The Differentiated State of Intestinal Lamina Propria CD4+ T Cells Results in Altered Cytokine Production, Activation Threshold, and Costimulatory Requirements

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The Differentiated State of Intestinal Lamina Propria CD4\(^+\) T Cells Results in Altered Cytokine Production, Activation Threshold, and Costimulatory Requirements\(^1\)

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Intestinal lamina propria (LP) CD4\(^+\) T cells are memory-like effector cells that proliferate at relatively low levels and require high levels of TCR signaling and costimulation for full activation in vitro. To study LP CD4\(^+\) T cell functional potential we used DO11.10 TCR transgenic (Tg) mice specific for the class II MHC-restricted OVA\(_{223–339}\) peptide and nontransgenic BALB/c mice. Activation of LP Tg\(^+\) T cells with Ag using mucosal explants induced high levels of IL-2, IL-4, and IFN-\(\gamma\). Culturing isolated LP cells with IL-12 enhanced IFN-\(\gamma\) production and down-regulated IL-4 and IL-2, whereas addition of IL-4 maintained IL-4 production without inhibiting IFN-\(\gamma\) production. Systemic administration of relatively high dose (HD; 100 nM) OVA\(_{223–339}\) peptide induced similar levels of bromodeoxyuridine (BrdU) incorporation by LP and splenic Tg\(^+\) T cells in vivo, whereas low dose (LD; 4.5 nM) peptide injections induced 4-fold greater levels of BrdU incorporation for LP compared with splenic Tg\(^+\) T cells. Coadministration of CTLA-4Ig reduced BrdU incorporation for splenic cells by 70% with HD and LD stimulation, but had little effect on LP responses to HD stimulation. Results of in vivo studies were confirmed in nontransgenic BALB/c mice using HD (200 \(\mu\)g) and LD (10 \(\mu\)g) anti-CD3 mAb+/− CTLA-4Ig. These results suggest that LP T cells are differentiated effector cells that respond at high levels when activated with relatively low levels of Ag- and B7-mediated costimulation in vivo. The reduced activation threshold of LP T cells may facilitate responses to low levels of Ag derived from mucosal pathogens. The Journal of Immunology, 1999, 163: 5937–5945.

1 The primary function of CD4\(^+\) T lymphocytes localized to the intestinal lamina propria (LP)\(^5\) is to participate in protective immune responses that maintain integrity of the mucosal barrier (1). Lamina propria CD4\(^+\) T cells promote Th1-like immune responses against intracellular pathogens and support Th2-like humoral responses against extracellular pathogens by providing help to mucosal B cells and plasmablasts (2). Most LP T cells express an activated helper-inducer phenotype. Flow cytometric analysis has shown that the majority of CD4\(^+\) LP T cells are negative for expression of L-selectin (3, 4). Furthermore, between 66–96% of LP lymphocytes express the CD45RO isoform of the CD45 molecule associated with previously activated, memory-like T cells (5–8). However, LP T cells do not express other surface markers typical of resting memory cells. In fact, expression of the early activation markers CD25 (IL-2R), CD69, the 4F2 lymphocyte activation Ag, and transferrin receptors suggest that CD4\(^+\) LP T cells are chronically stimulated (9). In vitro mitogenic activation of LP T cells with CD28 or CD40-mediated costimulation induces low proliferative responses with relatively high production of IFN-\(\gamma\), IL-4, and IL-5 (5, 9–12). Taken together, these findings suggest that the activation state of LP CD4\(^+\) Th cells alters their functional responses to local Ags.

Repeated stimulation induces the transition of naive CD4\(^+\) T helper precursors to mature, activated Th cells that mediate secondary immune responses (13–16). Ag–primed subsets of Th cells have been referred to as effector T cells by analogy to CD8\(^+\) cytolytic T lymphocytes (13, 14, 17, 18). These Th cell subsets express an activated surface phenotype, secrete high titers of a wide range of cytokines (IL-2, IL-4, and IFN-\(\gamma\)), and can mediate helper activity for B cells (19–21). Earlier work has also demonstrated that previously activated CD4\(^+\) T cells required lower levels of costimulation and Ag compared with naive subsets (22, 23). These studies showed that naive T cells failed to produce cytokine when activated with low concentrations of Ag or mitogen or with low costimulation-competent APC such as resting B cells. In contrast, previously activated effector populations produced higher levels of cytokine at low Ag concentrations and could use a wide variety of APC populations, including resting B cells. Together, these data suggest that effector T cells may be distinguished from naive T cells by the requirement for lower levels of activation to induce functional responses. Observations that Ag experience reduces the activation threshold of CD4\(^+\) effector T cells have not been supported by studies of LP T cell activation. Results of in vivo studies

