Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 3920–3925, April 1997
Immunology

Functional differentiation of T cells in the intestine of T cell receptor transgenic mice

STEPHEN D. HURST, STEPHANIE M. SITTERDING, SANDY JI, AND TERRENCE A. BARRETT*

Departments of Medicine and Microbiology/Immunology, Lakeside Veteran's Administration Hospital and Northwestern University Medical School, Chicago, IL 60611

Communicated by K. Frank Austen, Harvard Medical School, Boston, MA, January 17, 1997 (received for review November 12, 1996)

ABSTRACT **The intestinal lamina propria (LP) is a major effector site of the mucosal immune system where antigen-specific and antigen-nonspecific factors shape the functional responses of $CD4^+$ T helper cells. To study the functional differentiation of LP T helper cells we utilized DO11.10 T cell receptor (TCR) transgenic (Tg) mice that expressed a clonotypic TCR specific for a class II major histocompatibility complex-restricted peptide of chicken ovalbumin. The majority of cells expressing Tg TCR (Tg^+) in peripheral lymphoid tissue expressed naive surface phenotypes whereas nearly all Tg^+ T cells in the intestinal LP expressed an activated/memory-like phenotype. Flow cytometric analysis of Tg^+ T cell populations revealed that a small proportion of cells in peripheral lymphoid tissue but nearly all cells in the LP expressed dual (Tg plus non-Tg) TCRs. In Tg × recombinase-activating-gene-1-deficient (Tg × *RAG-1*$^{-/-}$) mice, splenic and LP T cells expressed naive surface phenotypes and produced cytokines equivalent to naive splenic cells from Tg × *RAG-1*$^{+/+}$ mice. In contrast, Tg LP cells from Tg × *RAG-1*$^{+/+}$ mice produced 35-fold greater levels of interferon-γ and 5-fold greater levels of interleukin 4 compared with naive splenic cells. These findings suggested that activation of Tg^+ T cells through endogenous non-Tg TCR had promoted the localization and differentiation of memory-like effector T helper cells in the intestine.**

The intestinal lamina propria (LP) is a classic effector site for mucosal immune responses. Within this compartment, $CD4^+$ T cells provide helper functions for committed B cell populations and mature plasma cells (1). Lamina propria cells participate in cellular immune responses to enteric pathogens such as *Salmonella* (2), *Giardia lamblia* (3), *Chlamydia trachomatis* (3), *Listeria monocytogenes* (1), and others (1). Studies in humans have revealed that $CD4^+$ LP T cells were an activated population of memory T cells that expressed CD45RO, transferrin receptors, class II major histocompatibility complex, and interleukin (IL) 2 receptor and lacked CD45RA (4, 5). In response to stimulation, LP T cells manifested low proliferative responses but produced IL-2, interferon γ (IFN-γ), IL-4, and IL-5 at high levels (3–7). These results suggested that $CD4^+$ LP T cells were a unique population of T helper cells that had differentiated to deliver effector functions needed in the mucosal microenvironment.

In the intestinal LP, $CD4^+$ LP T helper cells are exposed to luminal antigen (Ag) and to the cytokine milieu generated by local immune cell activation (8). Activation of mucosal mast cells, macrophages, and natural killer cells exposes LP T cells to oxygen radicals, eicosanoids, chemokines, and cytokines such as IL-1, tumor necrosis factor α, IL-6, IFN-γ, and others (9). Under normal conditions, LP T helper cells cooperate to induce and regulate activities of local immune cells resulting in a state of "controlled inflammation." However, in states of chronic tissue inflammation such as inflammatory bowel disease (IBD), the balance of T cell cytokines shifts in favor of T helper type 1-like cells in Crohn disease and T helper type 2-like responses in ulcerative colitis (10). Results in animal models of IBD have suggested that IBD results from the dysregulation of intestinal T helper cell functional differentiation (11). The importance of enteric Ag in driving this inflammation has been highlighted by the failure of IBD to occur in these models when transferred to germfree facilities (11–13). Thus these studies have suggested that the regulation of LP T cell functional differentiation was an important factor in normal and pathologic states of inflammation in the bowel.

To further understand the potential mechanisms involved in T cell functional differentiation in peripheral lymphoid and extralymphoid sites, we utilized DO11.10 T cell receptor (TCR) transgenic (Tg) mice. The transgene encoded a Vα13, Vβ8.2 TCR specific for a class II major histocompatibility complex-restricted peptide of chicken ovalbumin (14). Our analysis of the T cells in the intestine revealed that Tg^+ LP T cells expressed an activated profile of surface markers and produced a cytokine profile typical of memory-like effector T helper cells. Flow cytometric analysis showed that activated Tg^+ LP T cells expressed dual TCRs: a Tg TCR identified by the clonotypic anti-TCR mAb KJ1–26.1 (15) and a second non-Tg TCR. Analysis of Tg mice crossed to mice deficient for the recombinase-activating gene (*RAG*)-1 (Tg × *RAG-1*$^{+/+}$) (16) revealed that Tg^+ T cells had localized to the LP but expressed naive surface and functional phenotypes in the absence of endogenous TCR. It was possible that Tg^+ LP T cells in Tg × *RAG-1*$^{+/+}$ mice were activated in a bystander fashion by non-Tg cells responding to exogenous antigens (gut flora, dietary, etc.). However, the relatively high proportion of dual TCR-expressing cells in the LP compared with peripheral lymphoid tissue suggested that activation via endogenous TCR expressed by Tg^+ LP T cells had led to the localization and functional differentiation of cells in this tissue.

