A Gnotobiotic Transgenic Mouse Model for Studying Interactions between Small Intestinal Enterocytes and Intraepithelial Lymphocytes

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The mouse intestinal epithelium undergoes continuous renewal throughout life. Intraepithelial lymphocytes (IELs) represent a significant fraction of this epithelium and play an important role in intestinal mucosal barrier function. We have generated a germ-free transgenic mouse model to examine the effects of a genetically engineered proliferative abnormality in the principal epithelial cell lineage (enterocytes) on IEL census and on IEL-enterocytic cross-talk. SV40 large T antigen (TAgWt) or a mutant derivative (TAgK107/8) that does not bind pRB was expressed in small intestinal villus enterocytes under the control of elements from the intestinal fatty acid binding protein gene (Fabpi). Quantitative immunohistochemical and flow cytometric analyses of conventionally raised and germ-free FVB/N Fabpi-TAgWt, Fabpi-TAgK107/8, and nontransgenic mice disclosed that forced reentry of enterocytes into the cell cycle is accompanied by an influx of thymically educated αβ T cell receptor (TCR)⁺ CD4⁺ and αβ TCR⁺ CD8α⁺ IELs and a decrease in intrinsically derived γδ TCR⁺ CD8α⁺ IELs. Real time quantitative reverse transcriptase-PCR studies of jejunal villus epithelium regenerated from germ-free nontransgenic and normal mice by laser capture microdissection and γδ TCR⁺ jejunal IELs purified by flow cytometry disclosed that the proliferative abnormality is accompanied by decreased expression of enterocytic interleukin-7 as well as IEL interleukin-7 receptor (IL-7R) and transforming growth factor β. The analysis also revealed that normal villus epithelium expresses Fms-like tyrosine kinase 3 (Flt3L), a known regulator of hematopoietic stem cell proliferation and neuronal cell survival, and its ligand (Flt3), a known regulator of hematopoietic stem cell proliferation and neuronal cell survival, and its ligand (Flt3). Epithelial expression of this receptor and its ligand is reduced by the proliferative abnormality, whereas IEL expression of Flt3L remains constant. Together, these findings demonstrate that changes in the proliferative status of the intestinal epithelium affects maturation of γδ TCR⁺ IELs and produces an influx of αβ TCR⁺ IELs even in the absence of a microflora.

The adult mouse small intestine is a complex, spatially diversified ecosystem that maintains distinctive cephalocaudal differences in its various functions. This regional variation in function is accompanied by regional differences in the differentiation programs of its four continuously renewing epithelial cell lineages, in the composition of its mucosal immune system, and in the composition of its resident society of commensal/symbiotic microorganisms (reviewed in Refs. 1–3). A full understanding of how this ecosystem is organized and functions in health and how it is reorganized or disorganized in various disease states requires knowledge about the nature and regulation of interactions between its microflora, epithelium, and gut-associated lymphoid tissue (1, 4, 5). The molecular nature and significance of the signals exchanged between these components have been difficult to decipher because of the dynamic quality and complexity of the system. One way of approaching this problem is to simplify the ecosystem using inbred strains of mice with defined microbiological status (gnotobiotic animals). For example, comparative functional genomics studies of mice containing no resident microorganisms (germ-free), conventionally raised mice harboring a complete microflora, and germ-free animals that have been colonized with a single species from the normal microflora (ex-germ-free) have shown that indigenous commensal bacteria play an important role in regulating host nutrient processing, fortifying the epithelial barrier, and organizing/educating the mucosal immune system (5, 6).

The intestine contains a large population of intraepithelial lymphocytes (IELs),¹ equivalent in size to the population of peripheral lymphocytes that resides in the spleen (7). IELs are distributed throughout the epithelium that overlies small intestinal villi (average of one IEL for every 6–10 epithelial cells (8)). Virtually all small intestinal IELs are T cells, but they are heterogeneous with respect to their surface phenotype. The majority are CD3⁺ and can be divided into αβ T cell receptor-positive (TCR⁺) and γδ TCR⁺ subsets (8). They can be further subdivided based on expression of CD8 (αα homodimer or αβ heterodimer) or CD4 coreceptors (i.e. (i) γδ TCR⁺ CD8α⁺ CD8β⁻; (ii) γδ TCR⁺ CD8α⁺ CD8β⁺ (abbreviated γδ TCR⁺ CD8αα); (iii) αβ TCR⁺ CD4⁺; (iv) αβ TCR⁺ CD8α⁺ CD8β⁻ (αβ TCR⁺ CD8αα); and (v) αβ TCR⁺ CD8α⁺ CD8β⁻ (αβ TCR⁺ CD8ββ)). Studies of Rag1⁻/⁻ mice injected with bone marrow from