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\(^7\) Abbreviations used in this paper: LP, lamina propria; PP, Peyer’s patch; MLN, mesenteric lymph node; Tg, transgenic; BrdU, bromodeoxyuridine; FCM, flow cytometry; HD, high dose; LD, low dose; MBP, myelin basic protein; NCS, normal calf serum.
suggest that LP T cells require relatively high levels of TCR-mediated and costimulatory signaling to induce responses (5, 9, 10, 12, 24, 25). Thus, studies of LP CD4⁺ T cell responses have not agreed with previous models of memory-like effector T cell populations generated from naive precursors.

To study the functional properties of LP CD4⁺ T cells in an Ag-specific model we used DO11.10 TCR transgenic (Tg) mice specific for the class II MHC-restricted OVA232–339 peptide (26). Previous studies from our laboratory suggested that Tg⁺ T cells in the intestinal LP of DO11.10 mice were activated by environmental Ag through endogenous TCRs (27). Activation of LP Tg⁺ T cells via endogenous TCRs led to the generation of a population of effector CD4⁺ T cells that expressed surface and functional phenotypes typical of CD4⁺ LP T cells in normal murine and human systems (1, 3, 4, 6–12, 25, 28). However, expression of Ag-specific TCR by LP Tg⁺ T cells allowed us to examine the effects of antigenic stimulation on an activated population of LP T cells. The current results show that CD4⁺ Tg⁺ LP T cells produce IL-4 and IFN-γ at relatively high levels and are resistant to additional differentiation in vitro. These results are consistent with those of Sarparov et al. (29), who detected relatively high numbers of Tg⁺ LP T cells in DO11.10 mice positive for IFN-γ or IL-10 using single-cell in situ hybridization. Furthermore, our studies of T cell responses in vivo using BrdU incorporation as a marker of activation suggest that LP cells are more responsive to low levels of TCR signaling and B7-mediated costimulation than are naive CD4⁺ splenic T cells. Collectively, these findings suggest that previous activation of CD4⁺ LP Th cells lowers the activation threshold and increases both expansion as well as cytokine production induced by antigenic stimulation.

**Materials and Methods**

**Animals**

BALB/c mice were obtained from the National Cancer Institute (Frederick, MD). DO11.10 Tg⁺ mice (a gift from Dr. Dennis Loh, Nippon Research Center, Kamakura, Japan) 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 for more than six generations, and the progeny was screened for expression of the TCR transgene by flow cytometry (FCM) using the clonotypic mAb KJ1-26.1 (27). DO11.10 Tg mice were between 5 and 8 wk of age.

**Cell isolation**

Spleenic cells were isolated as previously described (30). Briefly, spleens were mechanically dissociated and RBC lysed in ACK lysis buffer. Cell suspensions were washed and stored in DMEM with 10% FCS (10% DMEM) on ice until used. Cell preparations were purified by mAb and complement lysis as described above. Lamina propria lymphocytes were isolated by density centrifugation over Histopaque-1077 (Sigma), collected from the interface, and washed before culture. Lamina propria lymphocytes were isolated by collagenase digestion as described above and enriched by density centrifugation over Histopaque-1077 (Sigma), collected from the interface, and washed before culture. Lamina propria lymphocytes were isolated by collagenase digestion as described above and enriched by density centrifugation, layering the single-cell suspension over 30% Percoll (Pharmacia, Piscataway, NJ) for a total of 72 h with stimuli added at culture initiation. To measure proliferation, [3H]thymidine incorporation was measured in harvested cells using a scintillation counter (Wallac, Gaithersburg, MD).

