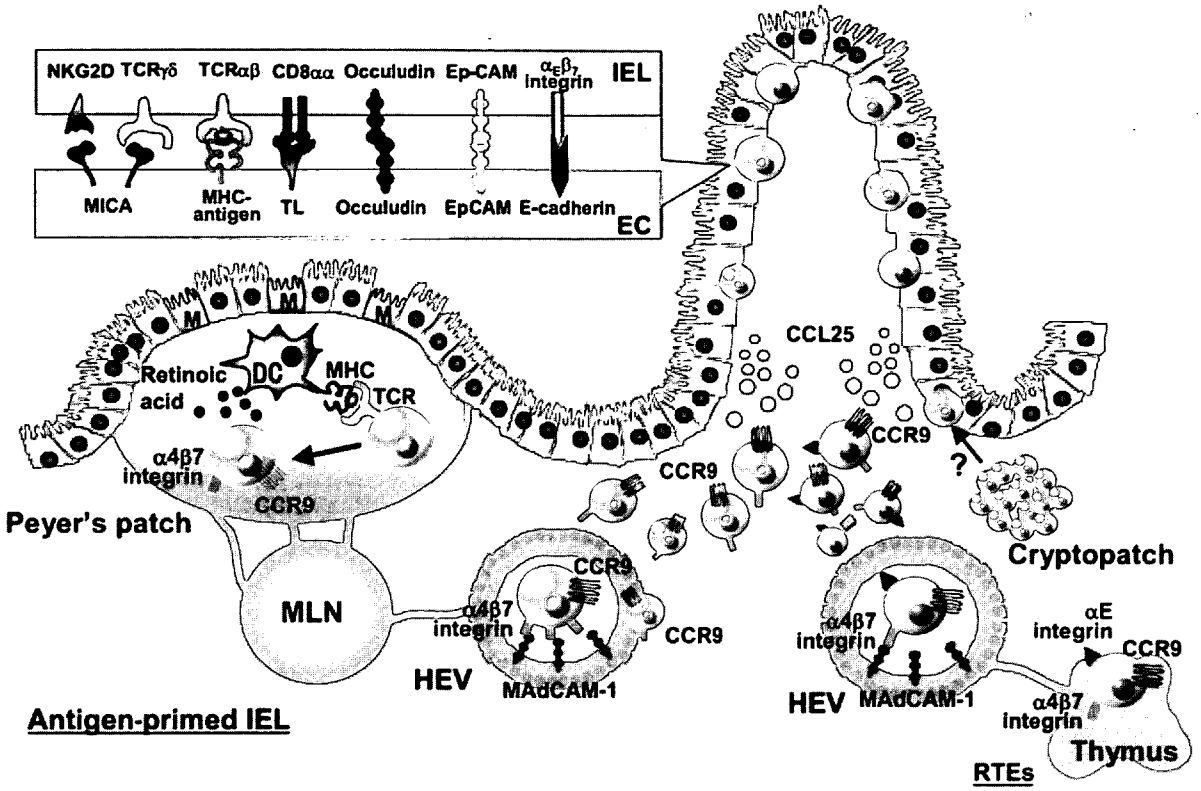

Fig. 1. Unique trafficking pathways for IELs in the intestinal compartments. In the antigen-primed IEL trafficking pathway, DCs in PPs take up antigens transported through M cells and present them to T cells in an MHC-dependent manner. Simultaneously, DCs produce retinoic acid, which causes antigen-primed T cells to express α4β7 integrin and CCR9. α4β7 integrin interacts with intestinal high endothelial venules (HEV) expressing MAdCAM-1, while CCR9 allows specific migration to the CCL25, a chemokine produced by intestinal ECs. In the other pathway, RTEs migrate into the intestinal epithelium without antigen priming at PPs and MLNs. Alternatively, RTEs have already expressed α4β7 integrin, CCR9, and αE integrin in the thymus. The other population of IELs may originate from the CPs, but their trafficking pathway has not been fully elucidated. Upon migration into the intestinal epithelium, IELs express several unique molecules capable of tethering ECs (e.g. αE integrin, Ep-CAM, and occludin) or of recognizing stressed or infected ECs (e.g. αβTCR, γδTCR, NKG2D, and CD8).

results obtained by the Study (32), one can surmise that almost half of the naive IELs in the small intestine are derived from RTEs, while PPs and MLNs contain a naive population of RTEs with much less frequency (less than 3%). With their diverse TCR repertoires, RTEs seem likely to play an important role in maintaining TCR diversity in the intestine. At present, however, it remains unknown whether or not RTEs are present in the large intestinal epithelium.

Differing compositions of naive and activated/memory IELs in the small and large intestine

Not surprisingly given the different environmental circumstances faced in the small and large intestine, IELs in the two environments differ with regard to the ratio between naive and activated/memory IELs. It is well established that small intestinal IELs are functionally characterized as antigen-experienced activated/memory cells expressing CD69 and that they contain few naive cells (less than 5%) (34–36). In contrast, the large intestine contains large numbers of naive-type cells expressing $CD62L^{high}$ and $CD44^{int}$ (more than 40%) but a far less abundant supply of activated/memory cells (34–36).

A correlation may exist between the abundant presence of activated/memory IELs and of CD8αα IELs in the small intestine. As mentioned above, the small intestine contains a large number of type b IELs expressing CD8αα as well as type a IELs expressing CD8αα together with CD4 or CD8αβ (*Table* 1). CD8αα, which can be expressed on activated conventional T cells of the lymph nodes and spleen, plays a key role in mediating the survival of those effector cells that further differentiate into mature memory T cells (37, 38). These surviving conventional T cells acquire the ability to migrate to and reside long-term in non-lymphoid tissues, including the gut, and they readily reinduce CD8αα expression after secondary restimulation. These observations indicate that the expression of CD8αα by the various subsets of mucosal T cells is not a marker to identify their origin or the specificity of their TCRs, but rather this expression reflects their effector/memory phenotype and plays a unique

role in maintaining immune homeostasis within the intestine.

Though both show a memory phenotype, type a and type b IELs represent distinct types of memory cells. Type a IELs resemble conventional memory T cells found in the periphery and *lamina propria* regions (39), and given that TCRβ clonotypes were comparable for type a IELs and lamina propria T cells (39), it is possible that they differentiate via the same pathway. In contrast, CD8αα (αβ IELs) are thought to be natural memory T cells. CD8αα αβ TCR IELs show oligoclonal TCR repertoires that are different from those of type a IELs (40), indicating that CD8αα αβ IELs differentiate via a distinct pathway. The accumulation of autoreactive TCRs in CD8αα αβ IELs (41), along with their early appearance in neonatal mice (42) and human fetal intestine (43), when exposure to exogenous antigen is minimal, provides evidence for the self-specificity of these T cells. In addition, most human fetal IELs already express an effector/memory cell phenotype (43), indicating that a differentiation pathway other than exposure to peripheral exogenous antigen resulted in the acquisition by these cells of an activated memory-like phenotype. However, unlike the CD8αα γδ TCR IELs (γδ IELs), the αβ IELs do not appear in the small intestine of germ-free mice lacking gut flora, although bacterial colonization of these animals restores the population of CD8αα αβ IELs (44). These observations indicate that local stimulation from the lumenal microflora is required for generation and maintenance of the CD8αα αβ IELs.

Antigen recognition by IELs

Type a IELs are reactive to classical MHC-restricted antigen presentation

Similar to peripheral T cells, type a IELs express αβTCRs together with the coreceptor CD8αβ, which recognizes MHC class I, or the coreceptor CD4, which recognizes MHC class II. In general, MHC class I presents peptide generated from cytoplasmic protein (endogenous antigen) (13), while antigens proteolytically processed after endocytosis/phagocytosis are presented by MHC class II molecules (45). Although both MHC class I and class II molecules express self- and non-self-antigen, type a IELs are specific for non-self-antigen because of the positive and negative selection in the thymus.

Antigen recognition by type b CD8αα αβ IELs

The MHC restriction of type b CD8αα αβ IELs is still enigmatic. CD8αα αβ IELs are drastically reduced in number in β2-microglobulin-deficient mice (46–49), but they are present in mice deficient either in the transporter associated with antigen processing (TAP) (49) or in the classical class I molecules ($K^{b-/-}D^{b-/-}$) (50, 51). This finding suggests that type b IELs require a TAP-independent non-classical MHC class Ib molecule.

It has been reported that mice deficient in Qa-2, a non-classical MHC class I molecule that is TAP dependent (52), possessed fewer CD8αα αβ IELs, suggesting that some CD8αα αβ IELs are reactive to Qa-2 (53). However, when they compared BALB/c mice with the Qa-2-null Bailey substrain of BALB/c mice, Cheroutre's group (54) were unable to confirm these findings. Furthermore, Qa-2 is broadly expressed by the IELs themselves (18), and its expression is mostly TAP dependent. Two other non-classical class I molecules, the thymic leukemia (TL) antigen (55) and CD1d (56), have been reported to be expressed by mouse intestinal ECs, although this is controversial in the case of mouse CD1d (57). Both TL and CD1d are TAP independent, but they require β2-microglobulin expression (58–60). However, the TCRs of this IEL subset in mice have never been shown to recognize either of these Ib molecules. Recent structural data indicate that the TL antigen has a very narrow and closed groove that makes interacting with αβTCRs unlikely (61).

Researchers using several MHC class-II-restricted TCR transgenic systems (62, 63) showed that CD8αα IELs were generated when agonist self-peptides were present, just as in the MHC class-I-restricted systems. Despite this finding, peripheral T cells in these same MHC class-II-restricted TCR transgenic mice were $CD4^{+}$. These observations indicate that CD8αα does not function as a TCR coreceptor and that expression of CD8αα on the αβ IELs does not necessarily imply MHC class I restriction. From these results, it can be surmised that CD8αα αβ IELs, unlike type a IELs, are self-reactive but are not selected for self-reactivity to a single antigen and that they may include cells reactive to classical class I molecules, non-classical class I molecules, and MHC class II molecules.

Specificity of γδTCR IELs (γδIELs)

It is still uncertain to what extent the γδTCRs require a selection event for their development, especially in mice. Murine γδ IELs make predominant use of the Vγ5 (also known as Vγ7) gene segment together with several Vδ genes (64). Their CDR3 regions are more diverse than the invariant γδTCRs expressed in the skin and reproductive tracts (65). Although the great majority of these cells express CD8αα, their numbers are not reduced in β2-microglobulin-deficient mice (47, 48). In humans, the γδTCRs expressed by IELs predominantly use Vδ1 (66), and they have an oligoclonal CDR3 repertoire that can be sustained over a period of many months (67). These TCRs recognize MICA and

MICB, class-I-like molecules that are induced by heat shock or stress on ECs (26, 68, 69). Like TL antigen in mice, MIC molecules are not capable of presenting peptides or other ligands (61). As mentioned above, MICA is also recognized by NKG2D. Although mice do not have a functional MIC gene ortholog, they do have NKG2D receptors that recognize class-I-like molecules such as H60 (70) and members of the RAE class-I-like family (71). There are no reports, however, indicating that significant populations of γδ IELs or other mouse γδ T cells recognize these class-I-like molecules.

TL antigen and MICA for CD8αα IELs

Given the MHC class I or class II specificity of the TCRs of CD8αα αβ IELs together with their requirement for β2-microglobulin, an MHC class I-molecule-mediated function other than peptide presentation must be required for CD8αα αβ IEL development and/or maintenance. In this regard, Leishman et al. (20) have shown that the mouse TAP-independent, β2-microglobulin-dependent, non-classical MHC class I molecule TL is a unique ligand for CD8αα. TL is constitutively and abundantly expressed by the ECs of the small intestine and so is in close proximity to the CD8αα IELs. The structural analysis of the molecule indicates the absence of an obvious antigen-binding groove (61). The specific interaction of TL with CD8αα on activated CD8αα αβ IELs modifies the TCR-activation signals to allow for regulated immune responses that are compatible with the gut environment (20). Unlike CD8αβ, CD8αα is not internalized with the TCR from the surface of activated T cells (72), and associated $p56^{lck}$ is inefficiently provided to the TCR complex, in part because of the inability of CD8αα to locate effectively into lipid rafts (73). It is, therefore, possible that the interaction of TL with CD8αα might sequester CD8-associated $p56^{lck}$ away from the TCR complex, consequently reducing activation signals while promoting survival. In contrast to the abundant expression of TL in the small intestinal epithelium, TL was only weakly expressed in the large intestinal epithelium (74), which might be consistent with the small numbers of CD8αα IELs in the large intestine.

Our previous study constructed a transgenic mouse with gut-specific MICA expression driven by a T3b promoter, and we surprisingly observed a clonal expansion of $CD4^+$ T cells also possessing CD8αα in the small intestine (75), suggesting that MICA positively regulates the expression of CD8αα (Fig. 2). Furthermore, studies in the inflammatory bowel disease model showed that transgenic MICA was able to attenuate the acute colitis induced by dextran sodium sulfate administration

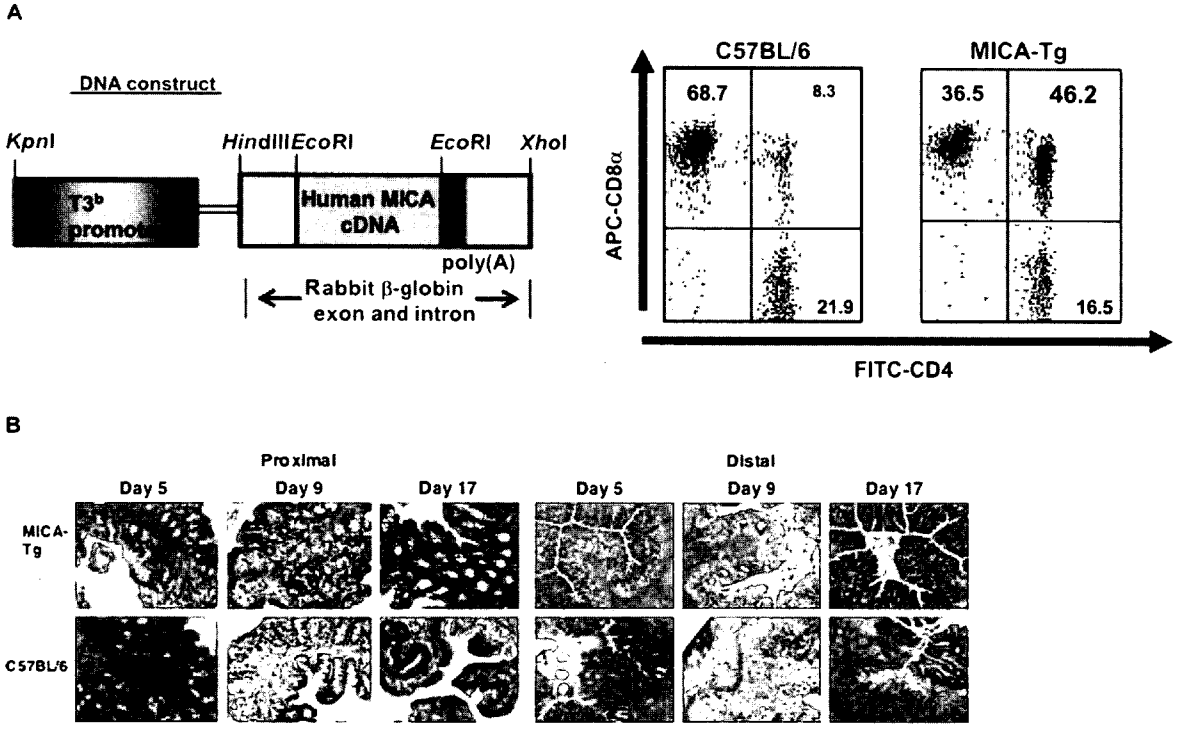

Fig. 2. CD8αα expression by mucosal T cells plays a unique role in maintaining immune homeostasis within the intestine. A transgenic mouse with gut-specific MICA expression driven by a T3b promoter was constructed. Surprisingly, a clonal expansion of $CD4^+$ T cells also possessing CD8αα was observed in the small intestine, suggesting that MICA positively regulates the expression of CD8αα (A). Furthermore, studies in the inflammatory bowel disease model show that transgenic MICA was able to attenuate the acute colitis induced by dextran sodium sulfate administration (B).