1 The abbreviations used are: IEL, intraepithelial lymphocyte; TCR, T cell receptor; IL-7, interleukin-7; IL-7R, interleukin-7 receptor; Tag, SV40 large T antigen; TAgK107/8; mutant Tag with Glu → Lys substitution at positions 107 and 108; FACS, fluorescence activated cell sorting; LCM, laser capture microdissection; qRT-PCR, real time quantitative reverse transcriptase-PCR; Flt3L, Flt3-like tyrosine kinase 3 receptor; Flt3, ligand for Fms-like tyrosine kinase 3 receptor; BrdUrd, bromodeoxyuridine; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PE, phycoerythrin; TGF, transforming growth factor.
nude mice or peripheral lymph node T cells from euthymic mice demonstrated that generation of αβ TCR+ CD4+ and CD8+ IELs is thymus-dependent, whereas γδ TCR+ CD8αα+ IELs appeared in the absence of a thymus (9). One site of extrathymic maturation may be the crypts of Lieberkühn. These distinct mucosal invaginations surround the base of each villus and contain long-lived multipotent stem cells (10) that give rise to the four epithelial lineages of the small intestine: enterocytes, goblet, and enteroendocrine cells, which differentiate as they migrate from the crypt up adjacent villi; and Paneth cells, which differentiate and remain at the crypt base (11–15). Crypts possess structures (cryptopatches) that contain clusters of c-Kit+ interleukin-7 receptor (IL-7R)+ Thy1+ lymphocytes (16). Mice with a truncated mutation of the common cytokine receptor chain (17) lack these cryptopatches and do not have γδ TCR+ CD8αα+ IELs but contain thymus-dependent αβ TCR+ CD4+ and αβ TCR+ CD8αβ+ IELs, suggesting a role for cryptopatches in maturation of extrathymically derived γδ TCR+ IELs (18–20).

The epithelium also appears to play a direct role in regulat-
ing IEL development. Epithelial cells produce stem cell factor (21), a ligand for the c-Kit receptor expressed on the surface of γδ TCR+ IELs (22). Mice deficient in either stem cell factor or c-Kit have reduced numbers of γδ TCR+ IELs (22). Furthermore, thyrotrop-releasing hormone stimulation of enterocytes results in local release of thyroid-stimulating hormone, which interacts with IEL-based thyroid-stimulating hormone receptor to promote IEL development (23) (e.g., hyt/hyt mice, which have a loss-of-function thyroid-stimulating hormone receptor mutation, have disrupted IEL maturation) (24, 25).

Epithelium-based IL-7 provides another regulatory signal for IEL proliferation (26). Studies of mice that lack IL-7 or the IL-7R have demonstrated that IL-7-mediated signaling is essential for γδ TCR+ IEL development (26, 27). Moreover, Laky et al. (28) used transcriptional regulatory elements from the rat intestinal fatty acid-binding protein (Fabpi) to express IL-7 in the villus enterocytes of IL-7−/− mice. γδ TCR+ IELs were restored in the intestinal epithelium but remained absent from all other tissues, indicating that local production of IL-7 was sufficient for proper development/survival of this IEL subset.

Interactions between intestinal epithelial cells and IELs are reciprocal; IELs can influence epithelial cell biology. One illustration of this reciprocity is provided by TCRα subunit-deficient mice. These animals have reduced numbers of dividing cells in their crypts of Lieberkühn and reduced crypt cellularity (29) and exhibit more severe intestinal epithelial damage following infection with the parasite *Eimeria vermiformis* (30). γδ TCR+ IELs produce keratinocyte growth factor, which affects epithelial cell growth and repair (31). These findings raise the question of whether γδ TCR+ IELs form part of a homeostatic surveillance mechanism that can detect and respond to perturbations in intestinal epithelial proliferation in order to maintain steady state cellular census in crypts and their associated villi.

