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Microbial Colonization Drives Lymphocyte Accumulation and Differentiation in the Follicle-Associated Epithelium of Peyer’s Patches

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Peyer’s patches (PPs) are lined by follicle-associated epithelium (FAE) with Ag-transporting M cells. To investigate the spatial relationships of B cells, T cells, and dendritic cells (DCs) in PPs during microbial colonization, their in situ redistribution was examined in germfree (GF) rats exposed to a conventional pathogen-free microflora (conventionalized, CV). Although occasional B and T cells occurred in the FAE of GF rats, it contained mainly immature DCs (CD45RD+CD86−), whereas mature DCs (CD86hi) were seen in the interfollicular zones even under GF conditions. In CV rats, DCs had disappeared from the FAE, which instead contained clusters by B and T cells associated with induction of putative M cell pockets. CD86 was seen neither in the FAE nor in the follicles under GF conditions, but it became apparent on intraepithelial B cells 5 wk after colonization. The level of CD86 on these B cells was comparable to that on germinal center B cells, although the B cell follicles did not show direct contact with the M cell areas. B cells in the follicular mantles acquired Bcl-2 after 12 wk in CV rats, whereas B cells in the FAE did not express Bcl-2 at a substantial level throughout the experimental period. The cellular redistribution patterns and phenotypic characteristics observed after colonization suggested that immature DCs, but not B cells, are involved in Ag presentation during primary immune responses against intestinal bacteria. However, the spatial cellular relationships sequentially being established among DCs, B cells, and T cells in PPs, are most likely important for the induction of post-germinal center B cells subsequently residing within the M cell pockets. The Journal of Immunology, 2003, 170: 816–822.
recall Ags and subsequent B cell survival as well as differentiation. This notion was supported by mechanistic in vitro studies with a similarly induced memory B cell phenotype from peripheral blood (16). However, it has remained unclear how the memory B and T cells establish their dominance in the M cell pockets.

The present study was undertaken to visualize in situ the impact of initial microbial colonization in the gut on 1) accumulation of FAE-associated lymphocyte subsets and 2) modulation of CD80/CD86 expression on B cells. Rats taken from GF to conventionalized (CV) animal house conditions were used for these experiments because this model is well established in our laboratory and has been used to study the effect of the normal microflora on the development of TCR αβ⁺ intraepithelial lymphocytes (IELs), both in terms of subset expansion (17) and shaping of the receptor Vβ repertoire (18). Moreover, the size of PPs is larger in rats than in mice, thus facilitating sample excision.

GF rats showed virtually no CD80/86 expression in their FAE or underlying lymphoid follicles. Although some scattered B and T cells were seen in the FAE under GF conditions, intraepithelial accumulation of CD86⁺ B cells and CD4⁺ T cells did not start until 1 wk after microbial colonization. Under GF conditions, CD4⁺ and CD86⁺ DCs dominated markedly over B and T cells in the FAE, but they decreased in number after microbial colonization. In the mantle zone of underlying lymphoid follicles, B cells started to express Bcl-2 at a high level 12 wk after microbial colonization. These initial cellular events suggested that immature DCs surveying the FAE are crucial for the primary immune response against commensal bacteria. After migration of these DCs into the PPs, the FAE are crucial for the primary immune response against commensal microbiota within 3 days (19). Such GF to CV (GF-CV) rats housed under ordinary pathogen-free conditions. This protocol ensured the establishment of a commensal microbiota, and rectal colonic fecal suspensions from rats housed under ordinary specific pathogen-free conditions. This protocol ensured the establishment of a commensal microbiota within 3 days (19).

**Results**

H&E-stained sections revealed well-developed PPs with B cell follicles in both GF and GF-CV rats, but no GCs occurred in the GF state (Fig. 1, A and B). Conversely, GF-CV rat PPs showed overt GC formation 1 wk after microbial colonization (Fig. 1C), and the GCs were maintained throughout the experimental period (Fig. 1, D and E).

The observed numerical fluctuations of B cells, T cells, and DCs in the FAE are summarized in Fig. 2. Small numbers of B cells had invaded the FAE of GF rats (Fig. 2A) but were virtually negative for CD86. Thus, without microbial colonization, neither the FAE

**Materials and Methods**

**Animals**

GF AGUS rats were bred at the Department of Medical Microbial Ecology (Karolinska Institute, Stockholm, Sweden). The GF rats were exposed to a conventional indigenous microbial flora at 2 mo of age by receiving oral and rectal colonic fecal suspensions from rats housed under ordinary specific pathogen-free conditions. This protocol ensured the establishment of a commensal microbiota within 3 days (19). Such GF to CV (GF-CV) rats were sacrificed 1 wk (n = 3), 5 wk (n = 6) or 12 wk (n = 6) later. Control GF rats were killed at the age of 2 or 5 mo (six rats in each group).