(Fig. 2). In this regard, it should be noted that MICA and TL, both of which belong to MHC class Ib molecules, are preferentially expressed in the intestinal ECs (55, 76). Thus, although MICA is both β2-microglobulin and TAP independent, while TL is TAP independent but β2-microglubulin dependent, it is likely that both molecules play a similar role in the development of IELs. Several years ago, Madakamutil *et al.* (38) demonstrated that TCR ligation and activation of CD8αα IELs by TL resulted in increased expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL, suggesting that TL may abrogate antigen-induced cell death.

Unique signal transduction through TCR and FcεRIγ in type b IELs

Type b IELs in both the small and large intestine exclusively express homodimers of FcεRIγ or heterodimers of FcεRIγ with CD3ζ (77–79), while type a IELs and peripheral T cells predominantly express homodimers of CD3ζ, which can form a complex with TCR that is important for signal transduction (77). Thus, most T cells from CD3ζ-deficient mice showed abnormal development (78, 80). However, type b IELs could express TCR in the CD3ζ-deficient mice because FcεRIγ compensated for the CD3ζ function in the TCR–CD3 complex (78–80). Because CD8αα molecules are not able to transfer signaling through TCR (73), selective expression of FcεRIγ on type b IELs might act as an alternative pathway for type b IEL activation.

Uniqueness of IEL developmental pathways

Agonist selection in CD8αα IELs

Although controversy remains as to what extent the γδ IELs require thymic selection (42, 81), much evidence exists in mice indicating that self-specific CD8αα αβ IELs are actively and preferentially selected in the thymus under agonist selection conditions (63, 82). The outcome of thymic selection of conventional αβ T cells is determined by the signal strength received through the TCR when interacting with MHC molecules presenting self-antigens. Intermediate TCR signal strength results in positive selection of conventional αβ T cells, whereas strong TCR-mediated signals delete cells that express those TCR from the conventional T-cell repertoire. By contrast, CD8αα αβ IELs preferentially accumulate under conditions that lead to the deletion of conventional αβ T cells, and, as a consequence, autoreactive TCRs are enriched in the TCR repertoire of CD8αα αβ IELs (41). The existence of an agonist-dependent selection of CD8αα αβ IELs has been shown using mice that express a transgenic TCR and cognate antigen during thymic selection. The H-Y TCR is specific for a Y-chromosome-encoded peptide presented by H-2D^b, and H-Y TCR transgenic mice provide a model for the self-based agonist selection of CD8αα αβ IELs (63, 83). When the H-Y TCR transgenic mice were crossed onto a recombination-activating gene (*Rag*)$^{-/-}$ background, CD8αβ thymocytes matured in the thymus and populated the peripheral tissues of H-Y antigen-negative female mice, whereas few CD8αβ IELs and no CD8αα IELs were detected in these female mice. By contrast, despite deletion of conventional CD8αβ H-Y TCR$^+$ T cells in *Rag*$^{-/-}$ male H-Y TCR transgenic mice, large numbers of H-Y TCR$^+$ IELs largely expressing CD8αα developed (83).

Similar observations have been made in TCR transgenic mice that coexpress the endogenous cognate antigen, such as 2C TCR transgenic mice expressing the H-2L^d alloantigen recognized by the 2C TCR (84) and double-transgenic mice expressing both the TCR and the cognate antigen. Examples of the latter include OT-1 TCR transgenic/RIPmOVA mice [membrane-bound ovalbumin (OVA) antigen transgene under the control of the rat insulin promoter (RIP)] (63), influenza virus nucleoprotein (NP)-specific F5 TCR transgenic mice that express NP peptides (85), and P14 lymphocytic choriomeningitis virus (LCMV)-specific TCR transgenic mice that coexpress the cognate LCMV-specific epitope GP33-41 (86).

The selection of CD8αα αβ IELs is most effective in the presence of high-affinity interactions between the TCR and the agonist self-antigens, as was shown using the clonotypic F5 TCR, which has a high affinity for the influenza virus epitope NP68. NP68/F5 TCR double-transgenic mice preferentially generated CD8αα αβ IELs, whereas double-transgenic F5 TCR mice coexpressing the antagonist peptide NP34 did not (85). Similarly, CD8αα IELs expressing the 2C TCR were readily generated in mice expressing the H-2L^d high-affinity ligand, whereas CD8αβ T cells developed preferentially in the presence of the low-affinity ligand presented by H-2K^b (84). Similarly, H-Y TCR$^+$ CD8αα IELs were more efficiently generated in H-2D$^{b/b}$ homozygous male mice than in their H-2D$^{b/d}$ heterozygous counterparts (87).

Similar to conventional αβ T cells, CD8αα αβ IELs require the expression of pre-Tα during the initial steps of αβTCR rearrangements (88). The fact that the efficient agonist selection of CD8αα αβ IELs also requires the expression of the TCRα chain connecting peptide domain (α-CPM) further suggests that the agonist and conventional selection processes have similar features and may have evolved simultaneously (63).

Agonistic selection in the thymus

The marked reduction in the numbers of CD8αα αβ IELs in congenic athymic nude mice (89, 90) and in mice that had been neonatally thymectomized (91) indicates that the thymus is

required for the generation of agonist-selected CD8αα αβ IELs. This requirement is further supported by the observation that thymus grafts from male H-Y TCR transgenic mice generated CD8αα H-Y TCR^+ IELs when transplanted into female recipient mice (63), and wildtype thymus grafts efficiently generated CD8αα αβ IELs when transplanted into recipient nude mice (42). In the latter case, it was shown that CD8αα IELs and conventional CD4 and CD8αβ IELs were generated with different kinetics. Fetal thymus grafts or grafts from mice up to weaning age mainly repopulated the intestine of the recipient mice with CD8αα αβ IELs, whereas thymus grafts from older mice were more efficient in generating the conventional αβ T-cell subsets (42). It should not be assumed, however, that the adult thymus had a reduced ability to generate CD8αα αβ IELs as the adult thymus grafts from the male H-Y TCR transgenic $Rag^{-/-}$ mice efficiently did so (63). It is possible that in the absence of conventional positive selection, as in the case of the male H-Y TCR transgenic mice on a $Rag^{-/-}$ background, the generation of CD8αα αβ IELs can continue in adult mice as long as a functional thymus is present.

Is it true that the type b IELs are developed in the thymus? Given that CD8αα αβ IELs continued to be efficiently generated under conditions that caused deletion of CD8αβ T cells in the thymus, some speculated that the precursors of these cells had not been submitted to thymic negative selection (41, 83). Taken together with observations that, in the absence of a thymus, these type b IELs nevertheless develop in lymphopenic mice grafted with normal bone marrow, fetal liver, or intestine (92, 93), this finding suggests that CD8αα αβ IELs are likely the progeny of extrathymic progenitor cells localized within the intestine itself.

Kanamori *et al.* (94) demonstrated that approximately 1500 cryptopatch aggregates (CPs) are dispersed throughout the wall of the small and large intestine in mice (Fig. 3). Cells within the CP are composed of mostly lymphoid progenitors expressing stem cell factor receptor, or c-kit, and IL-7Rα, but lacking the lineage markers (CD3, B220, Mac-1, Gr-1, and TER-119). They possess transcripts for germline TCR genes, messenger RNA (mRNA) for CD3ε as well as proteins (i.e. RAG-2 and pre-Tα) involved in TCR gene rearrangement (95), and are able to generate TCR^+ IELs, type b CD8αα αβ IELs, as well as γδ IELs, albeit with a strong bias toward the generation of γδ T cells, in irradiated severe combined immune-deficient mice. These findings demonstrated that c-kit^+ CP cells are committed to the T-cell lineage and are competent for the generation of IELs (93, 96). Both IL-$7R\alpha^{-/-}$ mice (94) and common cytokine receptor γ-chain (γ_c)-deficient mice (95) lack CP structure, but local

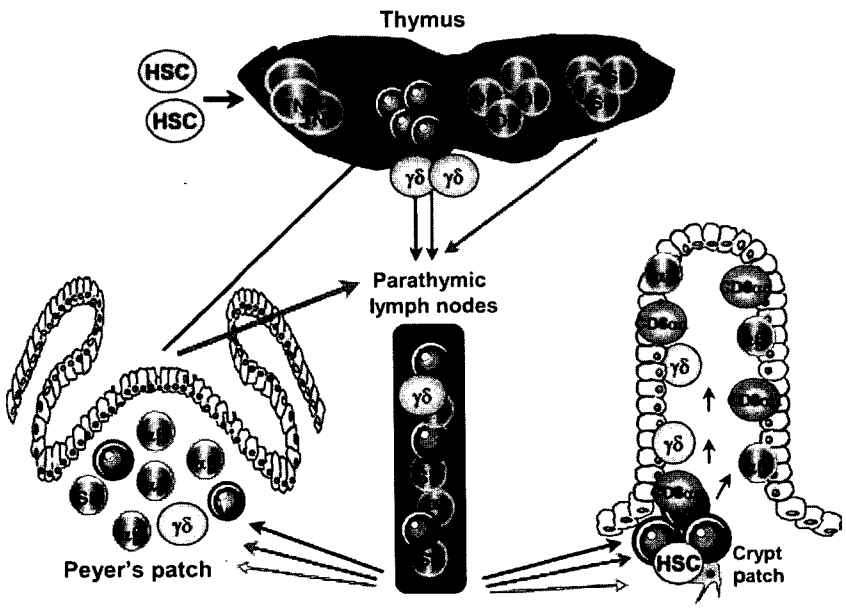

Fig. 3. Thymus-dependent versus thymus-independent IEL development. Bone marrow-derived or fetal-liver-derived hematopoietic stem cells (HSCs) enter the thymus as T-committed cells. Signals from the stroma and from other thymocytes ($CD4^+$ $CD8^+$ DP thymocytes?) induce differentiation toward type b unconventional T cells. Other immature $CD4^-CD8^-CD3^-$ (TN) cells gradually switch off unconventional T-cell genes and differentiate toward conventional $CD4^+$ or $CD8^+$ single-positive (SP) αβ T cells. Both immature and mature cells exit from the thymus. Unconventional T cells and their progenitors (TN2-3 cells) more readily populate the gut epithelium, possibly via CPs, where the progenitors may complete their differentiation into CD8αα IELs. Some TN2-3 progenitors may circulate back to the thymus. The gut epithelium could also be populated by bone marrow-derived HSCs that differentiate into type b γδ T cells in CPs.

(fatty-acid-binding protein promoter) transgene expression of IL-7 by mature enterocytes of IL-$7^{-/-}$ mice restored the development of CPs and γδ IELs, whereas γδ T cells remained absent from all other tissues (97), suggesting an important role for IL-7R signaling in their generation. These observations indicate a correlation between the presence of CP cells and lymphopoiesis in the gut, especially of γδ IELs.

Lambolez *et al.* (88) reported difficulty identifying specific T-lineage-committed precursors among the CP cells. Using multiple lineage markers, they performed a detailed characterization of the progenitor cells isolated from CPs. They also transferred bone marrow from nude mice to irradiated thymectomized γ_c and *RAG-2* double-deficient recipient mice to establish the sequence of appearance of these precursors during lymphoid development in the gut. This analysis provided little evidence that CP lineage $(Lin)^-$ cells are T-cell committed, a finding consistent with data showing that several gene mutations that affect T-cell differentiation, including pre-Tα, TCRα, and CD3ε, had no effect on the appearance or ratio of CP precursor cells (88). However, CP Lin^- cells did express GATA-2, a transcription factor found in pluripotent hematopoietic progenitor cells, indicating that these CP cells might represent precursors of other hematopoietic cells. There is relatively little cell turnover in CPs, and very few CP cells express *RAG-2* mRNA (88). In contrast, potential precursor cells isolated from the epithelium were more committed to the T-cell pathway, and TCRα mutation dramatically altered subtype ratios within the immature Lin^- cells of the epithelium (88).

In seeking to investigate which immune cells synthesize *RAG-2*, subsequent studies (98) have shown that RAG-2 production is detected exclusively in the thymus in normal mice with an intact thymus (euthymic), but RAG-2 is found in the MLNs of the gut and minimally in the PPs of nude or other athymic mice. Their findings suggest that extrathymic T-cell development is totally repressed in normal euthymic mice and becomes active only in cases of severe T-cell depletion. Whether in normal and athymic mice, neither CP cells nor IELs showed fluorescence-tagged *RAG-2*. To evaluate these findings, Nonaka *et al.* (99) generated athymic nude mice that lacked all lymph nodes and PPs by administering lymphotoxin-β receptor Ig and tumor necrosis factor (TNF)-R55-Ig fusion proteins into pregnant *nu*/+ mice and *nu*/*nu aly*/*aly* (alymphoplasia) mice that lacked all lymph nodes, PPs, as well as intestinal ILFs. These two kinds of mice, which harbored numerous DN as well as CD8αα γδ IELs possessing TCR Vγ1, Vγ4, and Vγ7 segments in the small intestines, were found to retain CPs. These findings provide compelling evidence that in the *lamina propria* of the intestine, at least some of the DN and CD8αα γδ IELs developed within the tiny clusters, that is, CPs, that are filled with c-kit^+IL-$7R^+Lin^-$ lymphopoietic cells.

Podd *et al.* (100) explored whether $CD3\varepsilon^+$ CP cells are linearly related to γδ IELs using laser capture microdissection analysis. They observed that in-frame TCR Vγ5 rearrangements were detected in the $CD3\varepsilon^+$ CP cells that are shared with a subset of γδ IELs. However, they did not detect mRNA for *RAG-1* gene expression in the CP aggregates. Thus, their data lend support to the hypothesis that CPs are perhaps reservoirs for some antigen-experienced γδ IELs in mice.

Eberl and Littman (101) performed interesting experiments in mice bearing two transgenes, a Cre recombinase transgene under the control of the retinoid-related orphan receptor γt (RORγt) promoter and a green florescent protein (GFP) transgene controlled by a ubiquitous promoter that is only transcribed when a lox-flanked stop sequence is removed by Cre. RORγt, an orphan nuclear hormone receptor, was detected in immature DP thymocytes, fetal lymphoid-tissue-inducer cells, and c-kit^+Lin^- cells in CPs. In these transgenic mice, all progeny of a Cre-expressing cell become constitutively GFP^+ because the stop sequence of the GFP transgene has been deleted. These experiments, also performed with a CD4–Cre transgene, showed that GFP was expressed by all DP thymocytes and also by all single-positive T cells and αβ IELs, including CD8αα αβ IELs, but not by any other immune cells such as γδ IELs, B cells, or NK cells. This finding elegantly demonstrates that all αβ IELs are of thymic origin and must have been selected by the DP pathway. However, these findings did not exclude an extrathymic origin for γδ and other IELs. They did suggest, however, that the contribution of intestinal precursors including c-kit^+Lin^- cells is minimal in healthy mice but could become more substantial in lymphopenic mice or in mice in which large numbers of T cells have been mobilized in response to intestinal inflammation (101). However, Eberl and Littman (102) mentioned elsewhere that the principal function of the CP cells is to induce the formation of lymphoid follicles in the intestinal *lamina propria* in a manner similar to the induction of lymph nodes and PPs by the RORγt-expressing fetal lymphoid tissue inducer cells.

