 3,4,5-triphosphate (PIP_3) recruits PKB, leading to the activation of the MAPKKK Tpl2, the inactivation of the proapoptotic protein BAD, and the O subgroup of Forkhead box transcription factors (FOXO) involved in cell cycle arrest, blocking of GISK activity, and phosphorylation and degradation of the tuberin complex TSC1-TSC2, which inactivate the mammalian target of rapamycin (mTOR). The degradation of mTOR inhibitors results in its activation and in turn in the activation of S6 kinase, the inhibition of the cell cycle inhibitor 4E-BP1, and the activation of glucose transporter synthesis.

in turn in the activation of S6 kinase—important for ribosome function and activation of the translation machinery. Additionally, mTOR inhibits 4E-BP1, whose repression allows the cell to enter the cell cycle. Another protein inhibited by PKB is the glycogen synthase kinase/3 (GSK3), which is involved in nuclear export of NFAT and β-catenin transcription factors. PKB also leads to the inactivation of the O subgroup of Forkhead box transcription factors (FOXO) involved in cell cycle arrest. Finally, PKB leads to the activation of the MAPKKK Tpl2, which leads to the activation of IKK and NF-κB (Fruman, 2004).

T cell activation is finely controlled at every step by an integrated series of negative regulators. These include phosphatases reversing the activities of kinases, inhibitory adaptors kidnapping signaling molecules (e.g., SLAP), the csk inhibitory tyrosine kinase that phosphorylates an inhibitory site in the Src kinases, and degradation-promoting ubiquitin-ligases (e.g., Cbl). Inhibitory receptors, including MHC-recognizing inhibitory receptors, first characterized in NK cells, the Fc receptor, and the co-receptor CTLA4 also

help to establish the threshold of necessary positive signals to overcome the regulation by negative signals in lymphocyte activation (Carter and Carreno, 2003; Krebs and Hilton, 2001; Rao *et al.*, 2002; Singer and Koretzky, 2002).

Once cells are activated, the secretion of cytokines and upregulation of cytokine receptors enhance the expansion of the activated clones and their differentiation into effector cells. The first cytokine to be secreted is IL-2, which delivers mitogenic signals at the start of the immune response. Cytokines delivered by antigen-presenting cells also determine the outcome of activation. In the presence of IL-12, IL-18, or IFN, T cells differentiate into type one, while IL-4 drives the differentiation toward type two cells (O'Garra and Murphy, 1996). When antigen presentation is accompanied by IL-10 or tumor growth factor (TGF)-β, however, T cells develop tolerance (Chen, 2003).

VI. T Cell Differentiation

A naive lymphocyte is not a functional immune cell. To exert its functions, T cells differentiate into two major populations of effector cells: type one and type two. Type one cells produce IFN-γ and coordinate systemic cell-mediated immune responses, controlled by cytotoxic T cells, activated macrophages, and NK cells. Type two cells secrete IL-4, IL-5, and IL-13 and induce a barrier immunity through the induction of neutralizing antibodies, activation of mast cells, eosinophilia, mucus production, and gut muscle hypercontractility to facilitate expulsion of parasites (Finkelman *et al.*, 1997; Maizels and Yazdanbakhsh, 2003; Santana and Rosenstein, 2003). T cell differentiation is a complex process involving four developmental stages (Grogan *et al.*, 2001; Santana and Rosenstein, 2003):

1. activation of cytokine gene expression;
2. commitment of the cells;
3. silencing of the opposing cytokine genes; and
4. physical stabilization.

A. Activation of Cytokine Gene Expression

The first stage is mediated by the signals from the TCR and coreceptor molecules and can occur in the absence of cytokines. The length and strength of the TCR signals and the combination of signals from the coreceptors determine the outcome of the cell response. Low-affinity interactions and

high antigen doses drive cell differentiation to the type two pattern, while high-affinity interactions result in type one differentiation (Hosken *et al.*, 1995). Different levels of lymphocyte activation result in differences in the duration of the activated state of signaling molecules and the recruitment of different molecules to cytoplasmic tails of the receptors. This affects the nature of the activated signaling cascades that appear to play a key role in the differentiation pathway of T cells. Calcium mobilization drives differentiation into the type one pattern, whereas PKC-mediated signals result in activation of the type two pathway (Noble *et al.*, 2000). The best known coreceptor molecule is CD28, which recognizes the costimulators CD80 and CD86, present in APCs. CD28 signals can drive differentiation toward the type one or type two patterns, depending on the ligands. Blocking CD80 leads mainly to type two development and protection in the type one-associated model disease experimental allergic encephalomyelitis, while blocking CD80 increases IFN-γ production and the severity of the disease (Kuchroo *et al.*, 1995). CTLA4 and ICOS, belonging to the CD28 family, are also important determinants of the differentiation pattern of T cells. CTLA4 engagement enhances the type one and ICOS signals the type two differentiation patterns (Dong *et al.*, 2001; Kato and Nariuchi, 2000). Another molecule involved in the determination of T cell differentiation is CD134 (OX40), a molecule of the TNF family, upregulated during cell activation, which promotes cell survival and type two development (Croft, 2003). The signals of the adhesion molecule LFA1 are implicated in T cell differentiation to the type one pattern, because the use of antibodies to block LFA1 interactions with its ligand ICAM1 leads to an overproduction of type two cytokines (Smits *et al.*, 2002).