MATERIALS AND METHODS

Animals. BALB/c and B6.*RAG-1*$^{-/-}$ mice (16) were obtained from The Jackson Laboratory. DO11.10 TCR Tg mice (a gift from D. Loh, Washington University) were bred in facilities at the Lakeside Veteran's Administration Medical Sciences Building and maintained under specific pathogen-free conditions. Transgenic mice were mated to BALB/c breeders (Tg × *RAG-1*$^{+/+}$) and the progeny were screened for expression of the TCR transgenes by flow cytometry using the clonotypic mAb KJ1–26.1 (15). Transgenic mice were also

Abbreviations: Ag, antigen; IFN-γ, interferon γ; IL, interleukin; LP, lamina propria; MFI, mean fluorescence index; MLN, mesenteric lymph node; PE, phycoerythrin; PP, Peyer's patch; TCR, T-cell receptor; Tg, transgenic.

*To whom reprint requests should be addressed at: Department of Medicine, Gastroenterology Section, Northwestern University Medical School, 303 East Chicago Avenue, Med/GI S208, Chicago, IL 60611. e-mail: tab926@lulu.acns.nwu.edu.

0027-8424/97/943920-6$2.00/0
PNAS is available online at **http://www.pnas.org**.

mated to B6.*RAG-1*$^{-/-}$ mice and the offspring were screened and mated to produce (Tg × *RAG-1*$^{-/-}$) F_2 mice. All Tg mice were used between 6 and 10 weeks of age.

Reagents and Flow Cytometric Analysis. Isolated cell populations were analyzed for cell surface markers by three-color flow cytometry. Viable cells were blocked for nonspecific staining with 2.4G2 (anti-Fc-receptor, American Type Culture Collection) and stained with anti-Thy-1 conjugated to R-phycoerythrin (PE) (PharMingen), biotin-labeled KJl-26.1 (ref. 15; anti-Tg TCR, a gift from Phillipa Marrack, National Jewish Center, Denver) and one of the following fluorescein isothiocyanate (FITC)-labeled mAb: control IgG2a (R35–95), anti-CD44 (IM7/Pgp-1), anti-CD45RB (16A), anti-CD69 (H1.2F3), or anti-L-selectin/CD62L (Mel-14) (all from PharMingen). In some experiments, cells were stained with reagents specific for TCR Vβ8.1/8.2 (MR5–2), Vα2 (B20.1), Vα11 (RR8–1), or Vα8 (B21.14) (PharMingen). Cell were washed with ice-cold fluorescence-activated cell sorting buffer (1× PBS/2% BSA/NaN_3). KJl-26.1 was purified over a column of protein A-Sepharose CL-4B (Pharmacia), dialyzed in PBS, and quantified by spectrophotometry at 280 nm. Purified mAb was then biotinylated (Pierce) or PE-coupled (Molecular Probes) by using the manufacturer's specifications. Biotin-coupled mAbs were visualized with Streptavidin-CyChrome (PharMingen). Events were collected and analyzed by using a Becton Dickinson FACScan and LYSIS II software.

Cell Isolation. Splenic cells were isolated as described (17). Briefly, spleens were mechanically dissociated and red blood cells were lysed in ammonium phosphate/chloride lysis buffer. Cell suspensions were washed and stored in DMEM (GIBCO/BRL) with 5% fetal calf serum (5% DMEM) on ice until used. Cell preparations were purified by mAb and complement lysis as described below. Mesenteric lymph node (MLN) cells were mechanically dissociated and fat eliminated by passage of the cell suspensions through nylon mesh. Cell suspensions were washed, pelleted, resuspended in 5% DMEM, and stored on ice. Lamina propria cells were prepared by modifications of a protocol previously described (7). Briefly, small intestines were removed and flushed with ice-cold PBS to remove fecal contents. After Peyer's patches (PPs) were excised, intestines were opened longitudinally, minced into 5- to 10-mm pieces, and washed extensively with ice-cold PBS. Mucosal pieces were then digested twice for 30 min at 37°C in trypsinizing flasks with 5 mM EDTA (Sigma) and 10% newborn calf serum (NCS) (GIBCO/BRL) in PBS. After each digestion with EDTA, mucosal pieces were washed with ice-cold PBS and the supernatants were discarded. The remaining tissue was then digested for four 30-min intervals in a buffer containing collagenase (Sigma; 100 units/ml), 25 mM Hepes, 7 mM $CaCl_2$, and 20% NCS in DMEM. After each 30-min interval, the cells released were centrifuged, washed, and stored in 10% DMEM on ice, and the mucosal pieces were returned to the collagenase buffer. After the fourth interval, the digestion buffer was supplemented again with collagenase (100 units/ml) and 10% NCS and digested for two additional 30-min intervals. Viable cells were isolated by centrifugation of the column elutant over Nycoprep 1.077 (Accurate Chemical). After centrifugation, cells were collected from the interface, washed, and pelleted. Cells were then purified further by mAb and complement lysis as described below. PP lymphocytes were isolated as LP with modifications. After EDTA digestions, excised PPs were digested with collagenase without NCS for 2 hr and vortex-mixed to liberate lymphocytes. The recovered cells were layered on Nycoprep 1.077 gradients to isolate viable cells. Cells were then washed, pelleted, and resuspended for mAb and complement lysis. Splenic, MLN, PP, and LP Tg$^+$ T cells were purified by mAb and complement lysis as described (18). Briefly, isolated cells were incubated on ice in a mixture of J11d.2 (anti-heat-stable Ag), 2.43 (anti-CD8), M5/114 (anti-class II), and 2.4G2 (anti-FcR). After a 30-min incubation with these mAbs, cells were washed and incubated on ice with MAR 18.5 (mouse anti-rat Ig) (all from American Type Culture Collection). Cells were then washed and incubated with 10% Rabbit Low-Tox C (Accurate Chemical) for 45 min at 37°C and viable cells were recovered on Nycoprep 1.077 gradients. Residual mAb-labeled cells were eliminated by incubation with anti-rat-coated magnetic beads; 40 beads/1 T cell (Dynal, Great Neck, NY), followed by magnetic-negative selection of the bound cells. The resulting T cell preparation were >90% pure for CD4$^+$ T cells by flow cytometry (data not shown). These cells were used as purified Tg$^+$ T cells.