Resolution of the thymus-dependent versus thymus-independent controversy

Just when researchers seemed to hit an impasse regarding thymus involvement in IEL production, a truly incendiary report by Lambolez *et al.* (103) was published. In this paper, they proposed a previously unknown pathway for T-cell

development that has implications for the thymus every bit as great as for the gut. Reexamining assumptions germane to the basic biology of T-cell development in the thymus, they noticed that neonatal thymus grafts leak specific subsets of early T-committed progenitor cells that will repopulate both the host thymus, in which they rapidly complete normal T-cell development, and the host gut, where they survive for many weeks and give rise via defined intermediates to type b IELs (103). This finding conflicted with previous assumptions that the thymus is a source solely of mature T cells and that it does not have the potential for long-term reconstitution. Furthermore, this report emphasizes that the leaked cells are early $CD4^-CD8^-CD3^-$ triple-negative stage 2 (TN2) and TN3 progenitors that are T-cell committed but that have not yet rearranged their TCR genes. However, CD8αα IELs could not be reconstituted even after adoptive transfer of other more mature DP or single-positive thymocyte populations (Fig. 3).

Using an indirect assay, the authors (103) also showed that the leaked TN cells are much more efficient at completing T-cell differentiation in the gut than are cells derived directly from the bone marrow (Fig. 3). These results strongly suggest that stroma cells in the thymus and/or other thymocytes could help 'persuade' progenitors to differentiate into unconventional CD8αα IELs. The thymic imprinting might include gene products that favor survival and/or are biased toward unconventional IEL development.

It should be underlined that recent studies using H-Y TCR transgenic mice seem to exclude the possibility that DP thymocytes could repopulate the intestine (104) because H-Y TCRβ and TCRα transgenes in this model are expressed abnormally in DN thymocytes and the mice have no TN2–TN4 cells. Those phenotypes were shown to be artifacts of the transgenic systems. In conditions in which the TCRα chain is physiologically expressed in DP thymocytes of the H-Y mice, the male mice have many DP thymocytes expressing the self-reactive αβ T cells, but no increase in *Smyc* peptide-specific CD8αα IELs is found in the gut. Thus, the accumulation of self-reactive TCR transgenic cells in the gut of conventional H-Y transgenic mice seems to be because of the anomalous expression of the TCRα transgene in early TN thymocytes.

It is not clear whether leaked TN2–TN3 cells home exclusively to the gut or to several mucosal sites. CPs may be special features of the gut that attract immature cells. In this regard, Onai *et al.* (105) reported that $CD11c^+$ DCs in CP aggregates express CCL25 and that the signaling via the chemokine receptor CCR9 that is present on $c\text{-}kit^+$ CP cells might play a central role in CP and CD8αα IEL development. When the expression of CCR9 on the surface of $c\text{-}kit^+$ bone marrow cells was blocked by a viral construct expressing an intracellular form of CCL25, bone-marrow-recipient mice largely failed to develop CP aggregates and CD8αα IELs. By contrast, the cellularity and composition of the thymus and spleen of these mice were well developed. These findings led Onai *et al.* (105) to hypothesize that CCR9–CCL25 interaction is required for CP maturation in the generation of TCR^- and TCR^+ IEL subsets.

The gut itself then seems to retain immature thymic emigrants and to nurture their development. As shown before, this nurturing includes local production of IL-7, which promotes local TCR gene rearrangement (97). A capacity for gene rearrangement might seem unexpected, given a report that a reporter gene expressed from the *Rag*-2 promoter is not detected outside the thymus (98). Nonetheless, it has been shown that *Rag*-1 is expressed in the gut, albeit in low numbers, from an alternative 5′ exon that may be conserved in the mouse (106). The reporter construct that has been used previously (95) might not register expression by an unorthodox *Rag* exon (98). In summary, the gut is a viable site for T-cell differentiation because it expresses *Rag*, locally produces IL-7, and can accommodate immature T-cell-committed progenitors.

Cytokine requirements for IEL development

IELs require cytokines for their development. As mentioned above, γ_c-deficient mice show impaired development of IELs expressing either αβTCRs or γδTCRs (107, 108). Several cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) share γ_c as a part of their receptors. Consistent with impaired CP development, IL-7- or IL-7 receptor (IL-7R)-deficient mice did not have γδ IELs but possessed substantial numbers of αβ IELs (109, 110). Local transgene expression of IL-7 by the ECs of $IL\text{-}7^{-/-}$ mice restored the development of γδ IELs associated with CP development (97). Although both αβTCR- and γδTCR-positive CD8αα IELs belong to type b IELs, the developmental pathway of each is distinct, at least in terms of IL-7 requirements. γδ IELs could be preferentially impaired at least in part because IL-7-mediated signaling is essential for the rearrangement of TCRγ genes (111). Additionally, the presence of substantial numbers of αβ IELs in IL-7- or IL-7R-deficient mice implies that other cytokines using γ_c play a pivotal role in the regulation of IEL development, particularly of αβ IELs.

Much attention is currently focused on IL-15, a cytokine that is produced by several kinds of cells including DCs and ECs and that includes an IL-2 receptor β chain (IL-2Rβ) as a part of its receptor. As one might expect from the elimination of αβ and γδ IELs from IL-2Rβ-deficient mice (112), disruption of the IL-15- or IL-15Rα-encoding gene also resulted in a significant

reduction in IELs expressing either αβTCRs or γδTCRs (113, 114). Reciprocally, IL-15 stimulates IEL proliferation and restricts the TCR repertoire of IELs by selectively regulating local Vγ gene chromatin modification (115, 116). In regard to IL-15-induced IEL development, a recent finding has indicated that MyD88 (myeloid differentiation factor 88), an adapter molecule associated with Toll-like receptors, mediates IL-15 production by ECs. As expected, the number of IELs significantly decreased in MyD88-deficient mice (117), suggesting that a MyD88 regulation pathway may help maintain the intestinal homeostasis against commensal microorganisms via the IEL and EC cross-talk system.

IEL trafficking pathway

Determination of IEL tropism by chemokine

It is generally accepted that chemokines play an important role in the regulation of lymphocyte trafficking (118, 119). For the regulation of IEL trafficking into the small intestine, CCR9 has been considered to be a key chemokine (120, 121) (*Fig.* 1). Its ligand, thymus-expressed chemokine (CCL25), is constitutively expressed on the ECs in the small intestine (122, 123) (*Fig.* 1). CCL25 has been shown to mediate the localization of type a IELs, especially $CD103^+$-naive cells and, more recently, to activate $CD69^+$ cells in the small intestine (124). CCL25 also regulates precursor differentiation during the generation of type b IELs, but it is less involved in steps that involve IEL retention (125). Mice with defective responses against CCL25 exhibited a significant reduction in CPs and IELs in the small intestine. In contrast to these implications, however, disruption of the CCR9 gene resulted in only a modest decrease in IELs, indicating that other chemokines might be involved in the IEL migration into the small intestine (126, 127). Furthermore, although there are $CCR9^+$ cells in the large intestine, the CCR9/CCL25-mediated pathway is not involved in IEL recruitment into the large intestine because its ligand (CCL25) is poorly expressed on large intestinal ECs (120, 122, 123). It would stand to reason then that chemokines other than $CCR9^+$ are involved in the regulation of IEL trafficking in the large intestine. Indeed, CCL28, which acts as a ligand for $CCR10^+$-IgA-secreting cells (128, 129), is the predominant chemokine expressed on ECs in the large but not the small intestine (130). Although memory-type T cells in the intestine did not express CCR10 (128), it is possible that a CCL28/CCR10-mediated pathway might contribute to the regulation of large intestinal IEL trafficking, especially naive-type IELs preferentially residing in the large intestine. In addition, the various chemokine receptors expressed on IELs, such as CCR3, CCR4, CCR5, and CXCR3 (131–133), may play a pivotal role in the alternative pathway of IEL trafficking. Although CCR6 is not expressed on mature IELs, CCR6-deficient mice showed impaired development of CD4 CD8αα αβ IELs and CD8αα αβ IELs but not CD8αα γδ IELs because of the dysregulated development of CPs (134). These findings suggest that CD8αα αβ IEL and CD8αα γδ IEL developments have different chemokine requirements.

In addition to chemokine-mediated pathways, we recently found that sphingosine-1-phosphate (S1P) plays a role in IEL trafficking, especially in the large intestine (Jun Kunisawa, unpublished data). S1P, a lipid mediator originating from sphingomyelin, has been linked to the regulation of lymphocyte emigration from the secondary lymphoid organs and thymus by an accumulating body of evidence (118). Our new finding suggests that some type a IELs also use S1P in their exit from the GALT (Jun Kunisawa, unpublished data). Because previous studies proposed a mutual interaction between S1P- and chemokine-mediated pathways in lymphocyte trafficking (135, 136), the cooperative pathway mediated by both S1P and chemokines may determine the selective trafficking of IELs into the small and large intestine.

Integrin-mediated IEL trafficking and distribution

In addition to chemokines, adhesion molecules also contribute to the regulation of IEL trafficking (*Fig.* 1). Among them, β7 integrin has been considered to be an important molecule in determining the localization of intestinal IELs (*Fig.* 1). Thus, β7-integrin-deficient mice showed critically reduced numbers of IELs in the intestine (137, 138). IEL numbers were equally reduced in the small and large intestine, indicating that a chemokine-mediated rather than an adhesion-molecule-mediated pathway determines whether IELs migrate to the small or large intestine.

Several lines of evidence have demonstrated that DCs derived from the GALT (PPs and MLNs) but not those derived from the spleen are capable of determining the gut tropism of intestinal T cells by the induction of high levels of α4β7 integrin and CCR9 expression (139–141). A recent study showed that retinoic acid, which induces the α4β7 integrin and CCR9 expression on T cells (142), is dominantly expressed by GALT DCs (*Fig.* 1). Thus, mucosal T cells activated by orally administered antigens presented by GALT DCs tend to migrate into distant intestinal effector sites, including the intestinal epithelium, by obtaining mucosal trafficking molecules (e.g. α4β7 integrin and CCR9). Regarding the integrated integrin- and chemokine-mediated induction pathway, CCL25, a ligand for CCR9, is expressed in the crypt region of the small intestine, which is close to the mucosal addressin cell adhesion molecule 1 $(MAdCAM\text{-}1)^+$ vessels (123, 127). These findings further indicate that α4β7

integrin–MAdCAM-1 and CCL25–CCR9 interactions synergistically recruit gut-tropic IELs into the small intestine.

The unique migration pathway of RTE into the small intestine

Because the migration of RTE into the small intestinal epithelium was detected in the lymphotoxin-α-deficient mice lacking organized secondary lymphoid organs such as PPs and MLNs, their migration must have been independent of the GALT structure (32). In contrast, the migration of RTEs was abolished in CCR9-deficient mice or mice treated with anti-CCL25 antibody or anti-α4β7 integrin antibody, suggesting that the migration was dependent on a CCL25/CCR9-mediated and α4β7-integrin-mediated pathway (32) (Fig. 1). As one might expect given the preferential expression of CCR9 on RTEs, RTEs homed to the small intestinal epithelium more efficiently than did non-RTE-naive T cells (32). Although RTEs share with type a IELs a similar dependency on CCL25/CCR9 and α4β7 integrin, RTEs do not require antigen-mediated activation. Thus, much unlike type a IELs, RTEs isolated from OT-I^{+}*Rag1*$^{-/-}$ mice can migrate into the small intestinal epithelium without antigen stimulation (32, 143).

Retention molecules on IELs

The intimate integrin-mediated interaction between ECs and IELs plays a pivotal role not just in the infiltration of the IELs into the intestine but also in the retention of IELs at the epithelium. Many attempts have been made at identifying the molecules that tether IELs and ECs together in the small intestine. CD103 (αE integrin), which interacts with the E-cadherin expressed on ECs, is expressed on IELs (144) (Fig. 1). It was previously reported that transforming growth factor-β (TGF-β) induced downregulation of α4β7 integrin and simultaneously upregulated CD103 (145). Thus, α4β7 integrin expression was reduced following IEL entry into the small intestinal epithelium, a reduction that coincided with an increase in CD103 expression (146). In addition to the TGF-β-mediated pathway, a recent study (146) proposed that CCR9-mediated signaling promoted the induction of CD103. In CCR9-deficient CD8^{+} T cells, upregulation of CD103 after migration into the small intestinal epithelium was markedly delayed (146). Additionally, the interaction between CD103 and E-cadherin was involved in the destruction of the intestinal epithelium during graft-versus-host disease (147), indicating that CD103 is a pivotal molecule regulating lymphocyte trafficking not only under natural conditions but also during pathological situations. Further, although CD103 was not required for the entry into the intestine (138), CD103-deficient mice exhibited a decreased number of IELs (148). However, the reduction was modest, suggesting that other molecules contribute to the retention of IELs at the epithelium. In this regard, several lines of evidence have demonstrated that IEL retention may be mediated by their expression of some integrins (e.g. α1β1 integrin, α4β1 integrin, and β2 integrin) (149–151). As in CD103-deficient mice, no or only a partial reduction in IELs was seen following the disruption of each of these integrins, suggesting that other integrins and/or molecules may compensate for their lack (151, 152). Additionally, we previously reported that IELs expressed the EC adhesion molecule (Ep-CAM) (153) (Fig. 1). Because Ep-CAM is also expressed on ECs and mediates homophilic cell-to-cell adhesion (154), interaction between IELs and ECs via Ep-CAM may play a pivotal role in IEL retention. Further, a recent study (155) indicates that IELs express tight-junction molecules such as occludins and zonula occludens-1 (Fig. 1). Taken together, the intimate biological interactions between ECs and IELs provide physiological and immunological barriers that act as a first line of defense at the intestine.

Physiological and biological functions of IELs

CD8αα αβ IELs in immune protection and mucosal homeostasis

Despite their high frequency, potentially autoreactive type b CD8αα αβ IELs are not self-destructive under normal conditions. Rather, their presence in the gut epithelium correlates with immune regulation and immune quiescence. Because endogenous, natural, specific self-antigens for these CD8αα αβ IELs have not yet been identified, studies analyzing their functional ability are limited to the use of TCR transgenic mice, which express a cognate antigen for this TCR either naturally or through expression of a second transgene.

These transgenic mice, which have few conventional αβ T cells expressing the transgenic TCR in the thymus, spleen, and lymph nodes, generate enhanced numbers of transgenic CD8αα αβ IELs specific for the self-antigen (63) that are not anergic (83, 86, 156). Upon a systemic LCMV infection in double-transgenic mice expressing both an LCMV epitope (GP33-41)-specific TCR transgene and the cognate antigen transgene (GP33-41) driven by an MHC class I promoter, CD8αα αβ IELs showed signs of virus-induced activation (86). This activation was antigen specific as the CD8αα αβ IELs from double-transgenic mice expressing an OVA-peptide-specific TCR and the OVA antigen did not show activation following LCMV infection. Unlike conventional (GP33-41-specific) CD8αβ T cells, which lose self-tolerance upon LCMV infection, the activation of these self-specific CD8αα αβ IELs did not result in

a loss of tolerance. The activated CD8αα αβ IELs did not show antigen-specific cytotoxicity nor did they promote an inflammatory response. Instead, the responding CD8αα αβ IELs secreted enhanced levels of TGF-β. The constitutive expression of measurable levels of mRNA for immunoregulatory cytokines, including IFN-γ, TGF-β, and IL-10, even without specific antigenic stimulation, further underscores that these type b IELs might exert regulatory functions rather than affording immune protection against specific pathogens.