**Cell proliferation**

For proliferation studies, spleens were teased apart, digested with collagenase (400 U/ml; Roche Molecular Biochemicals) for 2 h and then filtered through a 100-μm pore size nylon mesh (Falcon, Franklin Lakes, NJ) to yield a single-cell suspension. Lymphocytes were isolated by density centrifugation over Histopaque-1077 (Sigma), collected from the interface, and washed before culture. Lamina propria lymphocytes were isolated by collagenase digestion as described above and enriched by density centrifugation, layering the single-cell suspension over 30% Percoll (Pharmacia Biotech, Piscataway, NJ). Splenic or LP mononuclear cells (1 × 10⁶ cells/well) were activated with APC and Ag alone or with exogenous IL-4 (Genzyme, Cambridge, MA) at 100 U/ml or IL-12 (a gift from Dr. Frank Fitch) at 5 U/ml for the first round of stimulation only. In some cultures the rat isotype control (anti-β-galactosidase) or neutralizing anti-IFN-γ mAb XMG-1.2 was added (10 ng/ml). The effects of cytokine added to initial cultures were determined by assessing cytokine levels 48 h after the first round of stimulation in the absence of exogenous cytokine.

**In situ LP culture**

Excised small bowel from immunized Tg⁺ mice was flushed with cold PBS and opened longitudinally. Uniform tissue samples (28 mm) (26) were isolated using a sterile, 6-mm diameter boring tool (Baxter Healthcare Corp., McGaw Park, IL), washed with PBS, and incubated with EDTA and 10% NCS in PBS for 30 min. Tissue explants were then washed in medium and cultured with OVA232–339 peptide (300 pM).

**Cytokine analysis**

To assess cytokine production for isolated cells, 5 × 10⁴ T cells were cocultured in 24-well plates with 2.5 × 10⁵ irradiated APC and Ag. Unless otherwise indicated, supernatants were collected at 48 h and were analyzed for IL-2, IL-4, and IFN-γ by ELISA. The ELISAs for IL-2, IL-4, IL-10, and IFN-γ were performed according to manufacturer’s specification (Endogen, Cambridge, MA). Wells were developed with ABTS soluble substrate (Zymed, South San Francisco, CA) and read at 415 nm on a spectrophotometer. Cytokine levels are presented as units per milliliter according to previously described conversion values where 1 U IL-2 = 14.2 pg; 1 U IL-4 = 0.7 pg; 1 U IL-10 = 2 pg, and 1 U IFN-γ = 100 pg (33). The limits of detection for these ELISAs were as follows: IL-2, 0.2 U; IL-4, 4 U; IL-10, 1 U; and IFN-γ, 0.5 U.
FIGURE 1. Mucosal explants produce cytokine in response to Ag in vitro. Mucosal tissue samples of equal size (28 mm²) (26) were incubated with EDTA to remove the epithelial cell layer as described in Materials and Methods. Explants were then cultured in medium alone or with OVA323-339 peptide (300 pM). Cytokine production was determined by ELISA at 48 h and is presented as units per milliliter. Data shown are representative of three experiments.

Bromodeoxyuridine incorporation analysis

Bromodeoxyuridine analysis was achieved by variations of protocols first presented by Carayon and Bord (34) and Toug and Sprent (35). Mice were given 200-μl i.p. injections of sterile, endotoxin-free PBS diluted with control myelin basic protein (MBP), OVA323-339 peptide, control hamster IgG (PharMingen, San Diego, CA), or anti-CD3 (2C11, PharMingen) at the amounts indicated. In some experiments mice were given i.p. injections of 0.5 mg of murine control-1g or CTLA-4 Ig 12 h before i.p. OVA323-339 or anti-CD3. Twelve hours before sacrifice, mice were given 1 mg of BrdU (Sigma) in 200 μl of sterile PBS i.p. This 12 h point was used to limit the background and maximize the signal observed in previously published methodologies. At sacrifice, lymphocytes were prepared as previously described (27). Cells were stained with anti-CD4-PE (PharMingen) and KJl-26.1-BIO (anti-Tg TCR, a gift from Dr. Phillipa Marrack, National Jewish, Denver, CO). Biotinylated KJl-26.1 was visualized with strepavidin-CyChrome (PharMingen). Cells were then fixed overnight in 1% electron microscopy grade parafomaldehyde (Polysciences, Warrington, PA) in PBS with 0.2% Tween-20 (Sigma), washed with cold PBS, and resuspended in 500 μl of Mg-Ca DNase buffer (5 mM MgCl₂ and 5 mM CaCl₂ in 1