Cytokine Analysis. T cells were cultured in DMEM (GIBCO/BRL) supplemented with 5% fetal calf serum (GIBCO/BRL), 2-mercaptoethanol, penicillin–streptomycin, Hepes, and L-glutamine as described (17). To assess cytokine production, 5×10^4 T cells were cocultured in 24-well plates with 2.5×10^5 irradiated anti-Thy-1 and complement-lysed splenic antigen-presenting cells and ovalbumin peptide Ag (0.3 μM). For cytokine analysis, supernatants were collected for analysis of IL-2, IL-4, and IFN-γ at 48 hr. Supernatants were stored at −20°C until analysis by enzyme-linked immunosorbent assay (ELISA). ELISAs for IL-2, IL-4, and IFN-γ were performed according to manufacturer's specification (Endogen, Cambridge, MA), including calibrated standards provided by the manufacturer. Wells were developed with 3,3′,5,5′-tetramethylbenzidine soluble peroxidase substrate (Zymed) and read at 450 nm on a spectrophotometer. Cytokine levels are presented as units per ml by using the following conversion values: 1 unit of IL-2 = 14.2 pg; 1 unit of IL-4 = 1 pg; 1 unit of IFN-γ = 10 pg (18). The limits of detection for these ELISAs were as follows: IL-2, 0.2 unit; IL-4, 4 units; IFN-γ, 0.5 unit.

RESULTS

Intestinal T Cells Express "Activated" Surface Phenotypes in Tg Mice. To assess the surface phenotype of Tg$^+$ T cells in peripheral lymphoid and extralymphoid sites, cells were isolated from splenic and intestinal PP and LP tissues. In Fig. 1, results of staining for Tg TCR (KJ1–26.1) (15) versus CD45RB, CD69, and L-selectin are shown for CD4-gated cells.

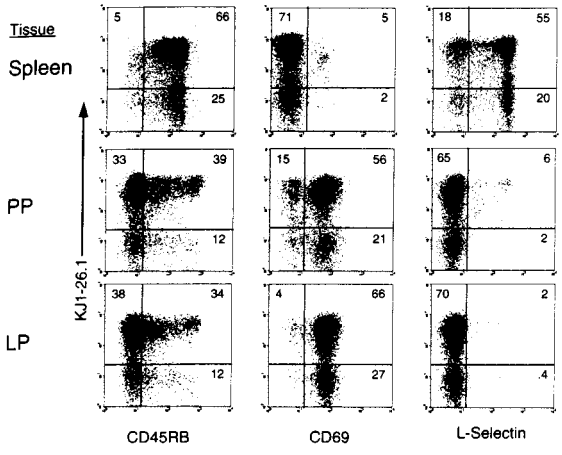

FIG. 1. Surface phenotype of CD4$^+$ T cells in lymphoid and extralymphoid sites. T cells from spleen, PP, and LP of 8-week-old DO11.10 TCR Tg mice were stained with FITC-labeled mAbs specific for CD45RB, CD69, or L-selectin; anti-CD4-PE; and KJl-26.1-BIO followed by Streptavidin-CyChrome. For all populations, lymphoid cells were gated on the basis of forward and 90° angle side scatter and staining for CD4. For each population, the percentage of positively stained cells is shown in quadrants. Quadrants were drawn based on staining with negative control (IgG2a) mAbs. Data shown are representative of more than five experiments.

The majority of $CD4^+$ T cells in each of these tissues expressed the Tg TCR with <1% of Tg^+ T cells expressing CD8 (data not shown and ref. 14). The results demonstrated that Tg^+ and non-Tg T cells from splenic and intestinal sites expressed distinct patterns of surface activation markers. The profile of surface markers expressed by splenic cells were typical of relatively naive cell types as were MLN T cells (data not shown). In the spleen, 93% of Tg^+ T cells expressed high levels of CD45RB and were $CD69^-$. In addition, the majority of splenic Tg^+ cells were L-selectin-positive. By comparison, greater proportions of previously activated Tg^+ T cells were detected in intestinal PP and LP tissues. In the PP, nearly half of the Tg^+ cells expressed low levels of CD45RB and 80% were $CD69^+$. Interestingly, the greatest proportion of activated cells was detected in the intestinal LP. The majority of LP Tg^+ T cells expressed low levels of CD45RB and nearly all cells were $CD69^+$. The pattern of expression for L-selectin also suggested that Tg^+ T cells in PP and LP tissues had shed L-selectin. In addition, the surface phenotypes expressed by non-Tg $CD4^+$ T cells in Tg (Fig. 1) and non-Tg BALB/c (data not shown) mice were typical of activated T cells and resembled the profiles expressed by Tg^+ T cells in these tissues. Overall, the numbers of LP $CD4^+$ T cells in unprimed Tg and non-Tg mice were equivalent as assessed by LP yields and immunohistochemical staining (data not shown). Thus these data suggested that in TCR Tg mice, Tg^+ cells in intestinal lymphoid and extralymphoid effector sites had been activated *in vivo*.