The regulatory ability of these self-reactive T cells was first showed by analysis of gene-targeted animals, including IL-2/IL-2R-deficient mice, which have specific defects in this mucosal T-cell subset. These mice, in which the CD8αα αβ IEL population is reduced, are highly susceptible to gut inflammatory diseases (157, 158).

Direct evidence that CD8αα αβ IELs could regulate immune responses by conventional mucosal T cells was provided by sophisticated adoptive transfer experiments of CD8αα αβ IELs into immune-deficient $Rag^{-/-}$ recipient mice that also received pathogenic $CD4^{+}CD45RB^{high}$ splenocytes (156). In the absence of CD8αα αβ IELs, transferred pathogenic $CD4^{+}$ T cells migrate to the intestine and induce an uncontrolled inflammatory immune response, whereas mice that had previously received CD8αα αβ IELs were protected against this $CD4^{+}$ T-cell-mediated pathology. Surprisingly, $CD4^{+}CD45RB^{high}$ T-cell-induced colitis was prevented by transfer of CD8αα αβ IELs isolated from H-Y TCR transgenic male donor mice (156). This protection, however, was observed only in $H\text{-}2D^{b}$ male recipient mice and not in female mice, indicating that TCR must recognize self-antigen if it is to mediate regulatory function.

CD8αα γδ IELs and immune protection

The antigen specificity of CD8αα γδ IELs is poorly defined, and cell-transfer studies have indicated that the CD8αα γδ IELs have only minimal pathogen-specific activity (159, 160). Nevertheless, in the case of recipient mice infected with *Toxoplasma gondii*, it was shown that successful protection by CD8αβ αβ IELs was, in part, dependent on the presence of CD8αα γδ IELs (160). Although these CD8αα γδ IELs show only minimal pathogen-specific activity, their ability to respond rapidly to unprocessed self-antigens expressed by stressed or transformed ECs and to control the activity of the non-self-reactive CD8αβ αβ IELs has led to the idea that these cells might provide the first line of defense against invading pathogens (54).

Alternatively, because activated γδ IELs can produce keratinocyte growth factor (KGF), which is important for epithelial growth and the repair of damaged tissue, some γδ IELs could be involved in the repair of tissue damage elicited during inflammatory immune responses (161). The synthesis of KGF by γδ IELs has led to the hypothesis that these cells are important for the integrity and healing of the epithelium. Consistent with this hypothesis, the epithelium of the intestine in TCRδ-chain-deficient mice had reduced numbers of crypts (162). By contrast, the epithelium had a higher mitotic index in the presence of γδTCR transgene (163). In addition, TCRδ-chain-deficient mice show increased susceptibility to epithelial damage caused by 2,4,6-trinitrobenzene sulfonic-acid-induced colitis; indeed, the transfer of γδ IELs to TCRδ-chain-deficient mice ameliorated the hapten-induced colitis, which correlated with decreased IFN-γ and TNF-α production and increased TGF-β production by IELs (164).

It remains to be determined if the effect of γδ IELs on ECs is mediated solely by KGF or if other factors are involved. γδ IELs synthesize mRNA for TGF-β1 (18) and TGF-β3 (165), and TGF-β has been reported to aid in the healing of epithelial damage (166). Furthermore, a lack of γδ T cells often results in severe pathological conditions mediated by autoantibodies and/or in destructive cell-mediated immune responses by αβ T cells (167). TCRδ-deficient mice that have been orally infected with *Eimeria vermiformis*, an enterocyte-specific parasite, can efficiently control the infection, but they also show severe bleeding in the small intestine because of uncontrolled immune responses by infiltrating $CD4^{+}$ αβ T cells (168). Transfer of normal IELs to these TCRδ-deficient mice could prevent mucosal injury. These results suggest that γδ IELs exert significant control over the immune responses mediated by mucosal αβ T cells.

Not all immune responses mediated by γδ IELs are beneficial. For example, in the case of celiac disease, it was shown that dysregulated IL-15 secretion by wheat gliadin elicited overexpression of MICA/MICB on ECs and IELs, possibly including γδ T cells. When isolated from patients with active celiac disease, γδ T cells exhibited NKG2D-mediated cytotoxicity of the EC line HT29 possessing MICA (30).

γδ IELs for mucosal IgA responses

γδ IELs appear to be necessary for the induction and maintenance of humoral immune responses in the intestine. Interestingly, in TCRα-chain-deficient mice, an increase in the numbers and overall proportion of γδ IELs is seen after environmental microbial challenge (169), which is accompanied by B-cell maturation and production of high levels of Igs (170–172). Collaboration between B and non-αβ T cells, including γδ T cells as well as ββ TCR^{+} T cells (ββ T cells), sustains the production of germinal centers, lymphoid follicles that ordinarily are the anatomical signature of αβ T-cell and B-cell interactions.

Murine γδ IELs, if administered in the periphery, can enhance antigen-specific IgA responses to orally ingested antigens and thus might be capable of abrogating oral tolerance (173, 174). However, because it is unlikely that γδ IELs recirculate from the intestinal mucosa, their potential role in inducing or suppressing systemic oral tolerance is questionable. Although antibody responses against some T-cell-dependent antigens are impaired in mice lacking αβ T cells, mucosal γδ T cells may enhance antigen-specific as well as polyclonal S-IgA responses in the *lamina propria* of the small intestine (175).

Conclusions and future perspectives

In the harsh environment of the gut, the mucosal immune system must prevent the invasion of pathogenic microorganisms from the lumen while remaining quiescent against commensal microorganisms and food antigens, if disordered immune responses are to be prevented. Remarkably, IELs play a pivotal role in both immune surveillance and immune regulation, helping to maintain intestinal homeostasis by adjusting their immunological function to the different circumstances that prevail in the small and large intestines. However, the origin and development of these key players remain a subject of debate, and so it is crucial that we work to define their precise function and delineate their developmental pathway. Further investigation of IELs in both the small and large intestine will undoubtedly be key to the development of novel mucosal vaccines against infectious diseases and to immune therapy for inflammatory bowel diseases, food allergies, and celiac diseases.

References

1. Kunisawa J, Fukuyama S, Kiyono H. Mucosa-associated lymphoid tissues in the aerodigestive tract: their shared and divergent traits and their importance to the orchestration of the mucosal immune system. Curr Mol Med 2005;**5**:557–572.
2. Kiyono H, Fukuyama S. NALT- versus Peyer's-patch-mediated mucosal immunity. Nat Rev Immunol 2004;**4**:699–710.
3. Neutra MR, Pringault E, Kraehenbuhl JP. Antigen sampling across epithelial barriers and induction of mucosal immune responses. Annu Rev Immunol 1996;**14**:275–300.
4. Kunisawa J, McGhee J, Kiyono H. Mucosal S-IgA enhancement: development of safe and effective mucosal adjuvants and mucosal antigen delivery vehicles. In: Kaetzel C, ed. Mucosal Immune. Defense: Immunoglobubin A. London: Kluwer Academic/Plenum Publishers, 2006; in press.
5. Berkes J, Viswanathan VK, Savkovic SD, Hecht G. Intestinal epithelial responses to enteric pathogens: effects on the tight junction barrier, ion transport, and inflammation. Gut 2003;**52**:439–451.
6. Tamagawa H, et al. Characteristics of claudin expression in follicle-associated epithelium of Peyer's patches: preferential localization of claudin-4 at the apex of the dome region. Lab Invest 2003;**83**: 1045–1053.
7. Bals R, et al. Mouse β-defensin 3 is an inducible antimicrobial peptide expressed in the epithelia of multiple organs. Infect Immun 1999;**67**:3542–3547.
8. Kunisawa J, Kiyono H. A marvel of mucosal T cells and secretory antibodies for the creation of first lines of defense. Cell Mol Life Sci 2005;**62**:1308–1321.
9. Mestecky J, Zikan J, Butler WT. Immunoglobulin M and secretory immunoglobulin A: presence of a common polypeptide chain different from light chains. Science 1971;**171**:1163–1165.
10. Halpern MS, Koshland ME. Noval subunit in secretory IgA. Nature 1970;**228**:1276–1278.
11. Hiroi T, et al. Deficiency of IL-5 receptor α-chain selectively influences the development of the common mucosal immune system independent IgA-producing B-1 cell in mucosa-associated tissues. J Immunol 1999;**162**:821–828.
12. Martin F, Kearney JF. B1 cells: similarities and differences with other B cell subsets. Curr Opin Immunol 2001;**13**:195–201.
13. Shastri N, Cardinaud S, Schwab SR, Serwold T, Kunisawa J. All the peptides that fit: the beginning, the middle, and the end of the MHC class I antigen-processing pathway. Immunol Rev 2005;**207**:31–41.
14. Das G, Janeway CA Jr. MHC specificity of iIELs. Trends Immunol 2003;**24**: 88–93.
15. Beagley KW, et al. Differences in intraepithelial lymphocyte T cell subsets isolated from murine small versus large intestine. J Immunol 1995;**154**: 5611–5619.
16. Van Houten N, Mixter PF, Wolfe J, Budd RC. CD2 expression on murine intestinal intraepithelial lymphocytes is bimodal and defines proliferative capacity. Int Immunol 1993;**5**:665–672.
17. Ohteki T, MacDonald HR. Expression of the CD28 costimulatory molecule on subsets of murine intestinal intraepithelial lymphocytes correlates with lineage and responsiveness. Eur J Immunol 1993;**23**:1251–1255.
18. Shires J, Theodoridis E, Hayday AC. Biological insights into TCRγδ$^+$ and TCRαβ$^+$ intraepithelial lymphocytes provided by serial analysis of gene expression (SAGE). Immunity 2001;**15**:419–434.
19. Klein JR. Ontogeny of the Thy-1$^-$, Lyt-2$^+$ murine intestinal intraepithelial lymphocyte. Characterization of a unique population of thymus-independent cytotoxic effector cells in the intestinal mucosa. J Exp Med 1986;**164**:309–314.
20. Leishman AJ, et al. T cell responses modulated through interaction between CD8αα and the nonclassical MHC class I molecule, TL. Science 2001;**294**:1936–1939.
21. Morrissey PJ, Charrier K, Horovitz DA, Fletcher FA, Watson JD. Analysis of the intra-epithelial lymphocyte compartment in SCID mice that received co-isogenic CD4$^+$ T cells. Evidence that mature post-thymic CD4$^+$ T cells can be induced to express CD8α in vivo. J Immunol 1995;**154**:2678–2686.
22. Tagliabue A, Befus AD, Clark DA, Bienenstock J. Characteristics of natural killer cells in the murine intestinal epithelium and lamina propria. J Exp Med 1982;**155**: 1785–1796.
23. Guy-Grand D, Cuenod-Jabri B, Malassis-Seris M, Selz F, Vassalli P. Complexity of the mouse gut T cell immune system: identification of two distinct natural killer T cell intraepithelial lineages. Eur J Immunol 1996;**26**:2248–2256.
24. Lundqvist C, Baranov V, Hammarstrom S, Athlin L, Hammarstrom ML. Intra-epithelial lymphocytes. Evidence for regional specialization and extrathymic T cell maturation in the human gut epithelium. Int Immunol 1995;**7**:1473–1487.

25. Bauer S, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 1999;**285**:727–729.
26. Groh V, Steinle A, Bauer S, Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial γδ T cells. Science 1998;**279**:1737–1740.
27. Das H, et al. MICA engagement by human Vγ2Vδ2 T cells enhances their antigen-dependent effector function. Immunity 2001;**15**:83–93.
28. Kinoshita N, Hiroi T, Ohta N, Fukuyama S, Park EJ, Kiyono H. Autocrine IL-15 mediates intestinal epithelial cell death via the activation of neighboring intraepithelial NK cells. J Immunol 2002;**169**:6187–6192.
29. Meresse B, et al. Coordinated induction by IL-5 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. Immunity 2004;**21**:357–366.
30. Hue S, et al. A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. Immunity 2004;**21**:367–377.
31. Ebert EC. IL-15 converts human intestinal intraepithelial lymphocytes to CD94 producers of IFN-γ and IL-10, the latter promoting Fas ligand-mediated cytotoxicity. Immunology 2005;**115**:118–126.
32. Staton TL, Habtezion A, Winslow MM, Sato T, Love PE, Butcher EC. $CD8^+$ recent thymic emigrants home to and efficiently repopulate the small intestine epithelium. Nat Immunol 2006;**7**:482–488.
33. Staton TL, Johnston B, Butcher EC, Campbell DJ. Murine $CD8^+$ recent thymic emigrants are αE integrin-positive and CC chemokine ligand 25 responsive. J Immunol 2004;**172**:7282–7288.
34. Camerini V, Panwala C, Kronenberg M. Regional specialization of the mucosal immune system. Intraepithelial lymphocytes of the large intestine have a different phenotype and function than those of the small intestine. J Immunol 1993;**151**: 1765–1776.
35. Camerini V, et al. Generation of intestinal mucosal lymphocytes in SCID mice reconstituted with mature, thymus-derived T cells. J Immunol 1998;**160**:2608–2618.
36. Ibraghimov AR, Lynch RG. Heterogeneity and biased T cell receptor alpha/beta repertoire of mucosal $CD8^+$ cells from murine large intestine: implications for functional state. J Exp Med 1994;**180**:433–444.
37. van Oers NS, Teh SJ, Garvin AM, Forbush KA, Perlmutter RM, Teh HS. CD8 inhibits signal transduction through the T cell receptor in $CD4^-CD8^-$thymocytes from T cell receptor transgenic mice reconstituted with a transgenic CD8α molecule. J Immunol 1993;**151**:777–790.
38. Madakamutil LT, et al. CD8αα-mediated survival and differentiation of CD8 memory T cell precursors. Science 2004;**304**:590–593.
39. Arstila T, et al. Identical T cell clones are located within the mouse gut epithelium and lamina propia and circulate in the thoracic duct lymph. J Exp Med 2000;**191**:823–834.
40. Regnault A, Cumano A, Vassalli P, Guy-Grand D, Kourilsky P. Oligoclonal repertoire of the CD8αα and the CD8αβ TCR-α/β murine intestinal intraepithelial T lymphocytes: evidence for the random emergence of T cells. J Exp Med 1994;**180**:1345–1358.
41. Rocha B, Vassalli P, Guy-Grand D. The Vβ repertoire of mouse gut homodimeric α CD8+ intraepithelial T cell receptor α/β + lymphocytes reveals a major extrathymic pathway of T cell differentiation. J Exp Med 1991;**173**:483–486.
42. Lin T, et al. Thymus ontogeny and the development of TCRαβ intestinal intraepithelial lymphocytes. Cell Immunol 1996;**171**:132–139.
43. Latthe M, Terry L, MacDonald TT. High frequency of CD8αα homodimer-bearing T cells in human fetal intestine. Eur J Immunol 1994;**24**:1703–1705.
44. Imaoka A, Matsumoto S, Setoyama H, Okada Y, Umesaki Y. Proliferative recruitment of intestinal intraepithelial lymphocytes after microbial colonization of germ-free mice. Eur J Immunol 1996;**26**:945–948.
45. Villadangos JA, Schnorrer P, Wilson NS. Control of MHC class II antigen presentation in dendritic cells: a balance between creative and destructive forces. Immunol Rev 2005;**207**:191–205.
46. Correa I, Bix M, Liao NS, Zijlstra M, Jaenisch R, Raulet D. Most γδT cells develop normally in β2-microglobulin-deficient mice. Proc Natl Acad Sci USA 1992;**89**:653–657.
47. Fujiura Y, et al. Development of CD8αα+ intestinal intraepithelial T cells in β2-microglobulin- and/or TAP1-deficient mice. J Immunol 1996;**156**:2710–2715.
48. Neuhaus O, Emoto M, Blum C, Yamamoto S, Kaufmann SH. Control of thymus-independent intestinal intraepithelial lymphocytes by β2-microglobulin. Eur J Immunol 1995;**25**:2332–2339.
49. Sydora BC, Brossay L, Hagenbaugh A, Kronenberg M, Cheroutre H. TAP-independent selection of CD8+ intestinal intraepithelial lymphocytes. J Immunol 1996;**156**: 4209–4216.
50. Park SH, Guy-Grand D, Lemonnier FA, Wang CR, Bendelac A, Jabri B. Selection and expansion of $CD8\alpha/\alpha^+$ T cell receptor α/β^+ intestinal intraepithelial lymphocytes in the absence of both classical major histocompatibility complex class I and nonclassical CD1 molecules. J Exp Med 1999;**190**:885–890.
51. Gapin L, Cheroutre H, Kronenberg M. Cutting edge: $TCR\alpha\beta^+$ $CD8\alpha^+$ T cells are found in intestinal intraepithelial lymphocytes of mice that lack classical MHC class I molecules. J Immunol 1999;**163**: 4100–4104.
52. Tabaczewski P, Stroynowski I. Expression of secreted and glycosylphosphatidylinositol-bound Qa-2 molecules is dependent on functional TAP-2 peptide transporter. J Immunol 1994;**152**: 5268–5274.
53. Das G, et al. Qa-2-dependent selection of CD8αα T cell receptor α/β(+) cells in murine intestinal intraepithelial lymphocytes. J Exp Med 2000;**192**:1521–1528.
54. Cheroutre H. IELs: enforcing law and order in the court of the intestinal epithelium. Immunol Rev 2005;**206**:114–131.
55. Hershberg R, et al. Expression of the thymus leukemia antigen in mouse intestinal epithelium. Proc Natl Acad Sci USA 1990;**87**:9727–9731.
56. Bleicher PA, Balk SP, Hagen SJ, Blumberg RS, Flotte TJ, Terhorst C. Expression of murine CD1 on gastrointestinal epithelium. Science 1990;**250**:679–682.
57. Brossay L, et al. Mouse CD1 is mainly expressed on hemopoietic-derived cells. J Immunol 1997;**159**:1216–1224.
58. Brutkiewicz RR, Bennink JR, Yewdell JW, Bendelac A. TAP-independent, β2-microglobulin-dependent surface expression of functional mouse CD1.1. J Exp Med 1995;**182**:1913–1919.
59. Holcombe HR, et al. Nonclassical behavior of the thymus leukemia antigen: peptide transporter-independent expression of a nonclassical class I molecule. J Exp Med 1995;**181**:1433–1443.
60. Teitell M, et al. Nonclassical behavior of the mouse CD1 class I-like molecule. J Immunol 1997;**158**:2143–2149.
61. Liu Y, et al. The crystal structure of a TL/CD8αα complex at 2.1 A resolution: implications for modulation of T cell activation and memory. Immunity 2003;**18**: 205–215.
62. Guy-Grand D, Pardigon N, Darche S, Lantz O, Kourilsky P, Vassalli P. Contribution of double-negative thymic precursors to CD8αα(+) intraepithelial lymphocytes of the gut in mice bearing TCR transgenes. Eur J Immunol 2001;**31**:2593–2602.
63. Leishman AJ, et al. Precursors of functional MHC class I- or class II-restricted CD8αα(+) T cells are positively selected in the thymus by agonist self-peptides. Immunity 2002;**16**:355–364.
64. Allison JP, Havran WL. The immunobiology of T cells with invariant γδ antigen receptors. Annu Rev Immunol 1991;**9**:679–705.