Results

Stimulation of cytokine production in mucosal explants

Results of previous studies suggested that IL-4 and IFN-γ cytokine production had been induced in LP T cells as a consequence of activation in the intestinal microenvironment (27). However, in that study LP Tg⁺ T cell cytokine production was assessed using splenic APC and OVA323-339 peptide. To assess cytokine production in response to Ag presented locally by mucosal APC we used an in situ explant system. After removal of the epithelial layer with EDTA digestion, uniformly small intestinal tissue samples were incubated in 24-well plates with control or OVA323-339 peptide (300 pM) and assayed for cytokine production at 24 h. This culture system preserved the normal microenvironment of LP APC and Tg⁺ T cells. As shown in Fig. 1, incubation with Ag stimulated the production of high amounts of IL-2, IL-4, and IFN-γ similar to levels we had previously observed using purified LP T cells (27), while cytokine production was not detected in control wells. These data suggest that cytokines produced by isolated LP Tg⁺ T cells with splenic APC and Ag reflect the cytokine profile produced by LP T cells in response to Ag and local APC.

Lamina propria Tg⁺ T cells resist Th2 differentiation to exogenous rIL-4 by maintaining IFN-γ but down-regulating IL-2 production

Based on studies by Bucy et al. (36, 37) as well as our own intracellular cytokine staining (data not shown) we suspected that activated Tg⁺ T cells in the LP were a mixed population of Th1- and Th2-like cells. Reports by Murphy and colleagues (38) and others have suggested that Th1 and Th2 cells exhibit distinct lineage commitment. In these studies the characteristic cytokine profile of Th2 cells was more stable than that of Th1 cells with repeated stimulation in the presence of differentiating cytokine in vitro. To address the lineage commitment of LP cells, freshly isolated splenic and LP T cells from DO11.10 mice were cultured with APC, OVA323-339 peptide, rIL-4, OVA323-339 peptide and rIL-12, or medium alone. After initial activation with OVA323-339 peptide and exogenous cytokine, the cells were stimulated twice without exogenous cytokine for a total of three stimulations in vitro. Following the tertiary stimulation, the cultures were activated with OVA323-339 peptide and splenic APC without exogenous cytokine to determine the cytokine production profile. Fig. 2 shows that, as we had previously described, splenic Tg⁺ T cells produced relatively high levels of IL-2 and low levels of IFN-γ and IL-4 upon primary stimulation. Addition of rIL-12 to splenic Tg⁺ T cell cultures promoted a Th1 profile of cytokines with high levels of IL-2 and IFN-γ without IL-4. In contrast, addition of exogenous rIL-4 induced a Th2 profile with high IL-4 and reduced IL-2 and IFN-γ. Activation of LP Tg⁺ T cells cultured with rIL-12 up-regulated the production of IFN-γ and down-regulated IL-4 production compared with the primary stimulation. However, very little IL-2 or IL-4 was produced from the rIL-12-stimulated LP T cell cultures, suggesting that IL-12 and IFN-γ had effectively suppressed IL-4 production. Addition of IL-2 during initial cultures failed to reverse the trend toward diminished IL-2 production in later passages (data not shown), suggesting that consumption of IL-2 did not account for the extinction of IL-2 production detected in LP cultures. The down-regulation of IL-2 by rIL-12 observed for LP, but not for spleen, cells suggested that previous activation of LP T cells in vivo may have predisposed this population to down-regulate IL-2 synthesis. Culture with exogenous rIL-4 induced high IL-4 production by Tg⁺ LP cells, but a lower level than that of rIL-4- treated splenic Tg⁺ T cells. In contrast to splenic Tg⁺ T cells, LP Tg⁺ T cells continued to produce high levels of IFN-γ upon addition of rIL-4. LP Tg⁺ T cell IFN-γ production was, in fact, up-regulated from levels observed in primary cultures and suggested that rIL-4 did not have effectively suppressed IFN-γ production, as had occurred in splenic cultures. We also found that addition of neutralizing anti-IFN-γ (XMG-1.2) mAb to initial cultures failed to reduce subsequent IFN-γ production in IL-4-treated wells of LP T cells (data not shown). These data indicate that LP Tg⁺ T cells resist the effects of exogenous rIL-4 on IFN-γ production. However, in response to rIL-12, Tg⁺ cells uniformly differentiated into a phenotype dominated by high IFN-γ and low IL-2 and IL-4 production. These findings are consistent with data reported by Saparov et al. (29), who used single-label in situ hybridization of cytokine mRNA to show that Th1, but not Th2, LP T cells are resistant to phenotype reversal in vitro. In this report the authors also detected IL-10 and IL-5 production by LP cells, which was detected at low levels in our studies (data not shown). Differences in the detection of IL-10 and IL-5 may have been due to differences in culture conditions and/or sensitivities of the cytokine assays. Taken together, these data were consistent with the hypothesis that the induction of Th1 cytokines produced by LP Tg⁺ T cells is resistant to suppression by Th2-promoting cytokines and
may explain why some cytokine knockout animal models that favor emergence of Th1 cells lead to chronic inflammation in the intestine.