**Antibodies**

Mouse mAbs against rat Igκ L chain (OX12, IgG2a) and CD4 (W3/25, IgG1) were obtained from the Medical Research Council Cellular Immunology Unit (Oxford, U.K.). Anti-rat CD3 (G4.18, IgG3), anti-rat CD80 (3H5, IgG1), anti-rat CD86 (24F, IgG1), and anti-rat DCs (OX62, IgG1) were purchased from BD Pharmingen (San Diego, CA). A mAb against rat follicular DCs (FDCs; OX2, IgG1) was obtained from Cytotech (Copenhagen, Denmark), and anti-rat Bcl-2 (10C4, IgG1) from Zymed Laboratories (San Francisco, CA).

**Immunohistochemistry**

Terminal ileum segments with visible PPs were opened longitudinally, embedded in OCT compound (Tissue-Tek; Miles Laboratories, Elkhart, IN), and snap frozen in liquid nitrogen. Cryosections cut at 4 μm were subjected to multicolor immunofluorescence staining as previously detailed (17). Briefly, the sections were first incubated with a mixture of mAbs anti-CD3 and OX62 (2.5 μg/ml), anti-CD3 and anti-CD4 (1/4), or anti-Igκ L chain and anti-CD80 or anti-CD86 (6.25 μg/ml) for 60 min at room temperature. The former two combinations of primary mAbs were followed by a mixture of Cy3-conjugated goat anti-mouse IgG1, biotinylated goat anti-mouse IgG2a (25 μg/ml; Southern Biotechnology Associates), and the rabbit antiserum against cytokeratin (to visualize the epithelium). The sections were finally incubated with Cy2-conjugated streptavidin (1/1000; Amersham, Aylesbury, U.K.) and amminomethyl coumarin acetic acid-conjugated goat anti-rabbit IgG (20 μg/ml; Vector Laboratories, Burlingame, CA) for 30 min. Anti-Bcl-2 (2.5 μg/ml) or anti-OX2 (1/50) was combined with anti-Igκ L chain followed by appropriate secondary and tertiary immunoreagents as above. Tissue sections incubated with irrelevant isotype- and concentration-matched primary mAbs served as negative controls.

Microscopy was performed with a Nikon E-800 fluorescence microscope (Nikon, Tokyo, Japan) equipped with a charge-coupled device video camera system (C5810; Hamamatsu Photonics, Hamamatsu, Japan). Digitalized images were captured with a computerized imaging system (Fotostation; Interfoto, Oslo, Norway). Recording of the immunophenotypes of infiltrating cells in the FAE was performed at ×600 magnification. In each immunostained section, infiltrating cells were counted from all FAE areas (median number, 5; range, 3–8) and recorded as the average number of positive cells per FAE area. Putative M cell pockets were identified by intraepithelial clustering of B and T cells as described previously (15, 16).

**FIGURE 1.** Histology of rat PPs under GF or GF-CV conditions (H&E staining). A and B, Well-developed lymphoid follicles (F) with specialized PPs were seen under GF conditions but GCs did not develop throughout the experimental period (A, 2-mo-old GF rat; B, 5-mo-old GF rat). C, GCs (+) were formed in 2-mo-old GF rat 1 wk after microbial colonization although being relatively diffuse. D and E, Well-demarcated GCs (++) were observed 5 and 12 wk after microbial colonization. A–C and E: Original magnification, ×40; D: ×20.
nor the epithelium lining the nearby intestinal villi contained CD86+ cells throughout the experimental period. Conversely, DC-like cells located in the interfollicular T cell zones of PPs in GF rats expressed CD86 (Fig. 3, A and B).

After microbial colonization, the GCs increasingly expressed CD86 (Fig. 3, C–E). In parallel, the number of CD86+B cells in the FAE increased dramatically, although the expression of this costimulatory molecule was weak over the first week (Fig. 3 F) and had numerically declined slightly at 12 wk (Fig. 2 A). The CD86+B cells were often localized to the lateral side of the FAE (Fig. 3, F–H) where the M cells are known to be situated (15). Compared with the lymphoid follicles in GF rats, the expression level of Ig κ-chain became down-regulated in GF-CV rats over time (Fig. 3, D and E). The expression of CD80 on B cells was hardly visible under both GF and GF-CV conditions (data not shown).