65. Asarnow DM, Goodman T, LeFrancois L, Allison JP. Distinct antigen receptor repertoires of two classes of murine epithelium-associated T cells. Nature 1989;**341**: 60–62.
66. Halstensen TS, Scott H, Brandtzaeg P. Intraepithelial T cells of the TCR γ/δ^+ CD8- and $V\delta1/J\delta1^+$ phenotypes are increased in coeliac disease. Scand J Immunol 1989;**30**:665–672.
67. Chowers Y, Holtmeier W, Harwood J, Morzycka-Wroblewska E, Kagnoff MF. The Vδ1 T cell receptor repertoire in human small intestine and colon. J Exp Med 1994;**180**:183–190.
68. Steinle A, Groh V, Spies T. Diversification, expression, and γδ T cell recognition of evolutionarily distant members of the MIC family of major histocompatibility complex class I-related molecules. Proc Natl Acad Sci USA 1998;**95**:12510–12515.
69. Wu J, Groh V, Spies T. T cell antigen receptor engagement and specificity in the recognition of stress-inducible MHC class I-related chains by human epithelial γδ T cells. J Immunol 2002;**169**:1236–1240.
70. Diefenbach A, Jamieson AM, Liu SD, Shastri N, Raulet DH. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. Nat Immunol 2000;**1**:119–126.
71. Cerwenka A, et al. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. Immunity 2000;**12**:721–727.
72. Cawthon AG, Alexander-Miller MA. Optimal colocalization of TCR and CD8 as a novel mechanism for the control of functional avidity. J Immunol 2002;**169**: 3492–3498.
73. Arcaro A, et al. CD8β endows CD8 with efficient coreceptor function by coupling T cell receptor/CD3 to raft-associated CD8/p56(lck) complexes. J Exp Med 2001;**194**:1485–1495.
74. Wu M, van Kaer L, Itohara S, Tonegawa S. Highly restricted expression of the thymus leukemia antigens on intestinal epithelial cells. J Exp Med 1991;**174**:213–218.
75. Park EJ, et al. Clonal expansion of double-positive intraepithelial lymphocytes by MHC class I-related chain A expressed in mouse small intestinal epithelium. J Immunol 2003;**171**:4131–4139.
76. Groh V, Bahram S, Bauer S, Herman A, Beauchamp M, Spies T. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proc Natl Acad Sci USA 1996;**93**:12445–12450.
77. Guy-Grand D, et al. Different use of T cell receptor transducing modules in two populations of gut intraepithelial lymphocytes are related to distinct pathways of T cell differentiation. J Exp Med 1994;**180**: 673–679.
78. Liu CP, et al. Abnormal T cell development in $CD3\zeta^{-/-}$ mutant mice and identification of a novel T cell population in the intestine. EMBO J 1993;**12**:4863–4875.
79. She J, et al. CD16-expressing $CD8\alpha\alpha^+$ T lymphocytes in the intestinal epithelium: possible precursors of FcγR-$CD8\alpha\alpha^+$ T cells. J Immunol 1997;**158**:4678–4687.
80. Ohno H, Ono S, Hirayama N, Shimada S, Saito T. Preferential usage of the Fc receptor γ chain in the T cell antigen receptor complex by γ/δ T cells localized in epithelia. J Exp Med 1994;**179**:365–369.
81. Schweighoffer E, Fowlkes BJ. Positive selection is not required for thymic maturation of transgenic γδ T cells. J Exp Med 1996;**183**:2033–2041.
82. Mintern JD, Maurice MM, Ploegh HL, Schott E. Thymic selection and peripheral activation of CD8 T cells by the same class I MHC/peptide complex. J Immunol 2004;**172**:699–708.
83. Cruz D, Sydora BC, Hetzel K, Yakoub G, Kronenberg M, Cheroutre H. An opposite pattern of selection of a single T cell antigen receptor in the thymus and among intraepithelial lymphocytes. J Exp Med 1998;**188**:255–265.
84. Guehler SR, Bluestone JA, Barrett TA. Immune deviation of 2C transgenic intraepithelial lymphocytes in antigen-bearing hosts. J Exp Med 1996;**184**:493–503.
85. Levelt CN, et al. High- and low-affinity single-peptide/MHC ligands have distinct effects on the development of mucosal $CD8\alpha\alpha$ and $CD8\alpha\beta$ T lymphocytes. Proc Natl Acad Sci USA 1999;**96**:5628–5633.
86. Saurer L, Seibold I, Rihs S, Vallan C, Dumrese T, Mueller C. Virus-induced activation of self-specific $TCR\alpha\beta$ $CD8\alpha\alpha$ intraepithelial lymphocytes does not abolish their self-tolerance in the intestine. J Immunol 2004;**172**:4176–4183.
87. Podd BS, Aberg C, Kudla KL, Keene L, Tobias E, Camerini V. MHC class I allele dosage alters CD8 expression by intestinal intraepithelial lymphocytes. J Immunol 2001;**167**:2561–2568.
88. Lambolez F, et al. Characterization of T cell differentiation in the murine gut. J Exp Med 2002;**195**:437–449.
89. Yoshikai Y, Reis MD, Mak TW. Athymic mice express a high level of functional γ-chain but greatly reduced levels of α- and β-chain T-cell receptor messages. Nature 1986;**324**:482–485.
90. Guy-Grand D, Cerf-Bensussan N, Malissen B, Malassis-Seris M, Briottet C, Vassalli P. Two gut intraepithelial $CD8^+$ lymphocyte populations with different T cell receptors: a role for the gut epithelium in T cell differentiation. J Exp Med 1991;**173**: 471–481.
91. Lin T, Takimoto H, Matsuzaki G, Nomoto K. Effect of neonatal thymectomy on murine small intestinal intraepithelial lymphocytes expressing T cell receptor αβ and "clonally forbidden Vβs". Adv Exp Med Biol 1995;**371A**:129–131.
92. Rocha B, Vassalli P, Guy-Grand D. Thymic and extrathymic origins of gut intraepithelial lymphocyte populations in mice. J Exp Med 1994;**180**:681–686.
93. Saito H, et al. Generation of intestinal T cells from progenitors residing in gut cryptopatches. Science 1998;**280**:275–278.
94. Kanamori Y, et al. Identification of novel lymphoid tissues in murine intestinal mucosa where clusters of $c\text{-}kit^+$ $IL\text{-}7R^+$ $Thy1^+$ lympho-hemopoietic progenitors develop. J Exp Med 1996;**184**: 1449–1459.
95. Oida T, et al. Role of gut cryptopatches in early extrathymic maturation of intestinal intraepithelial T cells. J Immunol 2000;**164**:3616–3626.
96. Suzuki K, et al. Gut cryptopatches: direct evidence of extrathymic anatomical sites for intestinal T lymphopoiesis. Immunity 2000;**13**:691–702.
97. Laky K, et al. Enterocyte expression of interleukin 7 induces development of γδ T cells and Peyer's patches. J Exp Med 2000;**191**:1569–1580.
98. Guy-Grand D, et al. Extrathymic T cell lymphopoiesis: ontogeny and contribution to gut intraepithelial lymphocytes in athymic and euthymic mice. J Exp Med 2003;**197**:333–341.
99. Nonaka S, et al. Intestinal γδ T cells develop in mice lacking thymus, all lymph nodes, Peyer's patches, and isolated lymphoid follicles. J Immunol 2005;**174**:1906–1912.
100. Podd BS, et al. T cells in cryptopatch aggregates share TCRγ variable region junctional sequences with γδ T cells in the small intestinal epithelium of mice. J Immunol 2006;**176**:6532–6542.
101. Eberl G, Littman DR. Thymic origin of intestinal αβ T cells revealed by fate mapping of RORγt+ cells. Science 2004;**305**: 248–251.
102. Eberl G, Littman DR. Comment on "Thymic origin of intestinal αβ T cells revealed by fate mapping of RORγt+ cells". Science 2005;**308**:1553b.

103. Lambolez F, et al. The thymus exports long-lived fully committed T cell precursors that can colonize primary lymphoid organs. Nat Immunol 2006;**7**:76–82.
104. Baldwin TA, Sandau MM, Jameson SC, Hogquist KA. The timing of TCRα expression critically influences T cell development and selection. J Exp Med 2005;**202**: 111–121.
105. Onai N, Kitabatake M, Zhang YY, Ishikawa H, Ishikawa S, Matsushima K. Pivotal role of CCL25 (TECK)-CCR9 in the formation of gut cryptopatches and consequent appearance of intestinal intraepithelial T lymphocytes. Int Immunol 2002;**14**:687–694.
106. Bas A, Hammarstrom SG, Hammarstrom ML. Extrathymic TCR gene rearrangement in human small intestine: identification of new splice forms of recombination activating gene-1 mRNA with selective tissue expression. J Immunol 2003;**171**: 3359–3371.
107. Cao X, et al. Defective lymphoid development in mice lacking expression of the common cytokine receptor γ chain. Immunity 1995;**2**:223–238.
108. DiSanto JP, Muller W, Guy-Grand D, Fischer A, Rajewsky K. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor γ chain. Proc Natl Acad Sci USA 1995;**92**:377–381.
109. Moore TA, von Freeden-Jeffry U, Murray R, Zlotnik A. Inhibition of γδ T cell development and early thymocyte maturation in IL-7-/- mice. J Immunol 1996;**157**: 2366–2373.
110. Maki K, et al. Interleukin 7 receptor-deficient mice lack γδ T cells. Proc Natl Acad Sci U S A 1996;**93**:7172–7177.
111. Maki K, Sunaga S, Ikuta K. The V-J recombination of T cell receptor-gamma genes is blocked in interleukin-7 receptor-deficient mice. J Exp Med 1996;**184**:2423–2427.
112. Suzuki H, Duncan GS, Takimoto H, Mak TW. Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor β chain. J Exp Med 1997;**185**:499–505.
113. Kennedy MK, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J Exp Med 2000;**191**:771–780.
114. Lodolce JP, et al. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. Immunity 1998;**9**:669–676.
115. Inagaki-Ohara K, Nishimura H, Mitani A, Yoshikai Y. Interleukin-15 preferentially promotes the growth of intestinal intraepithelial lymphocytes bearing γδ T cell receptor in mice. Eur J Immunol 1997;**27**: 2885–2891.
116. Zhao H, Nguyen H, Kang J. Interleukin 15 controls the generation of the restricted T cell receptor repertoire of γδ intestinal intraepithelial lymphocytes. Nat Immunol 2005;**6**:1263–1271.
117. Yu Q, Tang C, Xun S, Yajima T, Takeda K, Yoshikai Y. MyD88-dependent signaling for IL-15 production plays an important role in maintenance of CD8αα TCRαβ and TCRγδ intestinal intraepithelial lymphocytes. J Immunol 2006;**176**:6180–6185.
118. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu Rev Immunol 2005;**23**:127–159.
119. Kunkel EJ, Butcher EC. Chemokines and the tissue-specific migration of lymphocytes. Immunity 2002;**16**:1–4.
120. Zabel BA, et al. Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. J Exp Med 1999;**190**:1241–1256.
121. Kunkel EJ, Campbell DJ, Butcher EC. Chemokines in lymphocyte trafficking and intestinal immunity. Microcirculation 2003;**10**:313–323.
122. Papadakis KA, et al. The role of thymus-expressed chemokine and its receptor CCR9 on lymphocytes in the regional specialization of the mucosal immune system. J Immunol 2000;**165**: 5069–5076.
123. Kunkel EJ, et al. Lymphocyte CC chemokine receptor 9 and epithelial thymus-expressed chemokine (TECK) expression distinguish the small intestinal immune compartment: Epithelial expression of tissue-specific chemokines as an organizing principle in regional immunity. J Exp Med 2000;**192**:761–768.
124. Svensson M, et al. CCL25 mediates the localization of recently activated CD8αβ(+) lymphocytes to the small-intestinal mucosa. J Clin Invest 2002;**110**:1113–1121.
125. Marsal J, et al. Involvement of CCL25 (TECK) in the generation of the murine small-intestinal CD8αα+CD3+ intraepithelial lymphocyte compartment. Eur J Immunol 2002;**32**:3488–3497.
126. Uehara S, Grinberg A, Farber JM, Love PE. A role for CCR9 in T lymphocyte development and migration. J Immunol 2002;**168**:2811–2819.
127. Wurbel MA, et al. The chemokine TECK is expressed by thymic and intestinal epithelial cells and attracts double- and single-positive thymocytes expressing the TECK receptor CCR9. Eur J Immunol 2000;**30**: 262–271.
128. Kunkel EJ, et al. CCR10 expression is a common feature of circulating and mucosal epithelial tissue IgA Ab-secreting cells. J Clin Invest 2003;**111**:1001–1010.
129. Lazarus NH, Kunkel EJ, Johnston B, Wilson E, Youngman KR, Butcher EC. A common mucosal chemokine (mucosae-associated epithelial chemokine/CCL28) selectively attracts IgA plasmablasts. J Immunol 2003;**170**:3799–3805.
130. Pan J, et al. A novel chemokine ligand for CCR10 and CCR3 expressed by epithelial cells in mucosal tissues. J Immunol 2000;**165**:2943–2949.
131. Luangsay S, et al. CCR5 mediates specific migration of Toxoplasma gondii-primed CD8 lymphocytes to inflammatory intestinal epithelial cells. Gastroenterology 2003;**125**:491–500.
132. Shibahara T, Wilcox JN, Couse T, Madara JL. Characterization of epithelial chemoattractants for human intestinal intraepithelial lymphocytes. Gastroenterology 2001;**120**:60–70.
133. Agace WW, Roberts AI, Wu L, Greineder C, Ebert EC, Parker CM. Human intestinal lamina propria and intraepithelial lymphocytes express receptors specific for chemokines induced by inflammation. Eur J Immunol 2000;**30**:819–826.
134. Lugering A, Kucharzik T, Soler D, Picarella D, Hudson JT 3rd, Williams IR. Lymphoid precursors in intestinal cryptopatches express CCR6 and undergo dysregulated development in the absence of CCR6. J Immunol 2003;**171**:2208–2215.
135. Henning G, et al. CC chemokine receptor 7-dependent and -independent pathways for lymphocyte homing: modulation by FTY720. J Exp Med 2001;**194**:1875–1881.
136. Kimura T, et al. The sphingosine 1-phosphate receptor agonist FTY720 supports CXCR4-dependent migration and bone marrow homing of human CD34+ progenitor cells. Blood 2004;**103**:4478–4486.
137. Wagner N, et al. Critical role for β7 integrins in formation of the gut-associated lymphoid tissue. Nature 1996;**382**:366–370.
138. Lefrancois L, et al. The role of β7 integrins in CD8 T cell trafficking during an antiviral immune response. J Exp Med 1999;**189**:1631–1638.
139. Johansson-Lindbom B, Svensson M, Wurbel MA, Malissen B, Marquez G, Agace W. Selective generation of gut tropic T cells in gut-associated lymphoid tissue (GALT): requirement for GALT dendritic cells and adjuvant. J Exp Med 2003;**198**: 963–969.
140. Mora JR, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. Nature 2003;**424**:88–93.