Proliferative responses of Tg\(^+\) LP T cells to Ag and anti-CD28 mAb

Previous publications had reported that LP T cells were hypoproliferative in response to activation in vitro (11) and needed high levels of stimulation via TCR and CD28 pathways for optimal responses (5). To compare T cell responses in DO11.10 mice to previously published data in nontransgenic systems, proliferative responses were measured for splenic and LP lymphocytes stimulated by Con A, anti-CD3 mAb, and OVA\(_{323-339}\) peptide/anti-CD28 mAb. The results presented in Fig. 3 indicate that DO11.10 LP cells proliferated at greatly reduced rates compared with splenocytes for all stimuli studied. Proliferative responses induced by OVA\(_{323-339}\) peptide and anti-CD3 mAb were similar in LP cultures, whereas splenic proliferative responses were greatest in cultures activated with OVA\(_{323-339}\) peptide. Although LP T cells proliferated at relatively low levels in all cultures, the provision of anti-CD28 mAb tended to enhance responses regardless of the stimulus. A more clear positive effect of anti-CD28 was seen in splenic cultures activated with anti-CD3 or OVA\(_{323-339}\) peptide. CD28 stimulation alone does not promote proliferation (data not shown). Thus, Tg\(^+\) LP T cells were hypoproliferative and dependent on anti-CD28 for optimal responses in vitro. These findings confirmed the idea that Tg\(^+\) LP cells in DO11.10 mice responded in a similar pattern as LP cells in nontransgenic mice as well as primate and human models (5, 10, 12, 39).

Decreased activation threshold for LP T cells

T cells may encounter intestinal Ag in vivo at concentrations far below that which is optimal for complete activation. Since previous work suggested that activated CD4\(^+\) T cells from peripheral lymphoid tissue respond to lower levels of Ag than naive T cells (17, 18, 23), we examined the differential sensitivities of LP and splenic T cells to a range of OVA\(_{323-339}\) peptide concentrations. At optimal levels of OVA\(_{323-339}\) (300 pM), IL-2 production was greater for splenic compared with LP T cells. However, at suboptimal Ag concentrations from 3–30 pM (Fig. 4), LP T cells produced greater levels of IL-2 compared with splenic T cells. In fact, at Ag levels 10-fold below the concentration used to maximally stimulate IL-2 production, LP Tg\(^+\) Tg\(^+\) T cells produced 5- to 20-fold the amount of IL-2 secreted by splenic Tg\(^+\) T cells. These results suggest that LP Tg\(^+\) T cells required a lower threshold of TCR-mediated activation to induce functional responses.