Activation of T lymphocytes leads to the activation of four main signaling pathways, as shown in Fig. 4, but the building blocks described for these signaling pathways are families of proteins and not individual molecules. Within these signaling pathways, a number of specific signaling molecules are involved in type one or type two differentiation, as summarized in Table I. The initial response to TCR activation signals results in the induction of three main families of transcription factors: AP-1, NFAT, and NF-κB (Fig. 4). The activation of AP-1 is the result of MAPK cascades, induction of NF-κB is a consequence of the activation of PKC, and a sustained increase in intracellular calcium results in NFAT migration to the nucleus. The AP-1 family includes as members Jun, Fos, Maf, and ATF. Although AP-1 activity is markedly induced in both phenotypes of differentiating cells, a specific complex of JunB with c-Fos has been identified only in differentiating type two cells. Transcription of JunB is similar in type one and type two differentiating cells, but the protein is more stable in type two cells, because of the induction, in type one cells, of the E3 ligase, Itch, which tags JunB for degradation (Fang *et al.*, 2002; Li *et al.*, 1999).

TABLE I

Effect of Specific Signalling Molecules on T Cell Differentiation[a]

Family of proteins	Specific member	Phenotype induced	Evidence
Src kinases	Lck	Type two	DN Lck fail to develop into type one but not type two cells (Yamashita *et al*., 1998)
Src kinases	Fyn	Type one	Fyn negative clones develop into type two but not type one cells (Tamura *et al*., 2001)
SYK kinases	SAP70	Type one	Inhibition of SAP70 activity with piceattannol induces type two differentiation (Tanaka *et al*., 2003)
SYK binding preoteins	SLAT	Type two	This protein inhibits SAP70 and is selectively induced in type two cells (Yamashita *et al*., 1999)
Ras GTPases	Ras	Type two	DN Ras shows a normal type one development but not type two (Yamashita *et al*., 1999)
Rho GTPases	Rac 2	Type one	Rac 2 is preferentially induced in type one cells and induces IFN-γ gene expression (Li *et al*., 2000)
MAPK	JNK 1	Type one	Mice deficient in JNK 1 increased the production of type two cytokines (Dong *et al*., 1998)
MAPK	JNK 2	Type one	JNK 2 is selectively induced in type one cells, and the JNK 2 negative cells show normal type two but not type one development (Yang *et al*., 1998)
MAPK	P38	Type one	p38 specifically induces IFN-γ expression, and inhibition of this enzyme impairs IFN-γ production (Rincon *et al*., 1998)
MAPK	ERK	Type one	The pharmacological inhibitor of ERK PD98059 results in an increased synthesis of type two cytokines (Jorritsma *et al*., 1993)
MAPK cascades	GADD45β	Type one	GADD45β-deficient CD4 T cells have impaired type one responses and all three MAPK activities are diminished (Lu *et al*., 2001)
Tek kinases	Itk	Type one/type two	Itk–deficient mice showed impaired type two responses without affecting type one differentiation (Miller *et al*., 2004); phosphorylation of T-bet by Itk results in a T-bet–GATA3 association and the inhibiton of GATA3-driven differentiation into type two cells (Hwang *et al*., 2005)
Tek kinases	Txk (Rlk in mice)	Type one	Txk is expressed only in undifferentiated and type Cero and type one cells, and antisense treatment diminished IFN-γ secretion (Hwang *et al*., 2005)
E3 ligase	Itch	Type one	Target JunB to degradation, which is a transcription factor involved in type two differentiation (Fang *et al*., 2002)

[a]Within the families of signal transduction proteins, the expression or activation of specific members results in type one or type two differentiation. In the case of Itk, two lines of evidence gave opposite results, which still remain controversial.

A basal NF-κB activity is important for the survival, differentiation, and full activation of T cells, whatever the cytokine profile. The NF-κB family has an intrinsic role in type one effector function, as two of the members of this family, c-Rel and RelB, are absolute requirements for synthesis of IFN-γ (Mowen and Glimcher, 2004). This correlates with a strong PKC activation leading to type one differentiation (Fig. 4; Noble *et al.*, 2000).

The NFAT family controls the expression of numerous cytokine genes; there is a fine balance in the roles played by the three members of this family in determining the differentiation pattern of T cells. Calcium signals are associated with type two development with a higher NFAT transcriptional activity observed in type two cells. The important NFAT member for type two development is NFATc (Hodge *et al.*, 1996; Ranger *et al.*, 1998a), while NFATp and NFAT4 promote the expression of type one cytokines and repress the expression of type two genes (Hodge *et al.*, 1996; Ranger *et al.*, 1998b). NFAT often binds to DNA in double consensus sites, that is, sites recognized by NFAT itself and another transcription factor, usually AP-1, interferon responsive factor-4 (IFR-4), or c-Maf, a transcription factor specifically induced in type two cells. Interaction of NFAT with any of these favors type two development by binding multiple complex sites in the IL-4 locus (Mowen and Glimcher, 2004).

B. Commitment of the Cells

T cell commitment into either pathway of differentiation requires the presence of cytokines, which are the strongest inducers of T cell differentiation. It is well known that an IL-4-rich environment promotes the differentiation of T cells to the type two pattern, while IL-12, type 1 interferons and IFN-γ promote type one differentiation. Other cytokines are also important in T cell differentiation; IL-6 promotes the type two development by inducing IL-4 expression, while IL18, IL-23, and IL-27 drive T cell differentiation to the type one pattern. The receptors for IL-23 and IL-27 are expressed at different T cell developmental stages. IL-23R is expressed only in memory CD4 T cells, and is considered important for the type one memory response. In contrast, IL-27R is expressed only in resting CD4 cells and is important for driving IFN-γ production during the primary immune response (Szabo *et al.*, 2003).