141. Stagg AJ, Kamm MA, Knight SC. Intestinal dendritic cells increase T cell expression of α4β7 integrin. Eur J Immunol 2002;**32**:1445–1454.
142. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. Immunity 2004;**21**:527–538.
143. Kim SK, Reed DS, Heath WR, Carbone F, Lefrancois L. Activation and migration of CD8 T cells in the intestinal mucosa. J Immunol 1997;**159**:4295–4306.
144. Cepek KL, et al. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the αEβ7 integrin. Nature 1994;**372**:190–193.
145. Shibahara T, Si-Tahar M, Shaw SK, Madara JL. Adhesion molecules expressed on homing lymphocytes in model intestinal epithelia. Gastroenterology 2000;**118**: 289–298.
146. Ericsson A, Svensson M, Arya A, Agace WW. CCL25/CCR9 promotes the induction and function of CD103 on intestinal intraepithelial lymphocytes. Eur J Immunol 2004;**34**:2720–2729.
147. El-Asady R, et al. TGF-β-dependent CD103 expression by CD8(+) T cells promotes selective destruction of the host intestinal epithelium during graft-versus-host disease. J Exp Med 2005;**201**:1647–1657.
148. Schon MP, et al. Mucosal T lymphocyte numbers are selectively reduced in integrin αE (CD103)-deficient mice. J Immunol 1999;**162**:6641–6649.
149. Roberts AI, Brolin RE, Ebert EC. Integrin α1β1 (VLA-1) mediates adhesion of activated intraepithelial lymphocytes to collagen. Immunology 1999;**97**:679–685.
150. Ebert EC, Roberts AI. Human intestinal intraepithelial lymphocytes bind to mucosal mesenchymal cells through VLA4 and CD11α. Cell Immunol 1996;**167**:108–114.
151. Huleatt JW, Lefrancois L. β2 integrins and ICAM-1 are involved in establishment of the intestinal mucosal T cell compartment. Immunity 1996;**5**:263–273.
152. Marsal J, Brakebusch C, Bungartz G, Fassler R, Agace WW. β1 integrins are not required for the maintenance of lymphocytes within intestinal epithelia. Eur J Immunol 2005;**35**:1805–1811.
153. Nochi T, et al. Biological role of Ep-CAM in the physical interaction between epithelial cells and lymphocytes in intestinal epithelium. Clin Immunol 2004;**113**:326–339.
154. Litvinov SV, Velders MP, Bakker HA, Fleuren GJ, Warnaar SO. Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule. J Cell Biol 1994;**125**:437–446.
155. Inagaki-Ohara K, Sawaguchi A, Suganuma T, Matsuzaki G, Nawa Y. Intraepithelial lymphocytes express junctional molecules in murine small intestine. Biochem Biophys Res Commun 2005;**331**:977–983.
156. Poussier P, Ning T, Banerjee D, Julius M. A unique subset of self-specific intraintestinal T cells maintains gut integrity. J Exp Med 2002;**195**:1491–1497.
157. Poussier P, Ning T, Chen J, Banerjee D, Julius M. Intestinal inflammation observed in IL-2R/IL-2 mutant mice is associated with impaired intestinal T lymphopoiesis. Gastroenterology 2000;**118**:880–891.
158. Kai Y, et al. Colitis in mice lacking the common cytokine receptor γ chain is mediated by IL-6-producing $CD4^+$ T cells. Gastroenterology 2005;**128**:922–934.
159. Muller S, Buhler-Jungo M, Mueller C. Intestinal intraepithelial lymphocytes exert potent protective cytotoxic activity during an acute virus infection. J Immunol 2000;**164**: 1986–1994.
160. Lepage AC, Buzoni-Gatel D, Bout DT, Kasper LH. Gut-derived intraepithelial lymphocytes induce long term immunity against *Toxoplasma gondii*. J Immunol 1998;**161**: 4902–4908.
161. Boismenu R, Havran WL. Modulation of epithelial cell growth by intraepithelial γδ T cells. Science 1994;**266**:1253–1255.
162. Komano H, et al. Homeostatic regulation of intestinal epithelia by intraepithelial γδ T cells. Proc Natl Acad Sci USA 1995;**92**:6147–6151.
163. Matsumoto S, et al. Physiological roles of γδ T-cell receptor intraepithelial lymphocytes in cytoproliferation and differentiation of mouse intestinal epithelial cells. Immunology 1999;**97**:18–25.
164. Inagaki-Ohara K, et al. Mucosal T cells bearing TCRγδ play a protective role in intestinal inflammation. J Immunol 2004;**173**:1390–1398.
165. Fahrer AM, et al. Attributes of γδ intraepithelial lymphocytes as suggested by their transcriptional profile. Proc Natl Acad Sci USA 2001;**98**:10261–10266.
166. Beck PL, Rosenberg IM, Xavier RJ, Koh T, Wong JF, Podolsky DK. Transforming growth factor-β mediates intestinal healing and susceptibility to injury *in vitro* and *in vivo* through epithelial cells. Am J Pathol 2003;**162**:597–608.
167. Born W, Cady C, Jones-Carson J, Mukasa A, Lahn M, O'Brien R. Immunoregulatory functions of γδ T cells. Adv Immunol 1999;**71**:77–144.
168. Roberts SJ, et al. T-cell $\alpha\beta^+$ and $\gamma\delta^+$ deficient mice display abnormal but distinct phenotypes toward a natural, widespread infection of the intestinal epithelium. Proc Natl Acad Sci U S A 1996;**93**:11774–11779.
169. Viney JL, et al. Lymphocyte proliferation in mice congenitally deficient in T-cell receptor $\alpha\beta^+$ cells. Proc Natl Acad Sci USA 1994;**91**:11948–11952.
170. Mombaerts P, Mizoguchi E, Grusby MJ, Glimcher LH, Bhan AK, Tonegawa S. Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. Cell 1993;**75**:274–282.
171. Mizoguchi A, et al. Cytokine imbalance and autoantibody production in T cell receptor-α mutant mice with inflammatory bowel disease. J Exp Med 1996;**183**:847–856.
172. Takahashi I, Kiyono H, Hamada S. $CD4^+$ T-cell population mediates development of inflammatory bowel disease in T-cell receptor α chain-deficient mice. Gastroenterology 1997;**112**:1876–1886.
173. Fujihashi K, et al. Immunoregulatory functions for murine intraepithelial lymphocytes: γ/δ T cell receptor-positive (TCR^+) T cells abrogate oral tolerance, while α/β TCR^+ T cells provide B cell help. J Exp Med 1992;**175**:695–707.
174. Takahashi I, Nakagawa I, Kiyono H, McGhee JR, Clements JD, Hamada S. Mucosal T cells induce systemic anergy for oral tolerance. Biochem Biophys Res Commun 1995;**206**:414–420.
175. Fujihashi K, et al. γ/δ T cell-deficient mice have impaired mucosal immunoglobulin A responses. J Exp Med 1996;**183**:1929–1935.

Role of Peyer's patches in the induction of *Helicobacter pylori*-induced gastritis

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***Helicobacter pylori* is a Gram-negative spiral bacterium that causes gastritis and peptic ulcer and has been implicated in the pathogenesis of gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. Although Th1 immunity is involved in gastritis and the accumulation of *H. pylori*-specific CD4$^+$ T cells in the *H. pylori*-infected gastric mucosa in human patients, how T cells are primed with *H. pylori* antigens is unknown because no apparent lymphoid tissues are present in the stomach. We demonstrate here that Peyer's patches (PPs) in the small intestine play critical roles in *H. pylori*-induced gastritis; no gastritis is induced in *H. pylori*-infected mice lacking PPs. We also observed that the coccoid form of *H. pylori* is phagocytosed by dendritic cells in PPs. We propose that *H. pylori* converts to the coccoid form in the anaerobic small intestine and stimulates the host immune system through PPs.**

CD4 T cells | coccoid form | dendritic cells | gastric epithelial cells | inflammation

Helicobacter pylori is a Gram-negative microaerophilic bacterium that infects human gastric epithelial cell (gEC) surfaces and the overlying gastric mucin. More than 50% of the world's population is infected by *H. pylori*, although most patients have no remarkable symptoms (1). However, in some of patients, *H. pylori* infection leads to active chronic gastritis or peptic ulcer (2). In addition, *H. pylori* has also been implicated in the pathogenesis of gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (3). When *H. pylori* colonizes gastric mucosa, effector molecules are injected into gastric epithelial cells or the submucosal area through the type IV secretion system (1, 4). For example, the CagA effector is phosphorylated in the target cells and activates a signaling pathway to elicit growth factor-like responses. Another effector molecule, VacA, causes the massive vacuolar degradation of epithelial cells, thus disrupting the gastric epithelial barrier. VacA also interferes with the activation and proliferation of T lymphocytes within the gastric lamina propria (gLP) (5).

It was originally proposed that effector molecules, including CagA, trigger the secretion of chemokines such as IL-8 and RANTES from gECs, which attract neutrophils and mononuclear cells into the gLP (4). However, it was later shown that *H. pylori* did not induce gastritis in lymphopenic SCID mice, although gastritis was induced after adoptive transfer of naive CD4$^+$ T cells (6). The importance of CD4$^+$ T cells was underscored by the fact that *H. pylori* is not eliminated from gastric mucosa in MHC class II-deficient mice (7).

Gastritis is more severe in Th1-prone mice than Th2-prone mice on infection with the mouse-adapted *H. pylori* strain SS1 (8). Furthermore, the accumulation of *H. pylori*-specific CD4$^+$ T cells in the *H. pylori*-infected gastric mucosa in human patients (9) suggests that CD4$^+$ T cell-mediated Th1 immune responses play a critical role in *H. pylori*-induced gastritis. However, how CD4$^+$ T cells are primed by *H. pylori* antigens in the stomach where no apparent lymphoid tissues are present and how the *H. pylori*-induced chronic inflammation is maintained by T cells is unknown.

Although *H. pylori* forms an actively dividing, spiral-shaped morphology in the stomach, it is able to convert to a nonculturable, but viable, coccoid form under unfavorable conditions such as an anaerobic environment, increased oxygen tension, and long-term culture (10, 11). The coccoid form is thought to be important for transmission to new hosts by an oral–oral or oral–feces route because this form is more resistant to environmental stresses. Although the coccoid form is not culturable *in vitro*, transcription and translation actively take place in the coccoid form (12, 13). However, it is unknown whether the coccoid form is involved in the pathogenesis of *H. pylori*-induced gastritis.

In this study, we demonstrate that *H. pylori* antigen-specific CD4$^+$ T cells are necessary and sufficient for the induction of gastritis by *H. pylori*. We also demonstrate that CD4$^+$ T cells are likely primed with *H. pylori* antigens captured in the small intestine, where the coccoid form of *H. pylori* is taken up by dendritic cells (DCs) in Peyer's patches (PPs).

Results

Adoptive Transfer of Naive CD4$^+$T Cells Induces Gastritis in *H. pylori*-Infected Rag2$^{-/-}$ Mice. The *H. pylori* SS1 strain induces more severe gastritis in Th1-prone C57BL/6 than Th2-prone BALB/c mice as demonstrated by the infiltration of neutrophils and lymphocytes into the gLP and the submucosal area (Fig. 1*a* and data not shown). In contrast, when C57BL/6-Rag2$^{-/-}$ mice lacking T and B cells were infected with *H. pylori*, no gastritis was observed (Fig. 1*b*), as previously shown with SCID mice (6). The clearance of bacteria in Rag2$^{-/-}$ mice was impaired because $>10^7$ cfu/g tissues of *H. pylori* colonized the gastric mucosa (Table 1), and the colonization of *H. pylori* was readily detected by anti-*H. pylori* antibody staining (Fig. 1*c*). However, adoptive transfer of naive splenic CD4$^+$ T cells into Rag2$^{-/-}$ mice 2 months after *H. pylori* infection induced severe gastritis, with massive infiltration of neutrophils and lymphocytes into the gLP and the submucosal area (Fig. 1*d*). This massive infiltration resulted in the exclusion of colonized *H. pylori* from gastric mucosa (Table 1).

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Abbreviations: DKO, double knockout; LP, lamina propria; gLP, gastric LP; gEC, gastric epithelial cell; DC, dendritic cell; PPs, Peyer's patches; OVA, ovalbumin; NK, natural killer; APC, antigen-presenting cell; β-Rag DKO, IL-2 receptor β chain (IL-2Rβ)$^{-/-}$Rag2$^{-/-}$ DKO; γc-Rag DKO, cytokine receptor common γ chain (γc)$^{-/-}$Rag2$^{-/-}$ DKO; GALT, gut-associated lymphoid tissue; ILF, isolated lymphoid follicle; BMDC, bone marrow-derived cell; SED, subepithelial dome; mLN, mesenteric lymph node.