Some workers have proposed that IELs are key elements in a “mucosal intranet,” where they function to control epithelial integrity and immunologic homeostasis (32). Recent comparative DNA microarray-based studies of gene expression in γδ TCR+ IELs harvested from the small intestines of conventionally raised adult C57Bl6/J mice and αβ TCR+ cells harvested from their mesenteric lymph nodes have provided a list of candidate factors, preferentially expressed by γδ TCR+ IELs, that may support this mucosal intranet (33, 34).

In the present study, we examine the cross-talk between IELs and epithelium using transgenic mice that express simian virus 40 large T antigen (TagW) in their villus-associated enterocytes. The rationale for our experimental approach was as follows. Fabp1-directed expression of TagW produces a proliferative abnormality restricted to villus enterocytes: Fabp1-reporter transgenes are not expressed in the IELs. Expression of the viral oncoprotein in postmitotic enterocytes induces their reentry into the cell cycle (35) and an associated p53-independent apoptosis (36) but is not accompanied by evidence of dysplasia during the 1–2-day interval that they take to complete their migration to the cellular extrusion zone located at the villus tip (36, 37). Fabp1-directed expression of a mutant Tag containing a Glu → Lys substitution at residues 107 and 108 (TagK107/8) disrupts pRB binding that does not produce this proliferative abnormality. Thus, a three-way comparison of FVB/N Fabp1-TagW and Fabp1-TagK107/8 transgenic mice and their age-matched nontransgenic littermates would allow direct assessment of whether a proliferative abnormality limited to the predominant intestinal epithelial lineage is accompanied by changes in the fractional representation of extrathymically educated or thymically derived IEL subsets. By performing this analysis in conventionally raised and germ-free mice, we could also determine whether the microflora contributed to any observed changes in IELs. Finally, by using laser capture microdissection (LCM) of small intestinal cryosections to harvest villus epithelium, flow cytometry to retrieve their IELs, and the DNA microarray-based data sets of IEL gene expression to direct quantitative reverse transcription-PCR measurements of the levels of specified mRNAs in each cell population, we could use this environmentally well defined system to identify enterocytic gene products affected by proliferative status that may impact on IEL development/survival.

Our results show that the engineered proliferative abnor-
malities is accompanied by a microflora-independent reduction in extrathymically educated γδ TCR+ CD8αα+ IELs. This change is accompanied by coordinate changes in the expression of enterocytic and γδ TCR+ IEL gene products that probably help legislate the observed change in IEL representation.

**EXPERIMENTAL PROCEDURES**

*Generation and Maintenance of Conventionally Raised and Germ-
free Transgenic Mice—FVB/N mice hemizygous for a transgene containing nucleotides 1178 to +28 of rat Fabpi linked to TagW or TagK107/8 are described in earlier reports (35, 36, 38). Conventionally raised and germ-free mice were maintained in microisolators in a specified pathogen-free state.*

Normal and transgenic mice were rederived as germ-free by Caesarian section of transgenic mothers and transfer of their embryonic day 19 fetuses to plastic gnotobiotic isolators (Standard Safety Equipment Co.) containing germ-free foster mothers. The protocol used for this rederi-
vation is described in a recent publication (6). Both conventionally raised and germ-free mice were given sterilized BeeKay Autoclaveable Diet (B & K Universal Inc.) ad libitum. All animals were maintained under a strict light cycle (lights on at 0600 h and off at 1800 h). Animals were genotyped using primers, tail DNA, and PCR conditions described in Ref. 36. Some mice received an intraperitoneal injection of an aque-
ous solution of 5-bromo-2′-deoxyuridine (BrdUrd; 120 mg/kg) and 5-fluoro-2′-deoxyuridine (12 mg/kg) (Sigma) 90 min prior to sacrifice. Only male mice were studied.