To test the responses of LP cells to high and low levels of Ag in vivo, we assessed BrdU incorporation in mice given i.p. injections of a range of OVA\(_{323-339}\) peptide dosages. The percentages of BrdU\(^+\) Tg\(^+\) Tg\(^+\) T cells detected in at least three separate experiments were analyzed at each dosage level and reported as the mean ± SEM. As shown in Fig. 5, high doses of systemic OVA\(_{323-339}\) peptide induced BrdU incorporation in relatively high proportions of Tg\(^+\) T cells recovered from both LP and splenic compartments. However, at the four lowest doses of OVA\(_{323-339}\) peptide examined (<40 nmol), the proportions of BrdU\(^+\) Tg\(^+\) Tg\(^+\) CD4\(^+\) T cells were significantly higher for LP compared with splenic populations. At the lowest level at which splenic Tg\(^+\) T
cell responses were detected (4.5 nmol), the proportion of LP BrdU$^+$ Tg$^+$ CD4$^+$ T cells was more than twice that of splenic cells. These in vivo data parallel IL-2 production observed in vitro (Fig. 4) and suggest that compared with resting, naive splenic CD4$^+$ T cell populations, LP CD4$^+$ T cells require a lower activation threshold to induce cytokine production and proliferation.

**Dependence of LP Tg$^+$ T cell cytokine production and in vivo proliferation upon B7-1/2-mediated costimulation**

Lamina propria T cells may encounter Ag presented by professional APC such as resident macrophages and dendritic cells as well as nonprofessional, costimulation-deficient APC such as naive B cells and epithelial cells (1, 11, 40–43). Previous studies, however, have reported that LP T cells are highly dependent on costimulation for activation (5, 24, 39). Since these systems used mitogen-induced, non-Ag-mediated systems for LP T cell activation, we assessed the role of B7-1/2-mediated costimulation in TCR-OVA Ag-mediated stimulation of LP Tg$^+$ T cells.

**FIGURE 3.** Effect of CD28 costimulation on proliferation of Tg$^+$ lymphocytes. Splenic and LP mononuclear cells were activated with anti-CD3 Ab (0.5 µg/ml) or OVA$_{323-339}$ peptide (0.5 µM) in the presence or the absence of a stimulatory anti-CD28 Ab (0.5 µg/ml). Concanavalin A (5 µg/ml) served as a positive control. Proliferation was measured using $[^{3}H]$thymidine incorporation as described in Materials and Methods and is presented as the mean ± SEM. Data are representative of four experiments.

**FIGURE 4.** Sensitivity of Tg$^+$ T cells to low levels of Ag. Purified splenic and LP Tg$^+$ T cells were activated with splenic APC and various concentrations of OVA$_{323-339}$ peptide as indicated. IL-2 production at 24 h was determined by ELISA. Data shown are representative of three experiments.

**FIGURE 5.** Reduction in the activation threshold of LP T cells in vivo. In vivo proliferation (BrdU incorporation) of Tg$^+$ T cells following administration of low amounts of Ag. Mice were given a range of OVA$_{323-339}$ peptide in decreasing amounts in PBS by i.p. injection. Thirty-six hours after OVA$_{323-339}$ peptide administration and 12 h before sacrifice, mice were pulsed with 1 mg of BrdU in PBS i.p. 12 h before sacrifice. Cell samples were stained for CD4 and KJ1-26 and intracellular BrdU and analyzed by flow cytometry. Data presented are the percentages of BrdU$^+$ Tg$^+$ T cells within a CD4$^+$ gate. Each point represents the mean of at least three experiments ± SEM. Control levels (dashed line) indicate the BrdU incorporation for Tg$^+$ CD4$^+$ T cells in mice given control (MBP) peptide with SEM ± 2%. *, p < 0.05 for differences between LP and spleen cells tested with paired two-tailed Student’s t test.
Cytokine levels were measured in splenic and LP cell cultures activated in the presence of murine control-Ig or CTLA-4Ig. CTLA-4Ig binds to B7-1 and B7-2 and blocks CD28 and CTLA-4 ligation (32). The results in Fig. 6 indicate that CTLA-4Ig reduced IL-2 levels for both splenic and LP populations by >90% with minimal effect on IFN-γ production. Addition of CTLA-4Ig reduced IL-4 production for splenic populations by >50%, but reduced LP IL-4 levels by about 5%. Thus, the production of both IL-4 and IFN-γ effector cytokines by LP Tg+ T cells was not significantly affected by inhibition of B7-mediated costimulation.