In Fig. 1, the level of surface activation marker expression was compared with the level of Tg TCR expression as assessed by staining with KJ1–26.1. In all tissues examined Tg^+ T cells

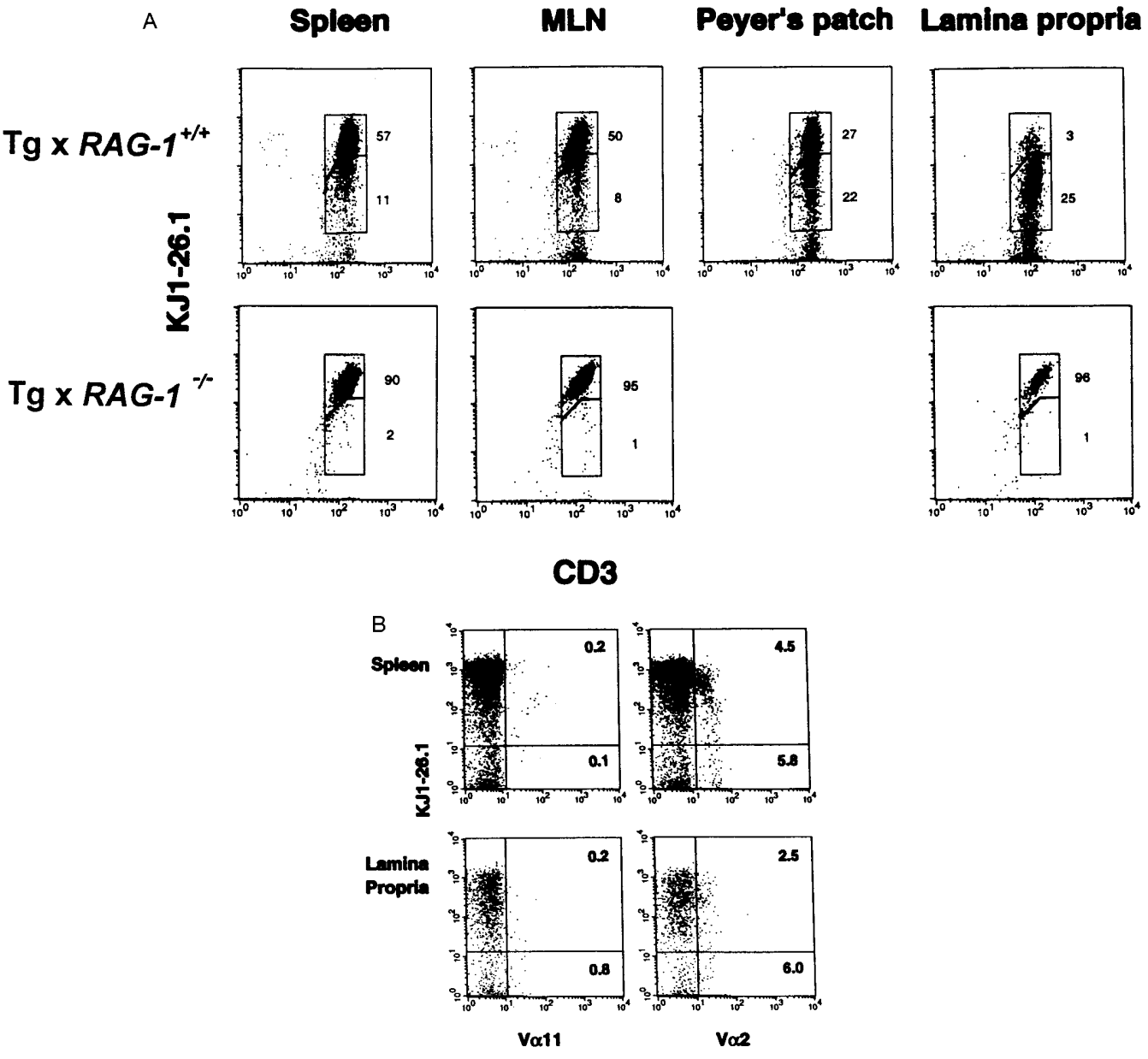

FIG. 2. TCR expression in Tg mice. Spleen, MLN, PP, and LP T cells were harvested from Tg × *RAG-1*$^{+/+}$ and Tg × *RAG-1*$^{-/-}$ mice (PP not detected in Tg × *RAG*$^{-/-}$ mice) and Tg TCR vs. CD3 expression was analyzed by staining with PE-coupled clonotypic anti-TCR mAb, KJ1–26.1-PE, and FITC-coupled anti-CD3 (*A*). Results of T helper cells gated on the basis of staining for CD4 are shown. Regions show the distribution of cells with higher (upper region) or lower (lower region) Tg TCR/CD3 ratios within each population. (*B*) Results are shown for CD4-gated populations of splenic and LP cells from Tg × *RAG-1*$^{+/+}$ mice stained with KJ1–26.1-PE and FITC-coupled mAbs specific for endogenous TCR Vα2 and Vα11 chains. Staining for these mAbs in Tg × *RAG-1*$^{-/-}$ mice was <0.1% on CD4-gated populations (data not shown). Data shown were representative of more than five experiments.

with surface phenotypes typical for activated cells ($CD45RB^{lo}$, $CD69^+$, or L-selectin-negative) also expressed lower levels of the Tg TCR compared with naive populations. In the LP, Tg TCR expression was slightly less for $CD45RB^{lo}$ compared with $CD45RB^{hi}$ cells [mean fluorescence index (MFI) for KJ1–26.1 staining: 323 compared with 360, respectively]. However, both populations were $CD69^+$, an early marker of activation, suggesting that subsets of LP Tg^+ cells may have been at distinct stages of activation. The correlation between low Tg TCR expression and expression of an activated surface phenotype was more apparent for populations in PP and especially spleen. The MFI of KJ1–26.1 staining was 15% lower for $CD45RB^{lo}$cells in the PP and nearly 50% reduced for $CD45RB^{lo}$ Tg^+ T cells in the spleen compared with parallel populations of $CD45RB^{hi}$ cells in these tissues. In the spleen, further analysis revealed that L-selectin-negative cells expressed 20% lower levels of the Tg TCR compared with L-selectin$^+$ cells (MFI of KJ1–26.1 staining: 411 compared with 514, respectively). An analysis of whole populations of splenic and intestinal cells suggested that Tg TCR expression was lowest for LP and PP cells and highest in the spleen (MFI of KJ1–26.1 staining: LP, 328; PP, 396; spleen, 490). Thus, these data suggested that activated Tg^+ T cells in both splenic and intestinal tissues expressed low levels of Tg TCR. The increased proportion of these cells in the intestine suggested that activated cells may have preferentially migrated to this tissue and/or been retained due to local factors.