Only a small number of T cells had invaded the FAE under GF conditions, mainly being CD3+CD4− and therefore presumably CD8− (Figs. 2B and 4, A and B). Accumulation of CD3+CD4+ T cells in the B cell follicles (Fig. 4, C–E) and the FAE (Figs. 2B and 5) clearly depended on microbial colonization. In GF rats, this subset was not seen in these two defined tissue compartments (Fig. 4, A and B).

**DC distribution in PPs after microbial colonization**

The limited mAb combinations available for multicolor immunofluorescence staining in rats rendered it difficult to distinguish between different subsets of DCs. Therefore, we attempted to trace numerical fluctuations of CD4+ DCs, OX62+ DCs, and FDCs in the FAE. Under GF conditions, the CD4+ cells appearing in the FAE were virtually all CD3− DCs (Fig. 4, A and B). The number of this DC phenotype remained unchanged in GF rats, but decreased strikingly after conventionalization (Fig. 2C). OX62+ DCs increased slightly 1 wk after conventionalization and then declined (Fig. 2D). The number of FDCs was less than five cells per FAE area in GF-CV rats, with little difference from the GF state (Fig. 2E). Because mAb OX2 recognizes a 47-kDa glycoprotein on rat thyocytes, brain tissue, endothelium, some smooth muscle cells, and B cells, it is difficult to identify strictly the rat FDC lineage outside of lymphoid follicles. Therefore, caution has to be exerted in an attempt to identify the distribution of FDCs in rat PPs. However, solitary polyhedral OX2+ cells, distinguished from B cells by lack of Ig κ-chain expression and from endothelial cells by morphology, were located in the subepithelial dome area, at the periphery of lymphoid follicles, and in the B cell follicles quite diffusely in PPs of GF rats (Fig. 6, A and B). Such putative FDCs gradually changed their location, along with OX2+ B cells, toward the serosal side and contributed to the formation of GCs after 5–12 wk of conventionalization (Fig. 6, C–E).

**Expression of Bcl-2 on PP B cells after microbial colonization**

Under GF conditions, B cells of lymphoid follicles or within the FAE did not express the apoptosis-preventing protein Bcl-2 (Fig. 7, A and B). After microbial colonization, however, B cells just...
beneath the FAE started to express Bcl-2, although the B cells of lymphoid follicles remained negative (Fig. 7, C, F, and G). Mantle zone B lymphocytes became faintly positive for Bcl-2 only at 5 wk after conventionalization (Fig. 7D) and more strongly positive at 12 wk (Fig. 7E). Throughout the experimental period, no substantial expression of Bcl-2 was observed on B cells within the FAE (Fig. 7, F–H). This was in contrast to the situation in M cell pockets of human adults in which B cells consistently expressed Bcl-2 at a high level along with the post-GC marker CD27 (16).

Discussion
The indigenous microbiota normally represents the most dramatic environmental challenge in the postnatal gut and elicits many morphological, functional, and immunological modulations. This was clearly revealed in the present study of PPs when GF rats became colonized with a conventional specific pathogen-free intestinal microbiota. We (17, 18) and others (20–22) have previously reported that the TCRαβ⁺, but not the TCRγδ⁺, subset of IELs is influenced by microbial colonization, resulting in oligoclonal expansion and phenotypic alterations both in rats and mice. GF mice are known to have reduced numbers of PPs and their lymphoid follicles, as well as M cells, show a numerical increase after transfer to a conventional specific pathogen-free animal facility (23). Repopulating GF mice either with a single nonpathogenic microorganism (Clostridium indolis) or with a single pathogen (S. typhimurium) is sufficient to establish the normal number of PPs with a FAE containing M cells (1, 13).