*Quantitative Immunohistochemical Analysis of the IEL Subsets—* FVB/N transgenic mice and their wild type littermates were sacrificed at 6–8 weeks of age (n = 3 conventionally raised or germ-free animals/ genotype/experiment; n = 3 independent experiments). The middle third of their small intestine (arbitrarily defined as jejunum) was im-
mediately flushed with PBS and subdivided into five equal length segments. All were segments placed together in a tissue cassette, over-
laid with OCT (Miles Scientific), and frozen in Cryocool II (Stephens Scientific). 100 serial 5-μm thick sections were cut parallel to the cephalocaudal axes of the segments. For each antibody surveyed, every 10th section was fixed for 20 min in methanol at −20 °C, washed three times in PBS (3 min/cycle), and treated with PBS-blocking buffer (1% bovine serum albumin, 0.05% Triton X-100 in PBS) for 2 h at room
temperature. Sections were subsequently treated three times with TNT wash buffer (0.1 M Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween 20; three cycles; 5 min/cycle) and then incubated overnight at 4 °C with each of the following monoclonal antibodies (all from BD PharMingen, diluted 1:1000 in TNB-blocking buffer (0.1 M Tris (pH 7.5), 0.15 M NaCl, and 0.5% blocking reagent from PerkinElmer Life Sciences)): (i) rat anti-mouse CD4 (clone H129.19); (ii) rat anti-mouse CD8 and 0.5% blocking reagent from PerkinElmer Life Sciences): (i) rat anti-mouse CD4 (clone H129.19); (ii) rat anti-mouse CD8 (clone 53-6.7); (iii) rat anti-mouse CD8α (clone Ly-32); (iv) hamster anti-mouse αβ TCR (β chain; clone H67-597); (v) hamster anti-mouse γδ TCR (6 chain; clone GL3); and (vi) hamster anti-mouse CD103 (integrin αEED chain; clone 2E7).

Following incubation with these primary antibodies, sections were washed in TNT buffer (three cycles, each 5 min). Biotin-conjugated mouse anti- rat IgG1/IgG2a (BD PharMingen) or biotin-conjugated mouse anti-hamster IgG mixture (BD PharMingen) was added (final dilution of each = 1:100 in TNB blocking buffer). After a 30-min incubation with the secondary antibodies at room temperature, sections were treated three times with TNT wash buffer (5 min/wash cycle). The sections were then incubated for 30 min at room temperature with streptavidin-horseradish peroxidase (PerkinElmer Life Sciences; 1:1000 in TNB) followed by three washes of 5 min each in TNT buffer. The final steps consisted of (i) adding biotinyl-tyramide (PerkinElmer Life Sciences; diluted 1:100 in 1% amplification diluent from the same manufacturer) for 10 min; (ii) washing three times with TNT buffer (5 min/cycle); (iii) incubating the section with indocarbocyanine (Cy3) conjugated streptavidin-horseradish peroxidase (PerkinElmer Life Sciences; diluted 1:500 in TNB) for 30 min, and (iv) performing three final rinses in TNT buffer. Two controls were performed to verify the specificity of the signals produced: (i) direct amplification of endogenous peroxidase activity alone without the addition of primary or secondary antibodies but with the addition of biotinyl-tyramide; (ii) direct amplification of endogenous peroxidase activity followed by omission of each primary antibody but with inclusion of all other steps and reagents.

Only well oriented jejunal crypt-villus units were scored. “Well oriented” was defined as sectioned parallel to the crypt-villus axis with an unbroken epithelial column extending from the crypt base to the villus tip. The data were compiled as the number of IELs of a particular type per 1000 villous epithelial cells or per 100 crypt epithelial cells. A minimum of 100 jejunal crypt-villus units were scored per mouse. Data obtained with each antibody from all mice of a given genotype (germ-free or conventional) were averaged.

Forced Expression of TAgWt in Villus Enterocytes Causes a Change in the Representation of IEL Subsets within the Small Intestinal Epithelium—As noted in the Introduction, transcriptional regulatory elements from the Fohpi gene were used to direct expression of TAgWt in small intestinal villus enterocytes of adult FVB/N transgenic mice (Fig. 1A). There was no detectable TAgWt in the crypt epithelium, the mesenchyme underlying crypt-villus units (Fig. 1A), the organized gut-associated
lymphoid tissue (Peyer’s patch lymphocytes plus smaller submucosal lymphoid aggregates), or in the spleen and thymus (data not shown). Other than villus enterocytes, the only other site of transgene expression was the follicle-associated epithelium overlying Peyer’s patches (Fig. 1B). An identical pattern of transgene expression was observed in FVB/N mice from the reference control pedigree containing Fabpi-TAgK107/108 (data not shown).