Frequency of Dual TCR-Expressing Tg^+ T Cells. Because data in Fig. 1 suggested that previously activated cells expressed low levels of Tg TCR, the potential for endogenous TCR expression by Tg^+ T cells was examined. Incomplete allelic exclusion of TCR gene rearrangement results in expression of dual TCRs by peripheral T cells in human subjects and in normal and TCR Tg mice (19–25). As the stoichiometry of total TCR to CD3 expression is fixed (25, 26), we utilized flow cytometric analysis of selective TCR expression to distinguish single and dual TCR-expressing cells. Thus, in TCR Tg mice, the ratio of Tg TCR/CD3 was higher for cells expressing only Tg TCR (Fig. 2*A*, upper gate) compared with cells expressing dual TCRs (Fig. 2*A*, lower gate). More than 84% of Tg^+ T cells in the spleen and MLN expressed high Tg TCR/CD3 ratios, whereas a small proportion of Tg^+ T cells in these tissues expressed lower Tg TCR/CD3 ratios. By comparison, greater proportions of dual TCR-expressing Tg^+ T cells were detected in PP and LP tissues. Nearly half of Tg^+ PP T cells expressed low Tg TCR/CD3 ratios whereas more than 85% of Tg T cells in the small intestinal LP expressed dual TCRs. Expression of CD3 was equivalent for Tg^+ T cells in all tissues examined indicating that Tg TCR had not been down-regulated due to activation. To examine Tg TCR/CD3 ratios in mice without endogenous TCR rearrangement, Tg mice were crossed to *RAG-1*$^{-/-}$ mice (16). Splenic, MLN, and LP Tg^+ T cells from Tg $\times$ *RAG-1*$^{-/-}$ mice expressed high Tg TCR/CD3 ratios without evidence for decreased Tg TCR expression or TCR down-regulation. (PPs were not detected in Tg $\times$ *RAG*$^{-/-}$ mice).

To further address whether Tg^+ T cells expressed endogenous TCR chains, cells were stained with mAbs specific for TCR Vα chains not encoded by the transgene. Results in Fig. 2*B* demonstrated that cells staining positive with the clonotypic mAb KJ1–26.1 also stained with reagents specific for Vα2 TCR chains and to a lesser extent for Vα11 TCR chains (anti-TCR Vα8 staining not detected, data not shown). The decreased ratio of Vα2/CD3 staining for Tg^+ T cells compared with nontransgenic T cells was consistent with dual TCR expression by Tg^+ T cells. Background levels of staining for Vα2 and 11 TCR chains in Tg $\times$ *RAG-1*$^{-/-}$ mice were $<0.1\%$ (data not shown). The equivalent proportions of Vα11- or Vα2-expressing Tg^+ T cells in LP and splenic tissues suggested that gut-specific expansion of endogenous TCR-expressing LP T cells had involved Vα chains not detected with the limited reagents available. We observed high ratios of Vβ8.1/8.2 to CD3 staining in Tg mice indicating that allelic exclusion of endogenous TCR Vβ expression was complete (data not shown). Thus with results in Fig. 1, these data suggested that in TCR Tg mice, rearrangement of endogenous TCR Vα genes led to generation of some dual TCR-expressing Tg^+ T cells that were selectively activated in the intestine.

Surface Phenotype of Tg^+ T Cells in Tg $\times$ *RAG-1*$^{-/-}$ Mice. To examine the surface phenotype of Tg^+ T cells in mice without endogenous TCR rearrangement, Tg mice were crossed to *RAG-1*$^{-/-}$ mice. As shown in Fig. 3, Tg^+ T cells in the spleen, MLN, and LP of Tg $\times$ *RAG-1*$^{-/-}$ mice expressed a profile of surface markers typical for naive T cells (27). In the spleen and MLN, Tg^+ T cells were $CD69^-$ and expressed dull levels of CD44 and high levels of CD45RB. Although Tg^+ T cells were largely L-selectin-positive in Tg $\times$ *RAG-1*$^{-/-}$ mice, a small subset of L-selectin-negative cells were detected. Overall the surface phenotype expressed by Tg^+ T cells in these mice were similar to those observed for cells in Tg $\times$ *RAG-1*$^{+/+}$ mice (Fig. 1), suggesting that splenic and MLN Tg^+ T cells in both mice were naive. In the LP of Tg $\times$ *RAG-1*$^{-/-}$ mice, however, LP Tg^+ T cells expressed a distinct profile of surface activation markers compared with LP cells from Tg $\times$ *RAG-1*$^{+/+}$ mice. In Tg $\times$ *RAG-1*$^{-/-}$ mice, Tg^+ LP T cells expressed a relatively naive profile of surface activation markers with low CD44, high CD45RB, and negative levels of CD69 that included expression of L-selectin by the majority of cells. Interestingly, the percentage of L-selectin-negative LP cells varied between 20 and 40% in mice <8 weeks of age (data not shown). These results were distinct from Tg $\times$ *RAG-1*$^{+/+}$ mice, where the vast majority of Tg^+ LP T cells were L-selectin-negative and expressed activated "memory-like" surface phenotypes (Fig. 1). Immunohistologic analysis of intestinal frozen sections of Tg $\times$ *RAG-1*$^{-/-}$ mice with KJ1–26.1 verified that high numbers of Tg^+ T cells were localized to the LP compartment (data not shown). Thus, these data suggested that without endogenous TCR expression, Tg^+ T cells had localized to the intestinal LP but had not been activated.