There are two theories for the mechanism of cytokine effects on T cell differentiation:

1. The instructive theory states that cytokine signals direct the differentiation pattern of naive cells.
2. The stochastic theory proposes that type one and type two precursors arise spontaneously and that the role of cytokines is to promote the selective proliferation and survival of either phenotype.

Both theories seem to be right and complementary. Activation of naive cells results in an intermediate cell population (type "0") expressing both patterns of cytokines—and cytokine signals drive the proliferation and survival of only one of them (Szabo *et al.*, 2003); however, cytokine signals also actively promote the commitment of T cells into one or other differentiation pattern.

Commitment of T cell differentiation is mediated by the cytokine-induced activation of STAT transcription factors. STATs induce cytokine gene transcription and also open the chromatin surrounding the cytokines that are to be expressed. This allows a much higher level of induction of the implied genes by transcription factors during cell activation. They can do so directly and also by inducing the expression of differentiation-specific transcription factors (Hebenstreit *et al.*, 2005). Chromatin modifications are brought by macromolecular remodeling complexes that use two major mechanisms: (1) movement of complete nucleosomes from one strand of DNA to another through the action of ATP-dependent translocases, and (2) nucleosome opening through the actions of histone acetylases and deacetylases. These two mechanisms respectively provoke relaxation or contraction of the DNA. Other chromatin modifications include methylation and ubiquitinylation. All these modifications are specific for histone residues and constitute what is called the chromatin code (Felsenfeld and Groudine, 2003). Macromolecular complexes are directed to specific genes by adaptor molecules that associate with transcription factors (GATA3, T-bet, STATs, NFAT) and with the macromolecular remodeling complexes. Adaptor proteins p300 and CBP are associated with the opening of chromatin (Avots *et al.*, 1999; Blobel, 2000), while IKAROS is associated with chromatin silencing (Avitahl *et al.*, 1999; Kim *et al.*, 1999).

The important STAT member that drives T cells to the type two pattern is STAT6, activated by IL-4 signals. STAT6 induces the expression of GATA-3 and c-Maf, the signature transcription factors of type two cells that are sufficient to drive type two differentiation (Kurata *et al.*, 1999). c-Maf, a member of the AP-1 transcription factors, directly drives the expression of IL-4 and inhibits the IFN-γ promoter. Overexpression of c-Maf in transgenic mice skews the T cell phenotype to the type two pattern and confers protection in type one-mediated autoimmune disease models (Ho *et al.*, 1998). GATA–3 expression is initially induced by STAT6, but later on initiates its own auto-activation loop, by driving its own transcription (Ouyang *et al.*, 2000). GATA-3 induces type two differentiation by uncoiling the chromatin of the IL-4 locus and the IL-4/IL-13 intragenic locus (Ansel *et al.*, 2003). GATA-3 also directly transactivates the type two cytokine genes for IL-5, IL-10, and IL-13, but not for IL-4.

T cells are committed to type one differentiation by STAT4 and STAT1, induced by IL-12 and interferon signals, respectively. The main role of

STAT4 may be to uncoil the chromatin surrounding the IFN-γ gene, through its association with CBP, and hence to help drive IFN-γ expression (Mullen *et al.*, 2001a). STAT1 acts directly on the IFN-γ promoter to induce its transcription and induces the expression of the signature transcription factor of type one cells, T-bet (Lighvani *et al.*, 2001). Just as GATA-3 does for type two cells, T-bet induces its own transcription, sustaining an amplification loop for type one cells. T-bet also induces chromatin remodeling of the IFN-γ locus and the expression of IL-12β2, which optimizes the IL-12/STAT4 signals (Afkarian *et al.*, 2002; Mullen *et al.*, 2001a). Another transcription factor induced by T-bet is the Hlx homeobox gene, which synergizes with T-bet in the induction of type one genes (Mullen *et al.*, 2002). T-bet also induces chromatin remodeling of the IFN-γ gene. Specific DNase I hypersensitive sites (indicative of chromatin relaxation and opening of the genes), which are specific for type one cells, are located in introns 1 and 3 of the IFN-γ gene, a region in which T-bet is critical for generating DNase I hypersensitive sites (Mullen *et al.*, 2001b).

C. Silencing of the Opposing Cytokine Genes

The mutual antagonism of type one and type two cells is expressed by several mechanisms that silence the opposite phenotype. IL-4 signals interfere with the association of the IFN-γ receptor with the TCR; this association is important for type one differentiation (Maldonado *et al.*, 2004). The implication is that there is a role for IL-4 in the inhibition of cross-talk between the TCR and the IFN-γ signalosomes. Cytokines can also silence opposing cytokine genes by inducing SOCS (suppressors of cytokine signaling). SOCS5 is induced in type one cells and binds to IL-4R, thereby blocking IL-4-mediated signals (Seki *et al.*, 2002). SOCS1, induced in type two cells, inhibits IFN-γ signaling (Alexander and Hilton, 2004).

The transcription of GATA-3 and T-bet is regulated, respectively, by IL-4 and IFN-γ signals. Other molecules mediate the posttranscriptional regulation of GATA-3. FOG (friend of GATA) potentiates GATA-3 activity, while ROG (repressor of GATA) inhibits its effects (Miaw *et al.*, 2000; Tsang *et al.*, 1997). T-bet and GATA-3 interact with each other when T-bet is phosphorylated. This interaction kidnaps GATA-3, which is then unable to bind to its target genes, driving type one differentiation (Hwang *et al.*, 2005). An effect of Itk in the inhibition of T-bet expression and the concomitant type two differentiation has also been described in Itk knockout mice (Miller *et al.*, 2004).