Age-matched 6–8-week-old Fabpi-TAgWt and Fabpi-TAgK107/108 male mice as well as their nontransgenic littermates were given an intraperitoneal injection of BrdUrd, 1.5 h prior to sacrifice (n = 2–3 mice/genotype). Expression of the wild type viral oncprotein induced villus enterocytes to reenter the cell cycle (Fig. 1A). In contrast, the jejunal villus epithelium and follicle-associated epithelium were not labeled with BrdUrd in either wild type or Fabpi-TAgK107/108 mice (data not shown). To determine whether the proliferative abnormality induced by TAgWt caused a change in the composition or spatial organization of IELs, these cells were isolated from the jejunal epithelium of each group of conventionally raised mice and subjected to flow cytometry. There were no statistically significant differences in the purity of the lymphocyte preparations from each group of mice; >80% of the gated lymphocytes expressed the IEL-specific marker, CD103 (Fig. 2A). The total yield of lymphocytes was similar in each group (5–7 × 10⁶).

The majority of the IELs were also positive for CD45, a pan-lymphocyte marker (Fig. 2B). However, there was a statistically significant increase in the fractional representation of αβ TCR⁺ IELs in Fabpi-TAgWt mice compared with their normal littermate controls (p < 0.05; Student’s t test) and a statistically significant decrease in γδ TCR⁺ IELs (p < 0.05) (Fig. 2, C and D). In contrast, there were no differences in the percentages of these IEL subsets in Fabpi-TAgK107/108 versus normal animals (Fig. 2, C and D).

We performed a quantitative immunohistochemical study of jejunal crypt-villus units to determine whether the change in αβ TCR⁺ and γδ TCR⁺ IEL representation in Fabpi-TAgWt mice was restricted to the villus epithelium, where the proliferative abnormality was evident, or whether the change extended to the crypt epithelium, where there was no change in proliferative status. An analysis of sections of jejunum indicated that there were no significant differences in the total number of CD103⁺ IELs per 1000 villus epithelial cells between age-matched Fabpi-TAgWt, Fabpi-TAgK107/108, and normal FVB/N mice (Fig. 3A). However, there was a significant increase in the density of αβ TCR⁺ IELs, and a significant reduction in the density γδ TCR⁺ IELs in TAgWt mice compared with the other two groups (p < 0.05) (Fig. 3, B and C).

**FIG. 1.** TAgWt expression in the jejunal villus epithelium of conventionally raised adult FVB/N Fabpi-TAgWt transgenic mice. Multilabel immunohistochemical study of a 6-week-old mouse that had received an intraperitoneal injection of BrdUrd (BrdU) 90 min prior to sacrifice. A, section of jejunum stained with rabbit antibodies to TAg, Cy3-labeled donkey anti-rabbit Ig, goat anti-BrdU, and FITC-conjugated donkey anti-goat Ig. TAgWt-positive nuclei appear red/orange. BrdUrd-positive nuclei appear green. Co-expression of TAg and BrdUrd produces yellow staining of nuclei (e. g. arrowhead). TAg is not expressed in the crypt epithelium (nuclei are green; arrows). B, TAgWt expression in the follicle-associated epithelium (FAE) overlying Peyer’s patches. The section was incubated with antibodies to TAgWt and Cy3-donkey anti-rabbit Ig, resulting in magenta-colored TAgWt-positive nuclei in the follicle-associated epithelium. The lymphoid population underlying the follicle-associated epithelium does not express detectable levels of TAgWt; their nuclei appear blue after counterstaining with bis-benzidine. Bars, 25 μm.

**FIG. 2.** Expression of TAgWt results in an increase in αβ TCR⁺ and a decrease in γδ TCR⁺ IELs. IELs from the jejunums of conventionally raised 6–8-week-old FVB/N Fabpi-TAgWt, Fabpi-TAgK107/108, and normal mice were analyzed by flow cytometry (n = 3 mice/group/experiment; three experiments). Mean values ± S.E. are plotted. A, gated lymphocytes positive for the IEL-specific marker, CD103. The results reveal no statistically significant differences in the purity of the lymphocyte preparations between groups. B, sorted IELs double positive for CD103 and CD45, a pan-lymphocyte marker. The total yield of lymphocytes is similar in each group. C, results showing a statistically significant increase in the percentage of αβ TCR⁺ IELs in Fabpi-TAgWt mice (asterisk; p < 0.05 relative to normal mice). D, evidence for a statistically significant decrease in γδ TCR⁺ IELs in Fabpi-TAgWt transgenics.
epithelium. To address this question, we rederived our pedigrees of Fabpi-TAg<sup>Wt</sup> and Fabpi-TAg<sup>K107/108</sup> transgenic mice and their normal littermates as germ-free. The cellular patterns of expression of TAg<sup>Wt</sup> and TAg<sup>K107/108</sup> were not affected when the microflora was removed. An epithelial proliferative abnormality extending from the base to the tips of the villi was evident in 6–8-week-old germ-free FVB/N Fabpi-TAg<sup>Wt</sup> but not in Fabpi-TAg<sup>K107/108</sup> or normal animals (Fig. 4A plus data not shown).