Functional Differentiation of Dual TCR-Expressing Cells. To examine how dual TCR expression and localization to the intestinal LP affected T cell functional differentiation, cytokines produced by splenic and LP cells from Tg $\times$ *RAG-1*$^{+/+}$ and Tg $\times$ *RAG-1*$^{-/-}$ mice were assessed after activation with antigen-presenting cells and Ag. Results in Fig. 4 show that in Tg $\times$ *RAG-1*$^{+/+}$ mice, LP Tg^+ T cells produced >5-fold greater levels of IL-4 and 35-fold greater levels of IFN-γ

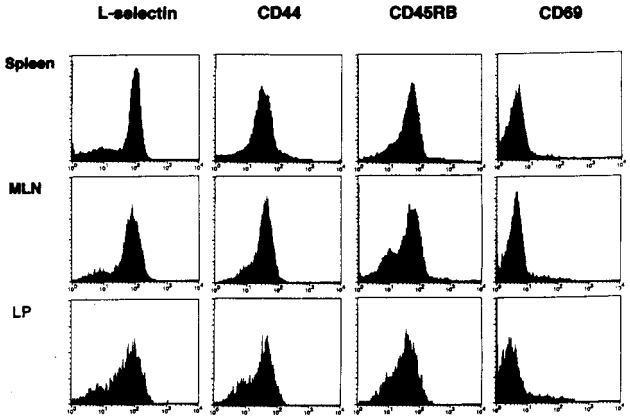

FIG. 3. Surface phenotype of $CD4^+$ T cells in Tg $\times$ *RAG-1*$^{-/-}$ mice. Tg T cells from spleen, MLN, and LP were gated on the basis of staining with anti-CD4-PE and KJ1–26.1-BIO followed by Streptavidin-CyChrome and results are shown for staining with FITC-conjugated mAbs specific for L-selectin, CD44, CD45RB, or CD69. Data shown are representative of four experiments.

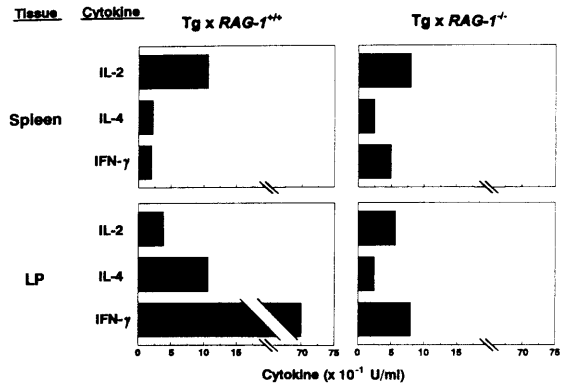

FIG. 4. Induction of cytokine production for LP Tg^+ T cells. Purified splenic and LP Tg^+ T cells were harvested from Tg × *RAG-1*$^{+/+}$ and Tg × *RAG-1*$^{-/-}$ mice and activated with antigen-presenting cells and Ag. Cytokine levels were determined by ELISA at 48 hr. Data shown are representative of four experiments.

compared with splenic cells. These results were also observed for Tg × *RAG-1*$^{+/-}$ littermate controls (data not shown). Thus with results of LP Tg^+ T cell cloning (S.D.H. and T.A.B., unpublished observations), these data suggested that Tg^+ LP T cells in these mice had become a T helper type 0-like effector population (28). To examine the functional differentiation of Tg^+ T cells in mice without endogenous TCR expression, cytokines produced by cells from Tg × *RAG-1*$^{-/-}$ mice were measured. Overall, Tg^+ T cells isolated from distinct tissues in Tg × *RAG-1*$^{-/-}$ mice produced cytokine profiles similar to naive splenic cells from Tg × *RAG-1*$^{+/+}$ mice. Thus, LP T cells expressing only clonotypic Tg TCR did not produce increased levels of IL-4 and IFN-γ compared with splenic T cells. These data suggested that without expression of endogenous TCR, Tg T cells localized to the intestinal LP had not differentiated to become effector T helper type 0 cells.

DISCUSSION

These data suggested that dual TCR-expressing Tg^+ T cells in the intestinal LP of DO11.10 TCR Tg mice had been activated by environmental Ag via endogenous TCR. Furthermore, we suspected that TCR-mediated activation played a direct role in the functional differentiation of cells in this extralymphoid effector site. The surface phenotype and cytokine profile produced by Tg^+ T cells in the LP of TCR Tg mice suggested that these cells were a population of activated/memory-like T helper cells. Previous studies have observed similar findings with LP cells from human (3–6) and murine systems (7, 8), suggesting that the development of effector T cells in DO11.10 mice resembled normal pathways for intestinal T cell functional differentiation. The profile of surface markers ($CD45RB^{lo}$, $CD69^+$, and L-selectin) along with the expression of high levels of CD44 (data not shown) by LP cells resembled the phenotype associated with activated memory-like T helper cells (27, 28). These cells mediate efficient help for both T helper type 1-like and T helper type 2-like responses (28). In particular, CD69 expression suggested that LP cells were an activated rather than a resting population of memory T helper cells. These findings suggested that previous activation may have played a role in promoting the unique cytokine profiles we detected. In studies by Bradley *et al.* (29–31), activation was shown to promote the development of effector T helper cells from resting $CD4^+$ memory cells. In these studies, activated/memory effector T helper cells produced greatly increased levels of IL-2, IL- 4, and IFN-γ upon re-exposure to Ag compared with resting memory T helper cells. In these and other studies, cytokines such as IFN-γ or IL-12 compared with IL-4 or IL-10 were shown to promote development of T helper type 1-like compared with T helper type 2-like effectors, respectively (28, 31–33). These results suggested to us that the high levels of cytokine produced by LP cells resulted from TCR-mediated activation along with exposure to IFN-γ or IL-12 produced by tissue macrophages or dendritic cells and IL-4 produced by mucosal mast cells or other T cells. The importance of activation in promoting this phenotype was further highlighted by results in LP of Tg × *RAG-1*$^{-/-}$ mice where LP cells did not express an activated surface phenotype and failed to produce the distinctive profile of cytokines observed in Tg × *RAG-1*$^{+/+}$ mice. Thus, recognition of enteric Ag by dual TCR-expressing cells in the LP may have induced activation and promoted the functional differentiation of this unique subset of IL-4 and high IFN-γ-producing effector T helper cells.