A major mechanism leading to gene silencing of opposing cytokine genes during T cell differentiation involves control of the accessibility of the genes to transcription factors. Nuclear structures separate active genes

from inactive DNA in the chromatin. Active genes extend their relaxed chromatin into the interchromosome areas where the transcriptional and splicing machinery are located. Inactive DNA is buried in condensed structures inside the chromosome territories or heterochromatin (Cremer and Cremer, 2001). The segregation of inactive and active DNA can be a transient process in gene expression, when chromatin-remodeling complexes become associated with precise DNA locations. These complexes act in two ways—by promoting the opening of cytokine genes of the selected phenotype, and by silencing the opposing genes. Staining of IL-4 and IFN-γ loci together with the marker of heterochromatin, the γ-satellite sequence, revealed that in type one cells, IL-4 alleles colocalized with heterochromatin while in type two cells IFN-γ alleles were inside heterochromatin domains (Grogan *et al.*, 2001).

D. Physical Stabilization

Differentiation into type one and type two cells can be reversed by exposure of differentiated cells to the opposing cytokine environment or by transfection with GATA-3 or T-bet, respectively. Beyond a certain point, however, cells cease to be able to change from one phenotype to another. This point is where genes originally silenced by deacetylation of histones are methylated (Grogan *et al.*, 2001). In human primary cells, reprogramming was easily observed in freshly induced differentiated clones and in T_{CM} cells, while the effector and T_{EM} memory subsets were more resistant to reprogramming (Sundrud *et al.*, 2003).

VII. T Cell Migration

One of the most striking characteristics of immune cells is their ability to work as a team, even though dispersed throughout the body. This is because they have meeting points and established routes for communication and move around to exert their immune functions (Iparraguirre and Weninger, 2003). Secondary lymphoid tissues, located close to antigen entrance points, are the sites of encounter between lymphoid cells and antigens. Thus the spleen is the lymphoid organ for blood antigens, lymph nodes receive cells from the skin, and the large area covered by mucosa is sampled by Peyer's patches, mesenteric lymph nodes, the appendix, solitary follicles in the intestine, and the tonsils and adenoids. An active cell circulation between lymphoid tissues and peripheral organs is essential for immune function.

The mucosa-associated lymphoid tissues differ from systemic lymphoid tissues in that they show phenotypically and functionally distinct B cells, T cells, and accessory cell subpopulations (Nagler-Anderson, 2001). These mucosa-associated lymphoid tissues communicate with each other by clear-cut routes, linking some sites with others (but not all). This is partially explained by the imprinting that mucosa-associated dendritic cells give to T cells during antigen presentation (Iwata *et al.*, 2004). Investigations of better ways for delivering vaccines have revealed that (1) oral immunization may induce a response in the small intestine, ascending colon and mammary and salivary glands but not the rectum, (2) rectal immunization evokes responses in the rectum only, (3) nasal and tonsilar immunization results in immune responses in the upper airway mucosa, without responses in the gut, and (4) the cervicovaginal mucosa responds to nasal and transcutaneous immunizations. This arises from the migratory routes followed by mucosa-associated lymphocytes during immune recognition and response (Holmgren and Czerkinsky, 2005).

The principal molecules involved in cell migration into lymphoid organs and peripheral tissues are chemokines, selectins, integrins, and their receptors. The processes involved in the exit from lymphoid organs is only starting to be characterized, but at least one molecule has been identified—sphingosine 1-phosphate (SIP) and its receptor (Cyster, 2005). All these molecules provide an immune cell with clues as to how and when to go to precise locations, depending on the cell's differentiation status, site of original activation, and phenotype (Figs. 8 and 9).

The development of microscopy and flow cytometry-based techniques has added to our knowledge of the dynamics and regulation of cell trafficking. Additionally, our understanding of this process *in vivo* has been boosted by our ability to follow up adoptively transferred cells in congenic mice. These transferred cells can be identified by congenic markers and labeled with specific dyes. We can then monitor their proliferation by following the expression of specific proteins. Histological analysis reveals the location of homed cells, in particular tissue microenvironments. Intravital, particularly two-photon, microscopy allows us to track cells within living tissues in real time (Iparraguirre and Weninger, 2003).

The lymphoid tissues and inflamed peripheral sites are marked for immune detection by leukocyte "danger signals," chemoattractants, and surface markers. These latter molecules are displayed on the surface of the high endothelial venules (HEV), which respond to cytokines produced by innate immune cells (Kluger, 2004). The most important chemoattractants for lymphocytes are chemokines, recognized in the lymphocytes by chemokine receptors associated with trimeric G proteins. Chemokines constitute a family of more than 40 small, mostly secreted proteins, grouped into four families (CC, CXC, C, and CX3C) named according to the number and