As T cell activation in vivo may be induced with suboptimal levels of Ag, we examined the effect of blocking costimulation in vivo using high dose (HD) and low dose (LD) OVA323-339 peptide in DO11.10 mice (Figs. 7A and 8A). CTLA-4Ig was used to assess the contribution of B7-1/B7-2 ligands to T cell responses to Ag stimulation in vivo. DO11.10 mice were administered either HD (100 nmol) or LD (4.5 nmol) of OVA323-339 peptide in PBS. Representative flow cytometric data shown in Fig. 8B indicate that the percentages of BrdU+ Tg+ T cells in mice given HD and LD control peptide (MBP) were relatively low for both LP and splenic cultures (1–3%). T cells were stimulated with or without CTLA-4Ig (0.2 mg) followed by injections of BrdU (1 mg) to label responding T cells. The results presented in Figs. 7A and 8B indicate that in response to LD OVA323-339 peptide administration, CTLA-4Ig reduced the proportion of BrdU+ Tg+ T cells in the spleen by 70% (p < 0.05) and 55% (p < 0.01) in the LP. Administration of CTLA-4Ig in combination with high dose OVA323-339 peptide also reduced the proportion of BrdU+ Tg+ T cells in the spleen by 66% (p < 0.01) vs OVA323-339 peptide alone. However, the effect of CTLA-4Ig on high dose OVA323-339 peptide stimulation was negligible (49% compared with 52%; nonsignificant) in the LP. Taken together, these data indicate that LP Tg+ T cells respond optimally in the absence of costimulation when activated with relatively high levels of TCR-mediated signaling. However, at suboptimal levels of Ag, costimulation is needed for complete activation.

Observation of in vivo T cell responses in DO11.10 mice suggested that LP cells responded better to LD stimulation than spleen and were less dependent on CD28-mediated costimulation with HD stimulation. To test whether these findings could be generalized to nontransgenic systems, BrdU incorporation was assessed in Thy-1 gated splenic and CD4+ LP T cells in normal BALB/c mice. BALB/c mice were injected with LD (10 μg) or HD (200 μg) anti-CD3 mAb (2C11) and sacrificed 48 h later (Figs. 7B and 8B). These data confirm results in DO11.10 mice and show that nontransgenic CD4+ LP cells respond nearly twice as well to LD stimulation as splenic cells. As in DO11.10 mice, CTLA-4Ig treatments reduced splenic CD4+ T cell responses to HD and LD stimulations, whereas no effect was detected on LP T cell responses activated with HD anti-CD3. CTLA-4Ig reduced LP T cell responses to LD stimulation by nearly 50%. These data suggest that the contribution of costimulation to LP CD4+ T cell activation was greater at lower levels of TCR signaling. Interestingly, CTLA-4Ig had little effect on splenic and LP CD8+ (Thy-1+, CD4−) T cell responses to HD stimulation. These data are consistent with previous observations (44) and suggest that CD8+ T cells are less dependent on costimulation than naive CD4+ T cells. Responses to control hamster mAb injections in BALB/c mice were similar to levels observed in DO11.10 mice given control (MBP) peptide (data not shown). Taken together these data confirm findings in
### Discussion