Quantitative immunohistochecmical studies also disclosed that Fabpi-TAg<sup>Wt</sup> mice, like their conventionally raised counterparts, had a reduction in the density of their villus γδ TCR<sup>+</sup> IELs when compared with age- and gender-matched FVB/N Fabpi-TAg<sup>K107/108</sup> or normal mice (p < 0.05; Fig. 4, B–D). There was also a modest increase in αβ TCR<sup>+</sup> IELs associated with the TAg<sup>Wt</sup>-induced proliferative abnormality, although the differences were not statistically significant compared with the other two groups of mice (Fig. 4E). The density of all IELs (i.e., CD103<sup>+</sup> cells) in the jejunal villus epithelium was similar in all three groups of germ-free mice (Fig. 3F) but sevelfold less than in conventionally raised animals (compare Figs. 3A and 4F). As in conventionally raised mice, production of TAg<sup>Wt</sup> in the villus epithelium did not result in any changes in the number of crypt-associated CD103<sup>+</sup>, αβ TCR<sup>+</sup>, or γδ TCR<sup>+</sup> IELs (data not shown).

FACS analysis of germ-free jejunal IELs confirmed the results of our quantitative immunohistologic survey and established that TAg<sup>Wt</sup> expression produced a statistically significant increase in αβ TCR<sup>+</sup> and a significant decrease in γδ TCR<sup>+</sup> IELs (p < 0.05 in comparison with age-matched normal or TAg<sup>K107/108</sup> mice (Fig. 5, A–C).

Based on these findings, we concluded that the alterations in these two IEL populations occurred independently of the microflora and were ascribable to the proliferative effects of TAg<sup>Wt</sup> rather than to other functions mediated by regions of the viral oncoprotein located outside of its pRB pocket protein binding domain.

Expression of TAg<sup>Wt</sup> Leads to a Decrease in Accumulation of Intestinally Derived γδ TCR<sup>+</sup> CD8αα IELs and an Increase in Thymically Derived αβ TCR<sup>+</sup> CD8αβ IELs—As noted in the Introduction, intestinal IELs are derived from two sources. The vast majority of αβ TCR<sup>+</sup> CD4<sup>+</sup> and αβ TCR<sup>+</sup> CD8αβ IELs are thymically derived, whereas all CD8αα<sup>+</sup> cells, whether they express αβ TCR or γδ TCR, are derived from extrathymic sites (42). The phenotype produced by TAg<sup>Wt</sup>-induced proliferation of villus enterocytes in germ-free mice could reflect subtle disruptions of epithelial barrier function with resulting presentation of nonmicrobial luminal antigens (e.g., from the diet) to components of the gut-associated lymphoid tissue. If this were the case, one would expect an increased influx of thymically derived, antigen-induced αβ TCR<sup>+</sup> IELs.

We addressed this hypothesis in two ways. First, germ-free Fabpi-TAg<sup>Wt</sup> mice and their normal littermates were given an intraperitoneal injection of BrdUrd 1.5 h prior to sacrifice to label intestinal epithelial cells in S phase. Sections of jejunal crypt-villus units were then stained with antibodies to BrdUrd and E-cadherin, the principal epithelial cadherin and an important regulator of cell adhesion in this system (43, 44). Expression of TAg<sup>Wt</sup> and/or entry of jejunal enterocytes into the cell cycle produced no detectable changes in the steady state cellular levels or intracellular compartmentalization of E-cadherin (data not shown; n = 2 germ-free mice/genotype). Second, FACS analysis of jejunal IELs demonstrated that the TAg<sup>Wt</sup>-associated increase in αβ TCR<sup>+</sup> IELs involved both the CD4 and CD8αβ subsets (Fig. 5, D and E). There were no changes in the CD8αα subtype of αβ TCR IELs (data not shown). These findings confirm that the proliferative abnormality engineered