In humans, PPs start to develop at 19 wk of gestation (12), and the local B cells apparently have a crucial role in the induction of M cells in the FAE (8–10). We have previously reported that B cells present in the M cell pockets of human PPs by far outnumber other types of cells with potential Ag-presenting function (15) and often coexist with CD4⁺CD154⁺ memory T cells (16). Therefore, we have proposed that cognate B-T cell interactions may take place in the M cell pockets (5). However, it has remained elusive when the B cells accumulate in the FAE to establish an ideal microcompartment for encountering luminal Ags and to start their putative interaction with memory T cells. Because human neonatal PP specimens generally are unavailable, we performed our study in rats reared under GF conditions to observe the redistribution of immune cells in PPs during initial microbial colonization. Interestingly, neither CD86⁺ B cells nor CD4⁺ T cells were present in the FAE of GF rats in any significant numbers. Notably, before microbial colonization, mainly CD4⁺ DCs occurred in the FAE and clearly outnumbered other leukocytes. These DCs could be
characterized as immature (or resting) because they did not express the CD80 or CD86 costimulatory molecules.

Liu et al. (24) reported that DCs present in rat intestinal lymph on their way to the mesenteric lymph nodes could be divided into two subsets by their CD4 expression; CD4<sup>+</sup>/H<sub>11001</sub>OX41<sup>+</sup>/H<sub>11001</sub> DCs had short fine processes and low levels of nonspecific esterase, whereas CD4<sup>+</sup>/H<sub>11002</sub>OX41<sup>+</sup>/H<sub>11002</sub> DCs had long pseudopodia and high levels of nonspecific esterase. The CD4<sup>+</sup>/H<sub>11001</sub> subset expressed MHC class II and CD11b/c and was better at stimulating an allogeneic MLR, presentation of Ag to sensitized T cells, and particularly Ag-specific activation of naive T cells than the CD4<sup>+</sup>/H<sub>11002</sub> subset (24). Recent experiments have shown that DCs are able to sample bacteria from the surface of a monolayered epithelium (25). Therefore, our study suggested that the CD4<sup>+</sup>/H<sub>11001</sub> DC subset observed in the FAE of GF rats has a role in eliciting primary PP immune responses against commensal bacteria after their initial colonization. Interestingly, this subset disappeared from the FAE soon after the conventionalization, probably as a result of rapid DC migration to the interfollicular T cell zone where DC activation/maturation and stimulation of naive T cells are known to take place. Such a compartmentalized interaction between APCs and T cells in PPs has recently been demonstrated in a peroral infection model with <i>S. typhimurium</i> in mice (26). That this could be the case also in GF rats was suggested by the presence of CD86<sup>+</sup>/H<sub>11001</sub> DCs in the interfollicular T cell zones of their PPs, although observations based on functional markers in tissue sections are only hypothesis generating and not conclusive in mechanistic terms. Nevertheless, our presumption is supported by a murine lymph node study showing that it is the CD11b<sup>+</sup>CD8<sup>-</sup> (presumably CD4<sup>+</sup>), and not the CD11b<sup>+</sup>CD8<sup>+</sup> DC subset that undergoes physical interactions with Ag-specific CD4<sup>+</sup> T cells just outside of the B cell follicles (27).

B cells are known to be semiprofessional APCs but are likely to tolerize naive T cells (28–30). We found that neither CD86<sup>+</sup> B cells nor CD4<sup>+</sup> T cells accumulated in the FAE without the presence of an indigenous microbiota, whereas such intraepithelial
that B cells can aid the expansion of the CD4 responses against initial microbial colonization, but it is possible of memory T cells. The cellular redistributions we observed in memory and Ag presentation was not essential to this end, because the presence of B cells. The transfer of B cells restored immunological sense of B cells. The transfer of B cells restored immunological

**FIGURE 8.** Hypothetical model for Bcl-2 modulation in B cells based on observations of different PP compartments in adult humans and GF or CV-GF rats. To avoid apoptosis due to lack of Bcl-2 expression, naive B cells need interaction with (? factors from) macrophages (Mφs) or DCs activated by microbial components. Induction of a high level of Bcl-2 in memory/effector B cells depends on cognate T cells and sustained Ag stimulation through a B cell receptor (BCR) with high affinity after somatic hypermutation of Ig variable region genes. Such positive B cell selection normally takes place on Ag-retaining FDCs in GCs, but this mechanism appears to be less robust in rats under clean, specific pathogen-free conditions than in adult humans.
function. Instead, the DCs that were observed to be leaving the FAE after microbial colonization could interact with naive T and B cells in PP parafollicular areas. However, subsequent to this event, B cells with strong CD86 expression accumulated in putative M cell pockets of the FAE; in these microcompartments, they were potentially able to present Ags to cognate memory T cells in recall responses as we previously observed in vitro for human B cells with a similar phenotype (16).

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References