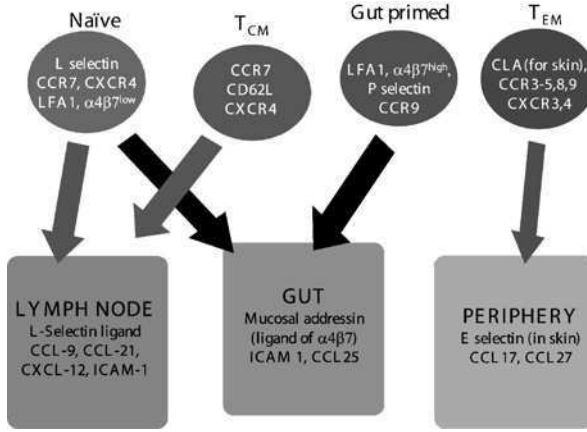

FIG. 8 Homing molecules in T cells. Lymphocytes migrate to lymphoid tissues or the periphery according to the chemokine receptors they express and also to the expression of integrins and selectins or selectin ligands they acquire. These molecules are expressed in the T cells after priming and differentiation into the two subsets of memory lymphocytes, T_{CM} or T_{EM}. Gut-primed T cells express molecules that allow them temporarily to go to the lymph nodes, by expression of CCR7, but, soon after, they express the homing molecules that allow them to return to the gut. The ligands of these molecules are expressed differently in the lymph nodes, the gut, and the periphery to direct the migration of the adequate T cells.

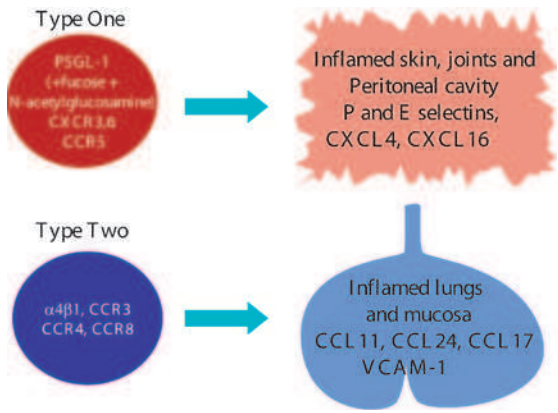

FIG. 9 Migration of type one and type two cells. T cells express different sets of homing molecules and migrate to different effector areas according to their differentiation profile. While type one cells migrate primarily to inflamed skin, joints, and the peritoneal cavity, type two cells are mainly directed to inflamed lungs and mucosa.

location of their N-terminal cysteine residues. The nomenclature adds a letter to distinguish chemokines from their receptors, so chemokines themselves have an L (ligand) and receptors have an R (receptor) in their name. Chemokines and their receptors are numbered, but the number in the receptor does not correspond to the number of the cytokine, and most chemokine receptors recognize more than one chemokine. The most important pairs of chemokines and, receptors for the control of T cell traffic, include the following (Moser *et al.*, 2004):

1. CCR7–CCL19 and CCL21 (controlling the traffic of T cells toward the lymph nodes, particularly the T cell areas).
2. CXCR5–CXCL13 (controlling the traffic of primed CD4 cells toward the lymphoid follicles for entry in contact with B cells).
3. CXCR4–CXCL12 (controlling to some degree the traffic of CCR7-independent naive T cell migration to lymph nodes and return to lymph nodes of effector lymphocytes).
4. CCR4–CCL17 and CCR10–CCL27 (controlling the traffic of effector and memory T cells to the skin).
5. CCR9–CCL25 (controlling the traffic of effector and memory T cells to the gut).
6. CXCR3, CXCR5, and CCR5–CXCL4, and CXCL16 (controlling the traffic of type one cells to inflamed skin, joints, and the peritoneal cavity).
7. CCR3, CCR4, and CCR8–CCL11, CCL24, and CCL27 (controlling the traffic of type two cells to inflamed lungs and mucosa).

Lymphocytes express more than one chemokine receptor. It is the balance between signals from all of them, rather than from any one of them, that determines the direction of movement of the cells. For instance, traffic of T cells to the lymphoid follicles for interaction with B cells can be determined by downregulation of CCR7 as well as by upregulation of CXCR5 (Moser *et al.*, 2002).

The process of lymphocyte entry into lymph nodes and Peyer's patches through endothelial venules involves four stages: (1) rolling, supported by selectins and sometimes integrins, (2) activation, mediated by integrins activated by chemokine receptors, (3) firm adhesion, mediated by integrins, and (4) transmigration, mediated by integrins and chemokines. In this way the selective expression of selectins, integrins, and their ligands also control the circulation of lymphocytes (Steeber and Tedder, 2000). Entry into the spleen is direct, passing from the bloodstream to the marginal sinus, from which cells migrate to the white pulp. Migration into the white pulp depends on CCR7 (Iparraguirre and Weninger, 2003).

Naive T cells express a restricted panel of traffic molecules, including a large amount of CCR7, CXCR4, L-selectin, the β_2 integrin LFA1, and moderate levels of the integrin $\alpha_4\beta_7$ (Fig. 8). The normal chemokines resident

in the lymph nodes, produced by the radiation-resistant follicular stromal cells, are CCL19, CCL21, and CXCL12 in the T cell areas and CXCL13 in the lymphoid follicles. L-selectin in naive T cells binds to peripheral node addressin and thereby mediates cell rolling on HEV. This low-strength adhesion brings together CCR7 and its ligands, with a resultant activation of the integrin LFA1. The subsequent firm adhesion involves the interaction of LFA1 on the T cell surface with ICAM1 and 2 on the surface of the endothelial cells (Campbell *et al.*, 2003).

In Peyer's patches, the $\alpha_4\beta_7$ integrin of the T cell rolls on a different ligand present on the HEV (the mucin-like domain of the mucosal addressin cell adhesion molecule). Both of the integrins, LFA1 and $\alpha_4\beta_7$, involved in the firm adhesion of the cells in mucosal HEVs, are the same as those activated by the signals of the chemokine receptor (Bargatze *et al.*, 1995).