In the crypt epithelium of conventionally raised normal male 6–8-week-old FVB/N mice, the densities of CD103<sup>+</sup>, αβ TCR<sup>+</sup>, and γδ TCR<sup>+</sup> lymphocytes are 10 ± 1, 5 ± 1, and 4 ± 1 per 100 epithelial cells, respectively. There were no statistically significant differences in the numbers of these cells among the three groups of mice, indicating that the proliferative abnormality produced by TAg<sup>Wt</sup> had a “local” effect on villus IELs that did not extend to the crypt.

The Increase in αβ TCR<sup>+</sup> and Decrease in γδ TCR<sup>+</sup> IELs Observed in Conventionally Raised TAg<sup>Wt</sup> Transgenics Is Recapitulated in Germ-free Mice—One question raised by these findings is whether the intestinal microflora was exerting an influence on the composition of the villus IEL population, e.g., from a potential epithelial barrier disruption associated with the engineered proliferative abnormality, or as a direct consequence of a cross-talk between components of the microflora and the intestinal flora. To address this question, we rederived our pedigrees of Fabpi-TAg<sup>Wt</sup> and Fabpi-TAg<sup>K107/108</sup> transgenic mice and their normal littermates as germ-free. The cellular patterns of expression of TAg<sup>Wt</sup> and TAg<sup>K107/108</sup> were not affected when the microflora was removed. An epithelial proliferative abnormality extending from the base to the tips of the villi was evident in 6–8-week-old germ-free FVB/N Fabpi-TAg<sup>Wt</sup> but not in Fabpi-TAg<sup>K107/108</sup> or normal animals (Fig. 4A plus data not shown).

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in enterocytes is associated with an influx of thymically derived IELs.

FACS analysis also established that expression of Tag\(^W\) in germ-free villus epithelium leads to a significant \((p < 0.05)\) reduction in intestinally derived \(\gamma\delta\) TCR\(^+\) CD8\(\alpha\)α IELs compared with normal littermate controls (Fig. 5F). Immunostaining of intestinally derived \(\gamma\delta\) TCR\(^+\) CD8\(\alpha\)α IELs and thymically derived \(\alpha\beta\) TCR\(^+\) CD4\(^+\) and CD8\(\beta\)β subpopulations obtained by flow cytometry revealed that they did not contain detectable levels of Tag (data not shown plus see below).

Taken together, these findings demonstrate that Tag\(^W\)-dependent reentry of villus enterocytes into the cell cycle produces a specific decrease in the \(\gamma\delta\) TCR\(^+\) CD8\(\alpha\)α IEL populations that normally develop in the intestine.

**qRT-PCR Analysis of Tag\(^W\)-dependent Regulation of IL-7 Expression**—Previous reports have established that the majority of intestinal IELs are maintained in G0 of the cell cycle (45). In addition, some reports have suggested that epithelial cells may act as antigen-presenting cells for induction and activation of these resting IELs (46, 47). Thus, the proliferative abnormality produced by Tag\(^W\) could result in suppression of critical trophic factors necessary for the appropriate development and activation of \(\gamma\delta\) TCR\(^+\) CD8\(\alpha\)α IELs, leading to their diminution in the epithelium.

IL-7 is one such trophic factor: it is produced by the epithelium and required for generation of mature \(\gamma\delta\) TCR\(^+\) IELs (see Introduction). \(\gamma\delta\) TCR\(^+\) IELs express the receptor for this cytokine, IL-7R (48). We tested the hypothesis that Tag\(^W\)-induced reentry of villus enterocytes into the cell cycle is accompanied by reduced IL-7 expression by performing a qRT-PCR analysis of RNAs isolated from intact jejenum as well as LCM jejunal villus epithelium (Fig. 6A). The results revealed a 12-fold lower steady state concentration of IL-7 mRNA in the intact jejenum of germ-free Fabpi-Tag\(^W\) mice compared with germ-free normal littermates and a 4-fold reduction in levels in their LCM villus epithelium (Fig. 6B). Control qRT-PCR assays of LCM epithelial RNA documented a 2-fold reduction in TCR\(^\delta\) mRNA (Fig. 6C), consistent with the reduced representation of \(\gamma\delta\) TCR\(^+\) IELs in transgenic mouse jejenum documented by quantitative immunohistochemical and flow cytometry analyses (Figs. 4D and 5F).