In the current system, an Ag-specific TCR Tg model was used to examine cells that expressed only clonotypic ovalbumin-specific TCRs and other populations that expressed clonotypic Tg TCRs and a second non-Tg TCR composed of the Tg TCR Vβ chain and a non-Tg TCR Vα chain. We suspected that the increased TCR repertoire of Tg^+ cells expressing endogenous TCR Vα allowed this population to recognize a wide spectrum of environmental Ags. By comparing the phenotype of Tg^+ cells in the periphery and intestine, we concluded that the endogenous TCR expressed by LP Tg^+ T cells likely mediated T cell activation by enteric Ags and played a role in the retention of these effector cells in the mucosa. Other reports have verified the expression of two functionally intact TCRs by peripheral T cells in human T cell clones (34, 35) and in up to 15% of T cells isolated from peripheral blood (25). Von Boehmer and colleagues (19) suggested that dual TCR expression usually resulted from incomplete exclusion of TCR Vα gene rearrangement and allowed developing T cells to test various TCR combinations during positive selection. The potential for dual TCR-expressing cells to recognize an increased diversity of environmental Ags was highlighted by Balomenos *et al.* (21), who found that dual TCR-expressing cells expressed activated phenotypes and expanded in the periphery of older TCR Tg and normal mice. In studies in 4- to 6-week-old DO11.10 mice, Macatonia *et al.* (36) detected dual TCR-expressing Tg^+ splenic T cells but found no correlation between TCR usage and expression of L-selectin. We found that in slightly older (8 week old) DO11.10 mice, the expression of dual TCRs by Tg^+ cells in peripheral lymphoid tissue (spleen and MLN) did seem to correlate with expression of surface activation markers (Fig. 1). It was possible that activation of some Tg^+ cells in the periphery may have been due to non-TCR-mediated activation or cross-reactivity of Tg TCR with environmental Ag. In fact, some cells in the LP of Tg × *RAG-1*$^{-/-}$ mice expressed lower levels of CD45RB and L-selectin and increased levels of CD44 and CD69, suggesting that Tg^+ cells had been activated in a bystander fashion or stimulated with exogenous antigens (gut flora, dietary, etc.). Thus, the data suggested that TCR-mediated, Ag-dependent, and bystander mechanisms helped to generate the activated LP T cell populations that we detected. However, the differential roles of bystander and direct TCR-mediated activation in this process remains to be determined.

Our results and results of others (37, 38) suggested that activation of T cells enhanced the migration of cells into the intestinal LP. However, this circulation pathway did not exclude the potential for the migration of naive cells to the mucosa. Previous studies by Poussier *et al.* (39) in Ly 5.1/5.2 parabionts had suggested that peripheral $CD4^+$ T cells circulated through the intestinal LP. Other investigators have suggested that subsets of naive and activated $CD4^+$ T cells exhibit distinct migration patterns. In general, naive cells circulate through peripheral lymphoid tissues, whereas activated memory populations circulate through lymphoid and extralymphoid immune effector sites such as the intestine (37,

38). These models are consistent with our contention that activation of Tg^+ cells promoted the migration of cells to the LP. The failure to observe high numbers of activated dual TCR-expressing cells outside the intestine suggested that enteric Ag may have helped retain effector cells in this site. Given these results, the observation that LP cells in Tg × *RAG-1*$^{-/-}$ mice expressed a relatively naive phenotype was puzzling. Although naive T cells express dull levels of the mucosal integrin $\alpha4\beta7$ their circulation pattern in adult mice is restricted to lymphoid sites (38). Mebius *et al.* (40) have suggested that prior to 24 hr after birth, the pattern of vascular addressin expression does not restrict circulation of naive and activated T cell subsets to distinct lymphoid and extralymphoid sites respectively. These results have suggested that after birth a switch occurred that facilitated tissue-selective lymphocyte migration. Thus, it was possible that naive T cells migrated into the intestinal LP during fetal and early neonatal development. The transition to a more tissue-selective distribution of vascular addressin expression may have occurred, in part due to Ag exposure. Thus, these findings suggested that TCR and non-TCR-mediated factors such as age and the activation of T and non-T cell populations operated to shape the migration of naive and activated T cells into the LP. Ultimately, the goal of these mechanism(s) may have been to expose a wide repertoire of naive T cells to enteric Ag early in development. However, once Ag-driven T cell activation had occurred, the priority of the immune system was to position appropriate populations of activated effector T cells in mucosal extralymphoid sites where they could effectively participate in protective immune responses.

We are grateful to Drs. Geoffrey Kansas, Syamal Datta, William Karpus, Louis Picker, and Philip Streeter for their helpful discussions and constructive comments. This work was supported by National Institutes of Health Grant DK 47073 and the Crohn's and Colitis Foundation of America.