Once activated, effector T cells must change their homing molecules to be able to leave the lymph nodes and gain access to the peripheral tissues where they exert their immune function (Ebert *et al.*, 2005). Type one and type two cells differ in their homing molecules (Fig. 9). In type one cells, TCR and IL-12 signals together bring about an enzymatic glycosylation of the P-selectin glycoprotein ligand. This specific ligand binds P- and E-selectins on endothelial cells. Type one cells preferentially migrate to inflamed skin, joints, and the peritoneal cavity (Iparraguirre and Weninger, 2003). Type two cells do not display P- or L-selectin binding, and their migration depends on the integrin $\alpha_4\beta_1$, which mediates the rolling and firm adhesion to VCAM-1. Type two cells preferentially migrate to inflamed lungs and mucosa (Cheroutre and Madakamutil, 2004; Voehringer *et al.*, 2004). Type one and type two cells also differ in their chemokine receptors with type one populations expressing CXCR3, CXCR6, and CCR5 and type two cells expressing CCR3, CCR4, and CCR8 (Iparraguirre and Weninger, 2003; Moser *et al.*, 2004). Clear definition of the phenotypes of type one and type two cells has been made with cells differentiated *in vitro*, under strongly polarized conditions that block any influence of the opposite polarizing environment. *In vivo*, however, a more complex pattern of chemokine receptor expression is obtained and the balance of the various signals is what determines the extent of "type one- or type two-ness" for individual cells, thus influencing the direction of cell migration.

Memory cells also have a distinct pattern of chemokine receptors. An initial distinction between central and effector memory subsets, made according to their expression of CCR7 and CD62L, tissue distribution, and expression of effector cytokines was later found to be oversimplified. Nevertheless, the concept of more than one population of memory cells, one with an undifferentiated cytokine profile and the other with the profile already fixed, still stands. Memory cells do, however, display a series of homing markers for their specific tissue distribution, related to the site where they were primed (Iparraguirre and Weninger, 2003; Iwata *et al.*, 2004).

Skin-directed memory cells display the cutaneous lymphocyte-associated antigen (CLA), again a structure generated by inducible enzymes leading to the carbohydrate modification of CD162. CLA binds to E-selectin, which is upregulated in the HEV of inflamed skin (Picker *et al.*, 1993). These CLA^+ cells also express the chemokine receptors CCR4 and CCR10; their corresponding ligands, CCL17 and CCL27, are also induced in the HEV of inflamed skin (Fig. 8) (Campbell *et al.*, 1999; Hudak *et al.*, 2002).

Memory cells primed in mucosa-associated lymphoid tissues return to the mucosa, and mucosa tissues have links between them, as we previously mentioned (Iwata *et al.*, 2004). Gut-homing memory cells display high levels of the integrin $\alpha_4\beta_7$, and type one cells can also be recruited to the lamina propria because their P-selectin interacts with the CD162 in endothelial cells. Small intestine-tropic T cells express CCR9, induced in cells primed in the mesenteric, and downregulated in cells primed in the peripheral, lymph nodes. The CCR9 ligand, CCL25, is expressed in the crypt epithelium in the jejunum and ileum (Zabel *et al.*, 1999).

The process whereby cells exit from the lymph nodes is also regulated. Both naive and all types of primed T cells must be able to leave the lymph nodes. One way in which exit is controlled is through the downregulation of CCR7, although other molecules are also important for cell exit. As mentioned above, induction of the sphingosine 1-phosphate receptor 1 in activated cells is necessary for such cells to exit from the lymph nodes. This receptor is also involved in the exit of mature T cells from the thymus. Exactly how this receptor works is not clearly understood, but regulation may involve a gradient of sphingosine 1-phosphate (Cyster, 2005).

VIII. Tolerance

A fundamental role of the immune system is to distinguish between self and nonself and to respond only to potentially harmful nonself antigens. T cell receptors arise by random recombination of gene segments and give rise to a variety of clones that recognize a random array of peptides presented by self-MHC, without distinction between self and nonself. Most clones strongly reactive against self-peptides are eliminated during the maturation stage of T cells in the thymus by a process known as negative selection or central tolerance (Starr *et al.*, 2003). During the maturation stage in the thymus, strong signals coming from the TCR induce a death program in the cell, instead of proliferation and differentiation as happens in mature lymphocytes. Some self-reactive clones, however, survive and enter the bloodstream. To control the activity of these self-reactive cells and the activation of clones against nonharmful foreign antigens (as food proteins), the immune

system has a number of mechanisms known as peripheral tolerance (Saouaf *et al.*, 2003).

Some of the peripheral tolerance mechanisms are implicit in T cell activation. A lymphocyte will be activated only if the antigen is presented by an APC that expresses costimulators and cytokines, resulting from its encounter with a pathogen or "danger" signals from a damaged tissue. T cell activation induces the transcriptional machinery of the cell and "positive cell responses" to give rise to an immune response. The same signals also induce, in a later phase with different kinetics, activation of phosphatases and ubiquitin ligases and the synthesis of inhibitory receptors, death receptors, and their ligands (Fas and FasL). All of these build a negative regulatory loop that shuts down the cell response, and induces the death of the effector clones to return to homeostasis (Coyle and Gutierrez-Ramos, 2003; Gett and Hodgkin, 2000; Heissmeyer *et al.*, 2004; Leibson, 2004). DCs and macrophages also change their signals to T cells during the immune response, from proinflammatory to anti–inflammatory cytokines or from type one induction to type two, so as to limit the inflammatory response (Langenkamp *et al.*, 2000; Ma *et al.*, 2003; Mosser, 2003; Patterson, 2000).