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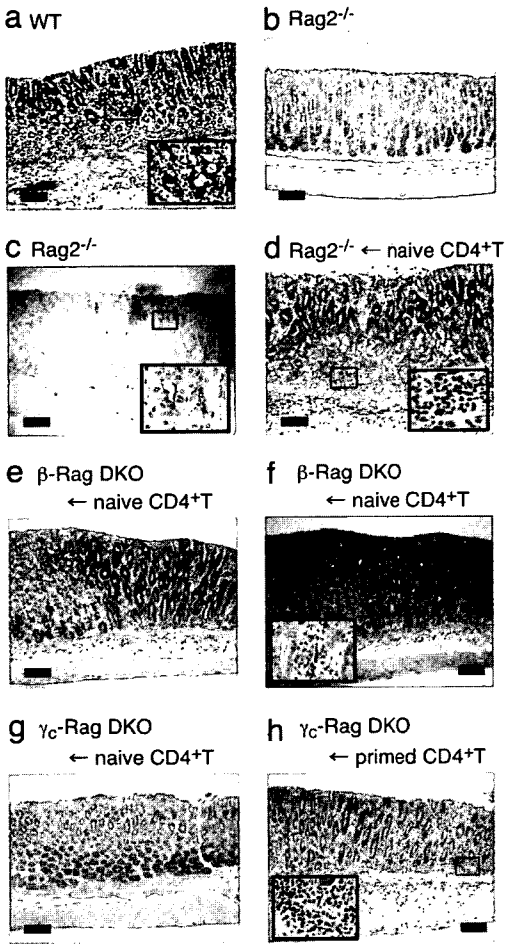

Fig. 1. Naive $CD4^-$ T cells did not induce gastritis in *H. pylori*-infected γ_c-Rag double knockout (DKO) mice. (*a–c*) Wild-type (*a*) or $Rag2^{-/-}$ (*b* and *c*) mice were infected with *H. pylori*. Two months after the infection, gastric specimens were prepared. (*d–h*) $Rag2^{-/-}$ (*d*), β-Rag DKO (*e* and *f*), and γ_c-Rag DKO (*g* and *h*) mice were infected with *H. pylori*. Two months after the infection, naive (*d–g*) or primed (*h*) splenic $CD4^+$ T cells were transferred. Two months after the cell transfer, gastric specimens were prepared. Specimens were stained with H&E (*a*, *b*, *d*, *e*, *g*, and *h*), anti-*H. pylori* antisera (brown) (*c*), or chloroacetate esterase (red) for infiltrated neutrophils and mast cells (*f*). (Scale bars: 200 μm.)

***H. pylori* Antigen-Specific $CD4^+$ T Cells Are Indispensable for Induction of Gastritis.** Primary gECs secrete MIP-2, a functional homolog of IL-8, on *H. pylori* infection *in vitro*, and the amount produced by $Rag2^{-/-}$ gECs was comparable to that produced by wild-type gECs [supporting information (SI) Fig. 5*a*]. $CD4^+$ T cells isolated from the gLP of *H. pylori*-infected mice were able to produce larger amounts of MIP-2 than splenic $CD4^+$ T cells from the same mice in response to *H. pylori* antigens (SI Fig. 5*b*). Moreover, the amounts of MIP-2 produced by gLP $CD4^+$ T cells were much larger than those produced by gECs (compare SI Fig. 5 *a* and *b*). The importance of $CD4^+$ T cells for neutrophil infiltration on *H. pylori* infection was further confirmed by the depletion of $CD4^+$ T cells from wild-type mice that had already developed gastritis by *H. pylori* infection. After depleting $CD4^+$ T cells by the i.v. injection of anti-CD4 mAb, the gastritis became milder (Table 1 and SI Fig. 6 *a* and *b*), and the number of bacteria in the gastric mucosa increased (Table 1). These results indicate the critical role of $CD4^+$ T cells for both triggering and maintaining gastritis. When $CD4^+$ T cells from OT-II transgenic mice on a $Rag2^{-/-}$ background (OT-II-Rag mice), specific for an ovalbumin ($OVA_{323-339}$ peptide on an MHC class II molecule $I\text{-}A^b$), were transferred into *H. pylori*-infected $Rag2^{-/-}$ mice, no gastritis was induced (SI Fig. 6*c*). Similarly, when OT-II-Rag mice were infected with *H. pylori*, no gastritis was induced despite the presence of $CD4^+$ T cells (Table 1 and SI Fig. 6*d*). Furthermore, when OVA protein or $OVA_{323-339}$ peptide was administered into *H. pylori*-infected OT-II-Rag mice, no inflammation was observed, although $CD4^+$ T cells were activated in these mice (Table 1, SI Fig. 6*e*, and data not shown). These results collectively indicate the importance of *H. pylori* antigen recognition by $CD4^+$ T cells in the induction of gastritis.

$CD4^+$ T Cells Are Not Primed with *H. pylori* Antigen in γ_c-Rag DKO Mice. IFNγ, a key cytokine for Th1 immune responses, is important for the pathogenesis of *H. pylori*-induced gastritis (14). Natural killer (NK) cells and antigen-presenting cells (APCs) including DCs are able to produce IFNγ to prevent bacterial infection (15). The interaction between DCs and NK cells enhances the production of IFNγ during *H. pylori* infection (16, 17). To test the importance of DC–NK interaction in the *H. pylori*-induced inflammatory response, we transferred splenic $CD4^+$ T cells into *H. pylori*-infected IL-2 receptor β chain $(IL\text{-}2R\beta)^{-/-}Rag2^{-/-}$ (β-Rag DKO) mice and cytokine receptor common γ chain $(\gamma_c)^{-/-}Rag2^{-/-}$ DKO (γ_c-Rag DKO) mice. These mice lack NK cells because of impaired IL-15 signaling, which is critical for NK cell development (18, 19). In addition, the production of IL-12 and IFNγ by APCs from these mice is impaired (20). As shown in Fig. 1 *e* and *f*, gastritis was induced in *H. pylori*-infected β-Rag DKO mice when naive $CD4^+$ T cells were transferred. Clearance of bacteria was also achieved by the naive $CD4^+$ T cell transfer (Table 1), indicating that NK cells and NK–DC interaction are dispensable for the induction of gastritis by *H. pylori* infection.

Surprisingly, there was no gastritis induced in γ_c-Rag DKO mice even after the transfer of naive $CD4^+$ T cells (Fig. 1*g*), NK cells (SI Fig. 7*a*), or NK cells with naive $CD4^+$T cells (SI Fig. 7*b*), suggesting that γ_c-Rag DKO mice have additional defects compared with β-Rag DKO mice. Interestingly, when splenic $CD4^+$ T cells isolated from *H. pylori*-infected wild-type mice were transferred, gastritis was induced in *H. pylori*-infected γ_c-Rag DKO mice (Fig. 1*h*), and the clearance of bacteria was evident (Table 1). These results suggest that $CD4^+$ T cells were not primed in γ_c-Rag DKO mice. In fact, splenocytes from these mice did not respond to DCs preincubated with *H. pylori* lysate, whereas splenocytes from wild-type mice infected with *H. pylori* strongly responded and produced IFNγ in response to the same DC preparation (data not shown). It should be noted that there were no apparent defects in DCs from γ_c-Rag DKO mice compared with those from wild-type mice with regard to their ability to induce T cell activation and present antigen as examined by the induction of CD69 expression and IFNγ production by splenic $CD4^+$ T cells (SI Fig. 8).

PPs Are a Critical Tissue for Priming $CD4^+$T Cells with *H. pylori* Antigen. One difference between β-Rag DKO and γ_c-Rag DKO mice is that the latter lack gut-associated lymphoid tissues (GALT) such as PPs and isolated lymphoid follicles (ILFs) due to impaired IL-7 signaling (21) (Fig. 2 *a–d*). Thus, we hypothesized that $CD4^+$ T cells are primed in GALT such as PPs or ILFs. To test this possibility, we generated PP-null mice by administration of anti-IL-7Rα mAb *in utero* (22) (Fig. 2 *e* and *f*). As observed in γ_c-Rag DKO mice, no gastritis was induced in PP-null mice 2 months after *H. pylori* infection, and a large number of *H. pylori* was detected in the gastric mucosa (Fig. 2 *h* and *i* and Table 1). We also generated PP-null mice on a $Rag2^{-/-}$ background (PP-null-$Rag2^{-/-}$ mice) (Fig. 2*g*). The adoptive transfer of $CD4^+$ T cells from *H. pylori*-infected wild-type mice, but not naive $CD4^+$ T cells, induced strong inflammation in PP-null-$Rag2^{-/-}$

Table 1. PP-dependent bacterial clearance in *H. pylori* infection

Mouse*	*n*	Cells transferred†	Bacterial colonization,‡ cfu/g tissue × 10^{-6}	Neutrophils, average (range)	Active inflammation, average (range)	GAIS,§ average (range)
Wild type	7	None	2.2 ± 1.3	1.6 (0–3)	1.4 (0–3)	13.6 (0–34)
Wild type	4	$CD4^+$ T cell-depleted	25 ± 7	0 (0)	1.0 (0–2)	0 (0)
$Rag2^{-/-}$	4	None	14 ± 4	0 (0)	0 (0)	0 (0)
$Rag2^{-/-}$	5	Naive $CD4^+$ T	0.15 ± 0.17	2.0 (2)	2.0 (2)	5.0 (2–8)
$Rag2^{-/-}$	5	OT-II-Rag $CD4^+$ T	34 ± 11	0 (0)	0 (0)	0 (0)
β-Rag DKO	4	Naive $CD4^+$ T	0.67 ± 0.39	1.5 (1–2)	1.3 (1–2)	9.2 (3–18)
γ_c-Rag DKO	6	Naive $CD4^+$ T	18 ± 11	0 (0)	0 (0)	0 (0)
γ_c-Rag DKO	6	Primed $CD4^+$ T	0.22 ± 0.39	0.66 (0–1)	1.5 (0–2)	1.6 (0–5)
γ_c-Rag DKO	3	Primed $CD4^+$ T from PPs	<0.01	1.0 (1)	2.0 (2)	1.0 (0–2)
γ_c-Rag DKO	3	Primed $CD4^+$ T from mLN	0.88 ± 0.78	0.5 (0–1)	1.5 (1–2)	4.0 (3–5)
γ_c-Rag DKO	3	Primed $CD4^+$ T by coccoid form	1.8 ± 1.6	0.33 (0–1)	0.66 (0–1)	2.0 (0–6)
PP-null-wild type	8	None	16 ± 9	0 (0)	0.37 (0–1)	0 (0)
PP-null-$Rag2^{-/-}$	3	Naive $CD4^+$ T	5.3 ± 4.0	1.0 (0–2)	1.0 (0–2)	4 (0–7)
PP-null-$Rag2^{-/-}$	3	Primed $CD4^+$ T	0.53 ± 0.28	2.6 (2–3)	2.6 (2–3)	21 (13–33)

*All mice were on a C57BL/6 background. Although not shown, the degrees of bacterial colonization in β-Rag DKO, γ_c-Rag DKO, and PP-null-$Rag2^{-/-}$ mice without $CD4^+$ T cell transfer were similar to those of $Rag2^{-/-}$ mice.

†Splenocytes were used for cell transfer unless otherwise stated. Five million cells were transferred except for the transfer of PP-derived cells, where 5×10^5 cells were used.

‡Mean ± SD.

§Gland active inflammatory score. See *Materials and Methods*.

mice just as in the γ_c-Rag DKO mice (Fig. 2 *j* and *k* and Table 1). These results strongly suggest that PPs are critical for priming $CD4^+$ T cells in *H. pylori* infection, but dispensable for the effector phase.

The Coccoid, but Not Helical, Form of *H. pylori* Is Phagocytosed by DCs in PP. Although *H. pylori* is helical in the stomach, it transforms to the coccoid form under anaerobic conditions, such as in the small intestine (23). Interestingly, the coccoid form of *H. pylori* induced higher IL-12 production from bone marrow-derived cells (BMDCs) than the helical form (SI Fig. 9). There is a possibility that *H. pylori* transforms to the coccoid form in the intestine and is then captured by DCs present in PPs to induce a Th1 response. To test this possibility, the coccoid and helical forms of *H. pylori* were inoculated into ligated small intestinal loops. As shown in Fig. 3*a*, immunofluorescence staining detected *H. pylori* in the subepithelial dome (SED) region of PPs in a time-dependent manner, and the number of bacteria in the SED region inoculated with the coccoid form was larger than that inoculated with the helical form of *H. pylori*. In addition, double immunofluorescence staining with anti-*H. pylori* antibody and anti-CD11c mAb demonstrated that the bacteria were captured by $CD11c^+$ DCs in the SED region (Fig. 3*b*). Although the helical form of *H. pylori* kept the rod shape 1.5 h after inoculation (SI Fig. 10 *a* and *b*), the bacteria phagocytosed by $CD11c^+$ DCs in PPs were round (SI Fig. 10 *c* and *d*). These results suggest that the coccoid, but not the helical, form of *H. pylori* is captured by DCs in PPs and activates immune responses by generating *H. pylori*-specific pathogenic $CD4^+$ T cells. Consistent with this observation, $CD4^+$ T cells from the PPs as well as mesenteric lymph node (mLN) of *H. pylori*-infected wild-type mice were also able to eliminate the bacteria in γ_c-Rag DKO mice infected with *H. pylori* (Table 1).

When wild-type mice were infected with the coccoid form of *H. pylori*, gastritis was not induced because the coccoid form of *H. pylori* was unable to colonize in the stomach (Fig. 4*a* and data not shown). However, $CD4^+$ T cells from these mice induced gastritis in γ_c-Rag DKO mice infected with the helical form of *H. pylori* (Fig. 4 *b* and *c* and Table 1). These results indicate that $CD4^+$ T cells primed with the coccoid form of *H. pylori* in the intestine are sufficient to induce inflammation in the stomach infected with the helical form of *H. pylori*.

Discussion

We showed here that *H. pylori* antigen-specific $CD4^+$ T cells are required to induce and maintain gastritis on infection with *H. pylori*. Because *H. pylori* interacts with and injects pathological molecules into gECs, it is generally thought that neutrophils infiltrating the gLP are attracted by chemokines produced by gECs. However, neutrophil infiltration was not observed in $Rag2^{-/-}$ mice, although gECs of $Rag2^{-/-}$ mice were able to secret MIP-2 on *H. pylori* infection. Thus, the secretion of chemokines by gECs seems insufficient for the induction of gastritis. In addition, adoptive transfer of $CD4^+$ T cells recognizing *H. pylori*-independent antigens did not induce gastritis, suggesting that *H. pylori*-specific $CD4^+$ T cells directly or indirectly regulate production of chemokines that attract neutrophils. In fact, a large amount of MIP-2 was produced by activated $CD4^+$ T cells derived from the gLP of *H. pylori*-infected mice. In addition, infiltrated neutrophils were located around $CD4^+$ T cells in the gLP of *H. pylori*-infected mice (data not shown). It is known that another keratinocyte-derived chemokine is able to recruit neutrophils. However, the amounts of keratinocyte-derived chemokine produced by both gECs and CD4 T cells were much lower than those of MIP-2 (data not shown).