1. Bloom, P. D. & Boedeker, E. C. (1996) in *Seminars in Gastrointestinal Disease*, eds. Strober, W. & Ehrhardt, R. O. (Saunders, Philadelphia), Vol. 7, pp. 151–166.
2. Tagliabue, A., Nencioni, L., Caffarena, A., Villa, L., Boraschi, D., Cazzola, G. & Cavalieri, S. (1985) *Clin. Exp. Immunol.* **62, 2,** 242–247.
3. Zeitz, M., Quinn, T. C., Graeff, A. S. & James, S. P. (1988) *Gastroenterology* **94(2),** 353–366.
4. Zeitz, M., Schieferdecker, H. L., James, S. P. & Riecken, E. O. (1990) *Digestion* **2,** 280–289.
5. James, S. P. (1991) *Gastroenterol. Clin. N. Am.* **20,** 597–612.
6. Targan, S. R., Deem, R. L., Liu, M., Wang, S. & Nel, A. (1995) *J. Immunol.* **154,** 664–675.
7. Harriman, G. R., Hornqvist, E. & Lycke, N. Y. (1992) *Immunology* **75,** 66–73.
8. Qiao, L., Schurmann, G., Meuer, S. C., Wallich, R., Schirren, A. & Autschbach, F. (1995) *Adv. Exp. Med. Biol.* **371A,** 31–34.
9. Fiocchi, C., Youngman, K. R., Simon, P. L., West, G. A., Cominelli, F., Rachmilewitz, D. & Klein, J. S. (1993) *Gastroenterology* **104,** 1202–1205.
10. Fuss, I. V., Neurath, M., Boirivant, M., Klein, J. S., de la Motte, C., Strong, S. A., Fiocchi, C. & Strober, W. (1996) *J. Immunol.* **157,** 1261–1270.
11. Powrie, F. (1995) *Immunity* **3,** 171–174.
12. Sadlack, B., Merz, H., Schorle, H., Schimpl, A., Feller, A. C. & Horak, I. (1993) *Cell* **75,** 253–261.
13. Taurog, J. D., Richardson, J. A., Croft, J. T., Simmons, W. A., Zhou, M., Balish, E. & Hammer, R. E. (1994) *J. Exp. Med.* **180,** 2359–2364.
14. Murphy, K. M., Heimberger, A. B. & Loh, D. Y. (1990) *Science* **250,** 1720–1723.
15. Haskins, K., Kobo, R., White, J., Pigeon, M., Kappler, J. & Marrack, P. (1983) *J. Exp. Med.* **157,** 1149–1169.
16. Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K. & Tonegawa, S. (1992) *Cell* **68,** 869–877.
17. Barrett, T. A., Gajewski, T. F., Danielpour, D., Chang, E. B., Beagley, K. W. & Bluestone, J. A. (1992) *J. Immunol.* **149,** 1124–1130.
18. Croft, M., Bradley, L. M. & Swain, S. L. (1994) *J. Immunol.* **152,** 2675–2685.
19. Borgulya, P., Kishi, H., Uematsu, Y. & von Boehmer, H. (1992) *Cell* **69,** 529–537.
20. Heath, W. R., Carbone, F. R., Bertolino, P., Kelly, J., Cose, S. & Miller, J. F. A. P. (1995) *Eur. J. Immunol.* **25,** 1617–1623.
21. Balomenos, D., Balderas, R. S., Mulvany, K. P., Kaye, J., Kono, D. H. & Theofilopoulos, A. N. (1995) *J. Immunol.* **155,** 3308–3312.
22. Lafaille, J., Nagashima, K., Katsuki, M. & Tonegawa, S. (1994) *Cell.* **78,** 399–408.
23. Katz, J. D., Wang, B., Haskins, K., Benoist, C. & Mathis, D. (1993) *Cell* **74,** 1089–1100.
24. Hardardottir, F., Baron, J. L. & Janeway, C. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 354–358.
25. Padovan, E., Casorati, G., Dellabona, P., Meyer, S., Brockhaus, M. & Lanzavecchia, A. (1993) *Science* **262,** 422–424.
26. Ashwell, J. D. & Klausner, R. D. (1990) *Annu. Rev. Immunol.* **8,** 139–167.
27. Sprent, J. (1994) *Cell* **76,** 315–322.
28. Paul, W. E. & Seder, R. A. (1994) *Cell* **76,** 241–251.
29. Bradley, L. M., Duncan, D. D., Yoshimoto, K. & Swain, S. L. (1993) *J. Immunol.* **150,** 3119–3130.
30. Bradley, L. M., Duncan, D. D., Tonkonogy, S. & Swain, S. L. (1991) *J. Exp. Med.* **174,** 547–559.
31. Bradley, L. M., Yoshimoto, K. & Swain, S. L. (1995) *J. Immunol.* **155,** 1713–1724.
32. Gollob, K. J. & Coffman, R. L. (1994) *J. Immunol.* **152,** 5180–5188.
33. Hsieh, C. S., Heimberger, A. B., Gold, J. S., O'Garra, A. & Murphy, K. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6065–6069.
34. Couez, D., Malissen, M., Buferne, M., Schmitt-Verhulst, A. M. & Malissen, B. (1991) *Int. Immunol.* **3,** 719–729.
35. Malissen, M., Trucy, J., Jouvin-Marche, E., Cazenave, P. A. & Scollay, R. (1992) *Immunol. Today* **13,** 315–322.
36. Macatonia, S. E., Hosken, N. A., Litton, M., Vieira, P., Hsieh, C. S., Culpepper, J. A., Trinchieri, G., Murphy, K. M. & O'Garra, A. (1995) *J. Immunol.* **154,** 5071–5079.
37. Schweighoffer, T., Tanaka, Y., Tidswell, M., Erle, D. J., Horgan, K. J., Luce, G. E., Lazarovits, A. I., Buck, D. & Shaw, S. (1993) *J. Immunol.* **151(2),** 717–29.
38. Butcher, E. C. & Picker, L. J. (1996) *Science* **272,** 62–66.
39. Poussier, P., Edouard, P., Lee, C., Binnie, M. & Julius, M. (1993) 1992. *J. Exp. Med.***176,** 187–199.
40. Mebius, R. E., Streeter, P. R., Michie, S., Butcher, E. C. & Weissman, I. L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 11019–11024.