DCs also play an important role in the induction of tolerance. As pointed out earlier, mature DCs induce effector T cells because, upon activation, they have high levels of MHC and costimulatory proteins on the cell surface; there is, however, an intrinsic risk in this process. For instance, apoptotic infected cells can be phagocytosed by immature DCs that will travel to the regional lymph node where they mature and present antigen to Tc and Th cells. It is likely, however, that not only microbial antigen would be presented, but also self-antigens, which are potentially recognized by auto-reactive T cells and induce autoimmune responses. Immature DCs phagocytosing normal apoptotic cells in the absence of stress or danger signals may travel to the lymph node and present self-antigens to autoreactive T cells (Steinman *et al.*, 2000). The low levels of MHC and costimulatory molecules, characteristic of immature DCs, together with the production of suppressor cytokines such as IL-10 and TGF-β would then induce anergy in T cells or their differentiation into regulatory T cells. In fact, it is very likely that presentation of self-peptides by immature DCs is continuously occurring to prevent the activation of autoreactive T cells in the immune responses against pathogen. The mucosa is one of the tissues in which this mechanism of tolerance by DCs is most actively occurring. An example of this is the gut mucosa where tolerance against food protein and commensal bacteria is extremely important to maintain homeostasis (Bilsborough and Viney, 2004). On the other hand, $CD4^+CD25^+$ T_{reg} cells can be activated by mature DCs in the absence of IL-10 and suppress the immune response of $CD4^+CD25^-$ and Tc cells in both an antigen-specific and nonspecific fashion (Mahnke and Enk, 2005). Finally, DCs in humans can express the inhibitory molecules ILT3 and ILT4 on their

surface; these molecules specifically inhibit the response of $CD4^+$ T cells (Chang *et al.*, 2002). ILT3 and ILT4 belong to the family of Ig-like inhibitory receptors and display a long cytoplasmic tail containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs). After coligation, the inhibitory receptors block Ca^{2+} mobilization and hence DC activation, decreasing expression of costimulatory molecules.

Deletion of peripheral autoreactive T cells is also an alternative mechanism of tolerance that can be induced by DCs. Tryptophan is an essential amino acid required for T cell proliferation, and its depletion in placenta correlates with prevention of fetus rejection (Mellor and Munn, 1999). The depletion of tryptophan is due to the presence of indoleamine 2,3-dioxygenase (IDO) (Grohmann *et al.*, 2003). DCs transfected with IDO both induced T cell death by limiting the tryptophan and inhibited T cell proliferation by direct cytotoxic action of the metabolites kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilinic acid (Funeshima *et al.*, 2005). A subset of human DCs has a constitutive IDO in parallel with increased synthesis of IL-10 (Munn *et al.*, 2002). This suggests that there may be a new role for IL-10 in the mechanism of induction of T cell tolerance.

Yet another mechanism of peripheral tolerance involves a specialized group of T cells capable of turning off the immune response; these are collectively named regulatory T cells. The existence of regulatory cells (initially called suppressor cells) was suggested in 1970, but the limited knowledge of molecular immunology and the lack of adequate technology at the time made it very difficult to isolate or characterize the regulatory clones (Gershon and Kondo, 1970). The availability of fluorescent dyes and flow cytometry and advances in molecular biology have allowed the isolation and characterization of various different regulatory T cells (Bach, 2003). The best characterized regulatory cells are the CD4 cells expressing the CD25 molecule and transcription factor FOXP3 (Fontenot and Rudensky, 2005; Grossman *et al.*, 2004). Within this population, two types of regulatory cells have been described—the so-called natural T_{reg} cells, from the thymus, and the acquired T regulatory cells (T_{R1}), which differentiate into regulatory cells in the periphery (Fontenot and Rudensky, 2005; Grossman *et al.*, 2004). Two main types of CD8 cells with regulatory activity have also been described—the $CD8^+CD28^-$ subtype and the Qa-1-restricted CD8 cells (Qa-1 is an MHC class Ib molecule, known as HLA-E in humans) (Chang *et al.*, 2002; Chess and Jiang, 2004). There are also regulatory NKT and $\gamma\delta$ T cells, which act by secretion of IL-10 and TGF-β immunosuppressor cytokines (Bach, 2003).

There are various mechanisms whereby tolerance is induced by suppressor cells (von Boehmer, 2005). Several mechanisms may occur within a single type of regulatory cell. T_{reg} cells secrete TGF-β, a cytokine that blocks T cell proliferation and the synthesis of TNF-α and IFN-γ. T_{reg} cells also induce

apoptosis of activated T cells by a granzyme A-mediated mechanism that requires calcium and interaction of the cells through the CD18 molecule. Acquired T_{R1} cells secrete the immunosuppressor cytokines IL-10 and TGF-β and also induce apoptosis of activated cells, but with granzyme B. The effectiveness in inducing apoptosis also differs from one type of suppressor cell to another. For T_{R1}, the suppressor-to-target ratio needed to induce apoptosis was 20:1, whereas the T_{reg} cells were effective at a 1:1 ratio (Grossman *et al.*, 2004). The immunosuppressor activity of both types of cells was induced by cross-linking the TCR with CD46, a receptor molecule of complement proteins. There is still some disagreement about the specificity of suppression by these cells. Some reports indicate that immunosuppression by T_{R1} cells is antigen restricted and that of T_{reg} cells is restricted to a discrete set of commonly found antigens. Others claim that both types of cells can induce bystander immunosuppression, probably due to the effect of the immunosuppressive cytokines in the tolerized area (Sakaguchi, 2004; von Boehmer, 2005).