These results support the view that prior activation of LP CD4⁺ T cells enhances the efficiency of effector immune responses in the intestine. In previous studies we reported that increased cytokine production correlated with expression of an activated profile of surface markers for Tg⁺ LP T cells in DO11.10 mice (27). Enhanced production of both IL-4 and IFN-γ by LP T cells compared with that by splenic T cells suggests that previous Ag experience in the intestine promotes both cellular as well as humoral effector responses to subsequent stimulations. Production of IFN-γ enhances class I and II MHC expression, up-regulates inducible nitric oxide synthase, and up-regulates microbial activities of macrophages (42, 45–47). These effects enhance responses to intracellular enteric pathogens. The dominance of IFN-γ responses for LP T cells was evidenced by the production of 35-fold greater levels of IFN-γ by LP compared with splenic cells (27). Given the high levels of IFN-γ produced by LP T cells one might expect that IL-4 production would be suppressed. However, relatively high levels of IL-4 were detected for freshly isolated LP cells activated in cultures as well as in mucosal explants. In studies by Saparov et al. (29), single-cell in situ hybridization was used to show that relatively high numbers of IL-10-positive CD4⁺ T cells were present in the LP. Thus, it is likely that mechanisms in the mucosa operate to support the coexistence of IFN-γ as well as IL-4 and IL-10 production. One possibility, suggested by Rennick and colleagues, is that endotoxin up-regulates IFN-γ and IL-12, whereas local APC as well as T cells produce IL-10 that impairs the effects of IFN-γ and IL-12 on Th cell differentiation (48, 49). Without IL-10, as in IL-10 knockout mice, the unopposed induction of Th1 responses leads to autoaggressive intestinal inflammation. Thus, our data suggest that activation of CD4⁺ T cells in the LP promotes the induction of helper cytokines that support effector immune responses as well as regulate the balance between Th1 and Th2 cytokines.

The results of in vivo studies suggest that the threshold of Ag- and B7/CD28-mediated costimulation is reduced for CD4⁺ LP T cells compared with that for naive splenic T cells. These findings parallel those of Swain and colleagues, who described the properties of an activated, memory-like Th cell population that made increased levels of cytokine, provided B cell help, and was less dependent on costimulation for activation compared to naive T cells (23, 50). Our in vitro results extend these findings by demonstrating that CTLA-4Ig had little effect on IL-4 and IFN-γ production. One possibility, suggested by Rennick and colleagues, is that endotoxin up-regulates IFN-γ and IL-12, whereas local APC as well as T cells produce IL-10 that impairs the effects of IFN-γ and IL-12 on Th cell differentiation (48, 49). Without IL-10, as in IL-10 knockout mice, the unopposed induction of Th1 responses leads to autoaggressive intestinal inflammation. Thus, our data suggest that activation of CD4⁺ T cells in the LP promotes the induction of helper cytokines that support effector immune responses as well as regulate the balance between Th1 and Th2 cytokines.

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The results of robust proliferative responses for LP T cells measured in vivo challenge current paradigms of mucosal TH cell activation. Our results suggest that LP T cells respond as well or better than splenic TH cells to in vivo antigenic stimulation. These results contrast observations of hypoproliferative responses detected in vitro for human (5, 10, 16) and murine LP T cells (Fig. 3). Low rates of proliferation reported in previous studies may be related to the relatively high level of apoptosis detected for LP cells activated
in vitro (51, 52). However, we found similar rates and kinetics of T cell apoptosis for LP and spleen in DO11.10 mice activated with OVA323-339 peptide in vivo (based on TUNEL staining of tissue sections; data not shown). Taken together these results suggest that factors present in the tissue enhance LP T cell proliferative responses and survival. Signals delivered from local APC through adhesion molecules (e.g., LFA-1) and costimulatory ligands (CD28, CD40) may reduce LP T cell activation requirements and cell death. It is also possible that soluble factors (e.g., IL-15, IL-7, IL-2, and IL-4) in the LP enhance expansion, lower the activation threshold, and block cell death pathways (53, 54). Taken together, the results of in vivo LP T cell activation suggest that elements of the local microenvironment contribute to potentiating mucosal T cell immune responses.

These results suggest that LP CD4\(^+\) T cells are a unique population of preactivated memory-like cells with the potential to deliver Th1- and Th2-like effector functions. We suspect that both prior activation as well as the mucosal environment combine to increase the level of responses while lowering the threshold of TCR signaling needed for complete stimulation. The relatively high level of cytokines produced by LP T cells may be required to drive highly potent mucosal responses against the type of pathogens encountered at this surface. The reduced activation threshold for stimulation of LP T cells could allow responses to be initiated to relatively low levels of enteric pathogens present early in local infections. Thus, these results suggest that LP CD4\(^+\) T cells possess a unique set of properties needed to rapidly initiate responses to invasion by life-threatening enteric organisms.

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References