To address the question of whether the Tag\(^W\)-induced proliferative abnormality in villus enterocytes produced changes in \(\gamma\delta\) TCR\(^+\) IEL gene expression, we purified these cells, using flow cytometry, from the jejunums of 6-8-week-old germ-free male Fabpi-Tag\(^W\) and normal mice (n = 50 mice/group). qRT-PCR studies indicated that the IELs from transgenic mice did not contain detectable levels of Tag mRNA, in agreement with the results of our immunohistochemical studies (see above). IL-7R\(\alpha\) mRNA levels were significantly decreased in \(\gamma\delta\) TCR\(^+\) IELs from transgenic compared with normal mice (5.5-fold; \(p < 0.05\); Fig. 7).
Fujihashi et al. (26) used IL-7 knockout mice to show that IL-7 signaling is necessary for IL-7R expression in γδ TCR+ IELs and for their subsequent activation and cell division. Based on this observation and the findings described above, we concluded that TAgWt expression in villus enterocytes results in decreased epithelial expression of IL-7, leading to a concomitant decrease in expression of the IL-7 receptor in γδ TCR+ IELs, and impeded intestinal development of γδ TCR+ CD8αα IELs.

IELs are important for their subsequent activation and cell division.

IELs and help regulate the extent of the p53-independent apoptotic response that occurs in villus enterocytes undergoing unscheduled, TAgWt-induced reentry into the cell cycle.

Flt3L—The DNA microarray studies revealed that the mRNA encoding the ligand for Flt3 is enriched in γδ TCR+ IELs compared with normal littermates. Flt3 was initially identified in hematopoietic stem cells (52). It is a member of the class III receptor tyrosine kinases that shares by other progenitors; Flt3 ligand inhibits EGF- and FGF2-stimulated division of neuronal stem cells (56). There is very little information about the regulation of expression of Flt3 ligand and its receptor or their functions in epithelia. One report indicated that Flt3 mRNA is present in mouse bile duct epithelium (57), whereas another identified the transcript in dividing neuroepithelial cells (56).

Our LCM/qRT-PCR studies revealed that the receptor is expressed in normal jejunal villus epithelium. Moreover, expression is down-regulated by the engineered proliferative abnormality; mRNA levels are reduced an average of 7.5-fold in LCM TAg Wt compared with nontransgenic epithelium (Fig. 6C). qRT-PCR/LCM analysis indicated that the mRNA encoding Flt3 ligand is also reduced in TAg Wt epithelium (Fig. 6C). qRT-PCR assays disclosed that TAg Wt expression in en-
terocytes does not have a discernible effect on IEL Flt3 ligand expression (Fig. 7). Since the extent of the reduction in Flt3 ligand mRNA in TAgWt epithelium was severalfold greater than the reduction of /H9253/H9254 TCR expression (5- versus 2-fold), and since IEL Flt3 ligand expression is unaffected by enterocyte TAgWt expression, we concluded that the proliferative abnormality reduces epithelial expression of the ligand. The response of Flt3 and its ligand to changes in the proliferative status of enterocytes raises the possibility that signaling through this system may normally serve to help suppress cell division as members of this lineage execute their terminal differentiation program.

Prospectus—These studies reveal that an engineered proliferative abnormality in postmitotic enterocytes impedes intestinal development of /H9253/H9254 TCR expression, and promotes accumulation of thymically educated CD4 and CD8αβ subsets of /H9253/H9254 IELs. Our findings highlight the interdependent contributions of enterocytes and /H9253/H9254 TCR expression to intestinal mucosal biology, a point illustrated by the diminution in enterocyte IL-7 expression associated with TAgWt production. The resulting diminution in intestinal maturation of /H9253/H9254 IELs “robs” the epithelium of IEL-derived factors known or postulated to support epithelial barrier function (e.g. TGF-β3). Gnotobiotic FVB/N Fabpi-TAgWt mice provide an environmentally and genetically defined, “sensitized” model system for genetic or pharmacologic tests of the role of enterocyte-derived factors postulated to promote maturation of /H9253/H9254 IELs, of IEL-derived factors that may affect epithelial barrier function, and/or of microbes or microbially derived products that may influence mucosal biology.

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