The discovery of the role of CD8 T cells in tolerance came from studies of the model autoimmune disease experimental allergic encephalomyelitis (EAE), which is much more severe in mice depleted of CD8 cells (Jiang *et al.*, 1992; Koh and Koh, 1966). CD8 suppressor cells are important in the secondary response and are induced by the Qa-1 molecule, that is, upregulated in CD4 cells during the primary response. This Qa-1 molecule presents hydrophobic peptides to the CD8 cells. Qa-1 can present a restricted repertoire of peptides, the best known of which is the MHC class I leader sequence that binds to the inhibitory receptor CD94 in NK cells and inhibits NK cell activation. Presentation of Qa-1 peptides to CD8 cells induces their development into suppressor cells, which act by suppressing CD4 activation, probably through tolerization of DCs (Chess and Jiang, 2004). Another type of suppressor CD8 cell is the so-called T_s with a $CD8^+CD28^-$ phenotype. Interaction of T_s cells with DCs or endothelial cells induces the expression of ILT3 and ILT4 membrane proteins and the downregulation of activation molecules. Antigen presentation of these tolerized cells leads to induction of tolerance in other T cells that develop contacts with them. Induction of T_s cells was associated with success in human heart transplantation (Chang *et al.*, 2002; Manavalan *et al.*, 2004).

IX. Concluding Remarks

It is clear that the cell biology of T lymphocytes is complex, because of their ability to circulate around the body and to interact with other cells. We have described basic processes that govern their responses. Controlling these

processes to regulate T cell responses could improve human health. This has prompted considerable research aimed at understanding the complex nature of the factors and signaling molecules involved in each of the processes of T cell biology. Recent major technological advances have been important tools for learning more about the cell biology of T cells and may well lead to improvements in medical treatments in the near future.

Among the most challenging diseases are those that are multifactorial, in which a genetic predisposition together with the presence of environmental factors and habits of the patient can lead to pathology (Tiret, 2002). These include diabetes, asthma, autoimmune diseases, and cancer. Genomic analysis of susceptible patients has identified some of the genes involved in the susceptibility of individuals. Proteins that determine the threshold for T cell activation and tolerance are a common factor in these studies (Matsui *et al.*, 1999). The identification of specifically directed drugs that will correct the imbalance in the biology of T cell function is a major goal in clinical immunology.

Vaccine development is focused now not only on the activation of the immune system, but also on the specific branch of immune response that confers resistance to disease. High levels of neutralizing antibodies can protect from infection, but eradication of intracellular parasites requires type one memory cells (Kourilsky *et al.*, 1998; Lycke, 2001; Morrison *et al.*, 1999).

Genetic shifts in the influenza viruses, HIV, and the propagation of new viruses are significant threats to our society (Epstein, 2003). Viruses are master regulators of immune function, able to divert immune cell function to type two immunity or suppressor responses (Wainberg and Mills, 1985). Coevolution with our parasites has generated a fine balance in which we coexist with our parasites by establishing efficient protective responses in most healthy individuals. This balance can be easily broken with the arrival of viruses from other species (Osterhaus, 2001), as is the case with avian flu, particularly in the very young, the elderly, and the immunosuppressed population. If we can determine the mechanisms of T cell activation and function and the molecules involved in the regulation of either type of acquired immune response, we will be better able to deal with viral infections.

Cancer induces local immunosuppression (Zou, 2005). Given the important role of DCs in the induction of effector T cells or T cell tolerance, DCs have been used in cancer immunotherapy for priming antitumor effector T cells. In the mouse model there have been spectacular results that initially suggested success in humans. Generally, in mice, DCs have been generated from bone marrow cells cultivated in the presence of GM-CSF, loaded with tumor antigens, and transferred into mice with malignant tumors. Among the DC antigen-loading techniques *in vitro* are

- MHC-binding peptides (Soares *et al.*, 2001),
- protein tumor lysates or apoptotic cells (Kim *et al.*, 2006),
- nucleic acid transfection (Sang *et al.*, 2005),
- antibodies or a ligand specific for the receptor for endocytosis DEC-205 conjugated with antigen (Bonifaz *et al.*, 2004), and
- fusion hybrids between DCs and tumor cells (Matsue *et al.*, 2004).

The transferred tumor antigen-loaded DCs reach the lymph nodes and induce Tc and Th cell responses, which can effectively attack the tumor cells. In humans, however, clinical trials using this methodology have been disappointing (Schultze *et al.*, 2004). Further studies that expand our knowledge of the biology of DCs in humans will help to improve the outcome.

Malfunctions of the immune system affect millions of people worldwide. Autoimmune diseases and allergies are due to the "polarization" of type one or type two immune responses, respectively (Singh *et al.*, 1999). The prospect of controlling these diseases by directing T cell response is an attractive idea.

Transplantation is another branch of medical intervention in which the knowledge of the cell biology of T cell function is expected to provide a new lease on life to affected patients. Drugs used for transplants are wide-range immunosuppressors (Aw, 2003). Thus transplanted patients have to live with antibiotics and antiviral drugs to be able to cope without a functional immune system. The understanding of how tolerance is induced could permit transplanted patients to have a normal life by restricting tolerance to the transplanted organ, instead of full immunosuppression.

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