Oral or intra-PP immunization with *H. pylori* antigens was effective in enhancing *H. pylori*-specific $CD4^+$ T cell responses and reducing *H. pylori* colonization in the stomach (24, 25). These reports are consistent with our current observation that PPs play critical roles in priming $CD4^+$ T cells, and *H. pylori* is indeed captured by DCs in PPs. *H. pylori* antigen-specific $CD4^+$ T cells would be primed by DCs in PPs or mLNs where DCs migrate after capturing antigens. Interestingly, $CD4^+$ T cells cannot be primed by DCs in the gLP or gEC, both of which are capable of expressing class II MHC and presenting antigens. The lack of antigen presentation is partly due to the fact that the helical form of *H. pylori* is resistant to phagocytosis in a type IV secretion system-dependent manner, although the molecular mechanisms of antiphagocytic activity remain to be determined (26). It should be noted that the transformation of the helical to the coccoid form is accompanied by changes in the composition

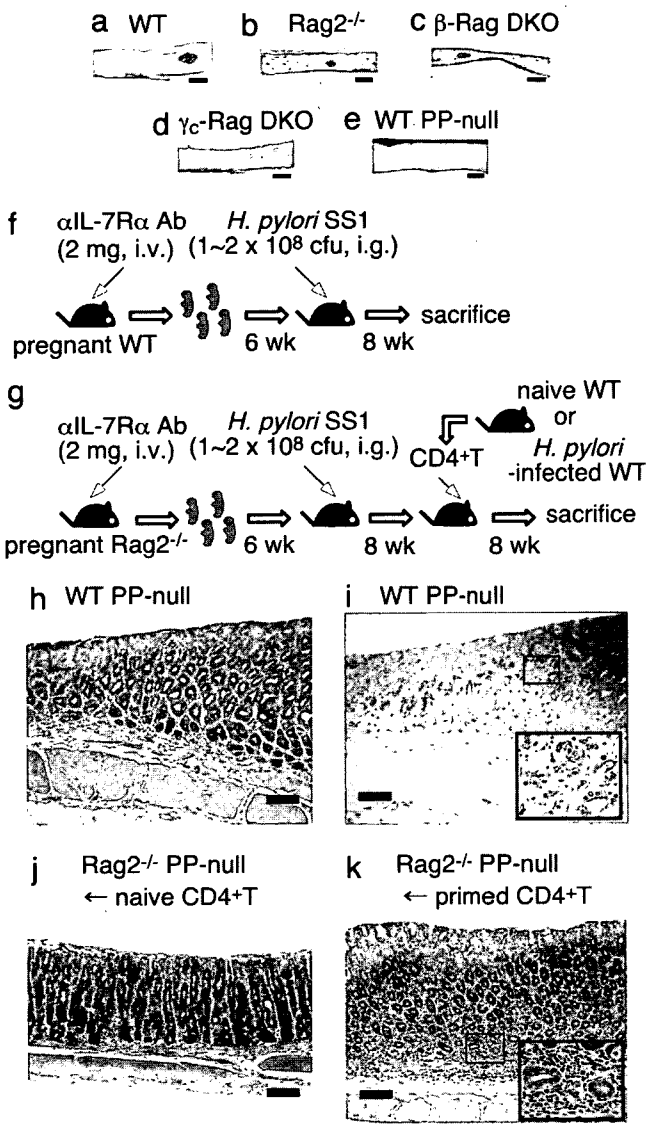

Fig. 2. PPs are critical for the priming of $CD4^+$ T cells in *H. pylori* infection. (*a–e*) Small intestines from wild-type (WT) (*a*) and PP-null (*e*) mice were stained with anti-B220 mAb, and small intestines from $Rag2^{-/-}$ (*b*), β-Rag DKO (*c*), and γ_c-Rag DKO (*d*) mice were stained with anti-CD45 mAb. (*f* and *g*) Schemes of the generation of PP-null WT (*f*) or PP-null-$Rag2^{-/-}$ (*g*) mice. (*h* and *i*) PP-null WT mice were infected with *H. pylori*. Two months after the infection, gastric specimens were prepared. (*j* and *k*) PP-null-$Rag2^{-/-}$ mice were infected with *H. pylori*. Two months after the infection, naive (*j*) or primed (*k*) splenic $CD4^+$ T cells were transferred. Two months after the cell transfer, gastric specimens were prepared. Specimens were stained with H&E (*h*, *j*, and *k*) or anti-*H. pylori* antisera (brown) (*i*). (Scale bars: 200 μm.)

of surface proteins and/or carbohydrates, which may make the bacteria susceptible to phagocytosis (27, 28), a subject worthy of further studies. It has been shown that mast cells are able to present *H. pylori* antigens to *H. pylori*-specific $CD4^+$ T cells, which in turn activate mast cells to degranulate (29). When we infected W/W^v and Sl/Sl^d mice lacking mast cells with *H. pylori*, gastritis was readily induced in both strains of mice on infection (S.N., T.Y., Y.B., and S.K., unpublished data), indicating that mast cells are not essential in priming $CD4^+$ T cells. In an *in vitro* experiment, BMDCs infected with the coccoid form of *H. pylori* produced larger amounts of IL-12 than those infected with the helical form of *H. pylori*, suggesting that the coccoid form of

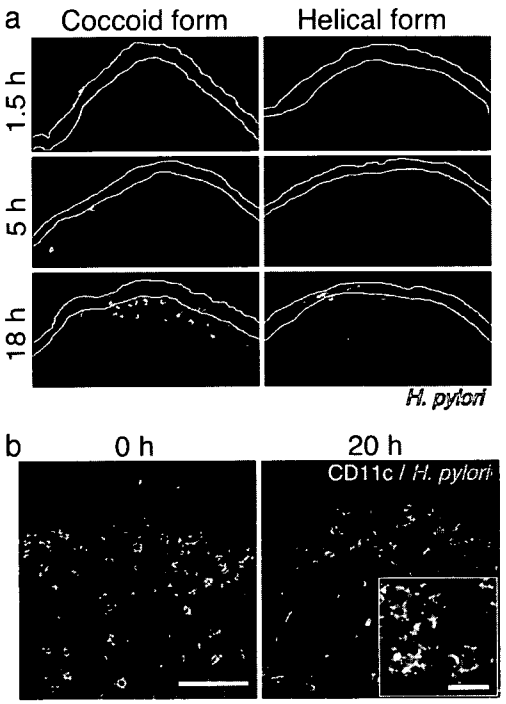

Fig. 3. The coccoid form of *H. pylori* is captured by DCs in PPs. The coccoid or helical form of *H. pylori* was inoculated into the ligated small intestines of wild-type mice. (*a*) After the indicated incubation times, PPs were stained with anti-*H. pylori* antibody. (*b*) Twenty hours after inoculation of the coccoid form, PPs were stained with anti-CD11c mAb (green) and anti-*H. pylori* antibody (red). (Scale bars: 0 h,100 μm; 20 h, 20 μm.)

H. pylori easily induces Th1 immune responses on *H. pylori* infection.

Importantly, $CD4^+$ T cells primed with the coccoid form of *H. pylori* were able to induce gastritis in *H. pylori*-infected GALT-null γ_c-Rag DKO mice where $CD4^+$ T cells are not

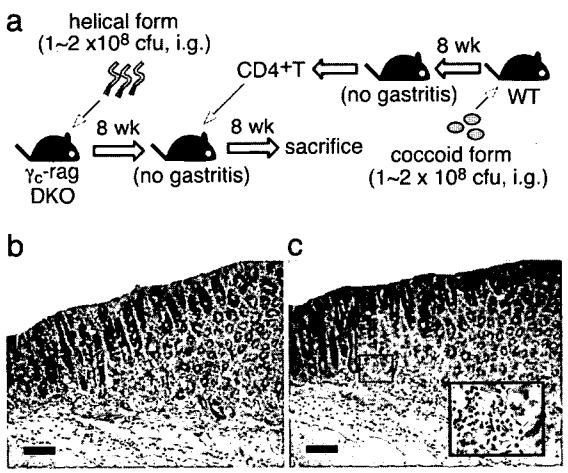

Fig. 4. Gastritis is induced by $CD4^+$ T cells primed by the coccoid form of *H. pylori*. Two months after infection of γ_c-Rag DKO mice with the helical form of *H. pylori*, splenic $CD4^+$ T cells from wild-type (WT) mice orally infected by the coccoid form of *H. pylori* were transferred to the infected γ_c-Rag DKO mice (*a*). Two months after the cell transfer, gastric specimens were prepared. Specimens were stained with H&E (*b*) or chloroacetate esterase (red) for infiltrated neutrophils and mast cells (*c*). (Scale bars: 200 μm.)

primed with *H. pylori* antigen at all. Thus, the following scenario emerges from our results: *H. pylori* transforms to the coccoid form when entering the intestinal tract and is captured by DCs in PPs. *H. pylori* antigens presented by DCs are recognized by $CD4^+$ T cells in PPs or mLN, and activated T cells migrate to the gastric mucosa to induce and maintain inflammatory responses. We noted that PP-null-$Rag2^{-/-}$ mice exhibited modest inflammation in the stomach after naive $CD4^+$ T cell transfer, compared with γ_c-Rag DKO mice, which showed no sign of inflammation. Because treatment by anti-IL-7Rα mAb *in utero* suppresses PP development, but leaves ILFs and recently discovered villous M cells (30) intact, ILFs and/or villous M cells may also participate in the capture of *H. pylori* in the intestine. Although luminal antigens can be taken up by M cells located over the follicular epithelia of ILF, the tissue is predominantly occupied by B cells (31). Furthermore, in a separate study, we have shown that no apparent organized lymphoid structure is developed under villous M cells (data not shown).

H. pylori is also implicated in the cause of other diseases such as idiopathic thrombocytopenic purpura (32) and Sjögren syndrome (33). Indeed, it has been shown that T cells migrate from the intestine to the salivary gland in Sjögren syndrome patients (34). It will be of interest to examine the functional relationship between these diseases and the coccoid form of *H. pylori* captured via PPs.

Materials and Methods

Mice. All mice used in this study were on a C57BL/6 background and were maintained at Taconic (Germantown, NY) or in our animal facility under specific pathogen-free conditions. Wild-type C57BL/6 mice were purchased from Sankyo Labo Service (Shizuoka, Japan) and CLEA Japan (Tokyo, Japan). $Rag2^{-/-}$ mice, γ_c-Rag DKO mice, and OT-II-Rag mice were obtained from Taconic. IL-2R$\beta^{-/-}$ mice (35) were generously provided by T. W. Mak (Ontario Cancer Institute, Toronto, ON, Canada). β-Rag DKO mice were obtained by crossing IL-2R$\beta^{-/-}$ with $Rag2^{-/-}$ mice (20). All experiments were approved by the Animal Care and Use Committee of the Keio University School of Medicine and were performed in accordance with institutional guidelines.

Antibodies. Fluorescein-conjugated antibodies for flow-cytometric analysis and biotin anti-mouse $CD2_c$ (HL3) were purchased from BD Bioscience (San Jose, NJ). Anti-*H. pylori* antibodies were purchased from Biomeda (Foster City, CA) or DAKO (Glostrup, Denmark).

Bacteria. *H. pylori* strain SS1, a mouse-adapted human isolate, was used for all experiments. To prepare the helical form of *H. pylori*, SS1 was grown on 5% sheep blood agar plates for 2 days. Before inoculating into mice, bacteria were grown in *Brucella* broth with 5% FCS overnight at 37°C under microaerobic conditions with gentle agitation. To prepare the coccoid form, SS1 was grown on 5% sheep blood agar plates under microaerobic conditions for 3 days at 37°C and then cultured under anaerobic conditions for 7 days at 37°C.

***In Vivo* Infection of Mice.** Bacteria were prepared from logarithmic phase cultures. Mice were intragastrically infected with $1–2 \times 10^8$ cfu *H. pylori* in 0.15 ml of broth. After the indicated time period, mice were killed and the stomach was aseptically removed. The stomach was then bisected along the greater and lesser curvatures. Half of the stomach was homogenized for the determination of bacterial colonization by a plate-dilution method. The rest of the stomach was sectioned transversely into two strips for frozen and paraffin-embedded sections.

***In Situ* Infection of Intestinal Loop.** Wild-type mice (6-week-old females) were anesthetized by an i.m. injection of 2 mg of ketamine hydrochloride (Sankyo, Tokyo, Japan) and 0.1 mg of Xylazine per mouse. An ≈4-cm-long piece of the small intestine containing one or two PPs was ligated at both ends with surgical thread. *H. pylori* (1×10^9) suspended in 0.2 ml of saline was inoculated into the loop. After the indicated time periods, PPs were removed and extensively washed with PBS. After fixation in 4% paraformaldehyde in PBS, specimens were processed for histopathological examination.

Generation of PP-Null Mice. PPs were depleted from a small intestine as previously described (22). Briefly, 14.5 days postcoitum pregnant wild-type or $Rag2^{-/-}$ mice were i.v. injected with 2 mg of anti-IL-7Rα mAb (A7R34; kindly provided by S.-I. Nishikawa, RIKEN CDB, Kobe, Japan). To confirm the depletion of PPs, a dissected small intestine from one of the offspring was fixed with acetone and stained with anti-B220 or anti-pan CD45 mAb (BD Biosciences).

Adoptive Transfer of $CD4^+$ T Lymphocytes. Naive or *H. pylori* antigen-primed $CD4^+$ T cells were purified from splenocytes, mLN, and PPs by using anti-mouse CD4-microbeads and AutoMACS (Miltenyi Biotech, Sunnyvale, CA) according to the manufacturer's instruction. The purity of isolated cells was >95%. Isolated cells (5×10^6 per mouse for splenocytes and mLN, 5×10^5 per mouse for PP-derived cells) were injected i.v. into recipient mice infected with *H. pylori* for 8 weeks. Eight weeks after the transfer, mice were killed for the indicated analyses.

Histological Analysis. An excised stomach was fixed in a neutral-buffered 10% formalin solution and cut into four strips. Samples were processed by standard methods, embedded in paraffin, and sectioned at 4 to 5 μm. Specimens were stained with H&E or used for cytochemical and immunohistochemical studies. The Leder method was used to assess naphtol-AS-D-chloroacetate esterase detection (36). Immunohistochemical analysis was performed with formalin-fixed and paraffin-embedded tissue sections by using heat-induced epitope retrieval and the ABC (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) method. Anti-*H. pylori* serum from DAKO was used for *H. pylori* staining. In some cases, frozen sections (7 μm) were prepared, fixed with 4% paraformaldehyde in PBS, and blocked with 2% BSA-PBS, and immunofluorescence was performed using the tyramide amplication method (TSA-Plus Fluorescein System; PerkinElmer Life and Analytical Sciences, Boston, MA) and then incubated with anti-*H. pylori* antibody from Biomeda, followed by Cy5 (GE Healthcare Bioscience AB, Uppsala, Sweden) or TRITC-linked rabbit IgG (Sigma–Aldrich, St. Louis, MO). The specimens were mounted with Vectashield (Vector Laboratories) and examined with a confocal laser-scanning microscope LSM510 by using version 3.2 software (Carl Zeiss, Thornwood, NY). The zymogenic zone of middle corpus ≈3 mm from the FS/Z transition zone was examined in each sample.

Histological Score. For assessment of gastric histopathology, blinded sections stained with H&E were examined by light microscopy. Neutrophil infiltration was assessed by the presence of neutrophils in the gastric mucosa. Active inflammation was assessed by the degree and area of damages of mucosal tissue and muscular layers because of infiltrations of neutrophils, lymphocytes, and/or macrophages. The scoring was graded as 0 (no), 1 (mild), 2 (moderate), or 3 (severe). The total number of glands with neutrophil infiltration in the crypt and lumen was also counted to produce a gland active inflammatory score.

Whole-Mount Immunohistochemistry. Small intestines were removed and stained with antibodies as described previously (37). Briefly, small intestines were washed, incubated twice in HBSS containing 5 mM EDTA at 37°C for 20 min, and fixed with ice-cold formalin