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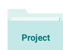

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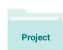

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Defects in CD8$^+$ Regulatory T Cells in the Lamina Propria of Patients with Inflammatory Bowel Disease[1]

Jens Brimnes, Matthieu Allez, Iris Dotan, Ling Shao, Atsushi Nakazawa, and Lloyd Mayer[2]

Mucosal tolerance is believed to be partly mediated by regulatory T cells. Intestinal epithelial cells (IECs) may play an important role in the generation of such regulatory cells, because they are able to process and present Ag to T cells. Furthermore, we have previously demonstrated that IECs are able to generate regulatory CD8$^+$ T cells in vitro. In the present study, we have analyzed lamina propria (LP) lymphocytes for the presence of such regulatory CD8$^+$ T cells in normal individuals as well as in patients with inflammatory bowel disease (IBD). The results of the present study show that LP CD8$^+$ T cells derived from normal controls possess regulatory activity, whereas both unfractionated LP lymphocytes and purified LP CD4$^+$ T cells do not. The LP CD8$^+$ T cells suppress Ig production by pokeweed mitogen-stimulated PBMCs by 31–80%, in a cell contact-dependent manner. No significant difference in suppression between CD28$^+$ and CD28$^-$CD8$^+$ LP T cells was observed. In contrast to CD8$^+$ T cells from normal LP, CD8$^+$ T cells isolated from LP of IBD patients, did not suppress Ig production by pokeweed mitogen-stimulated PBMC (five of six ulcerative colitis specimens; six of six Crohn's disease specimens). Furthermore, we demonstrate that the frequency of TCR Vβ5.1-positive CD8$^+$ T cells, which we previously have demonstrated to be regulatory and to be expanded by IECs in vitro, is decreased in IBD LP compared with normal LP. In conclusion, this study demonstrates that CD8$^+$ T cells with regulatory activity are present in the LP of normal healthy individuals, but not in patients with IBD, suggesting that these cells might play an active role in mucosal tolerance. *The Journal of Immunology,* **2005, 174: 5814–5822.**

The intestine harbors a complex microflora composed of a large variety of indigenous aerobic and anaerobic bacteria (1). These bacteria are in close proximity to a vast number of intestinal lymphocytes, yet it is unclear how the mucosal immune system is able to mount a rapid and effective response against pathogenic bacteria, viruses, and parasites, while remaining tolerant to the resident enteric microflora and dietary Ags. The importance of maintaining this tolerance is illustrated in inflammatory bowel diseases (IBD),[3] because these diseases are believed to be the result of a breakdown of tolerance to resident enteric bacteria (2, 3).

Accumulated data suggest that regulatory T cells play a central role in maintaining tolerance in the mucosal immune system (4). Several subsets of both CD4$^+$ and CD8$^+$ T cells have been shown to act as regulatory cells. In both humans and rodents, it has been demonstrated that naive CD4$^+$CD25$^+$ T cells possess regulatory activity (5–7). The CD4$^+$CD25$^+$ cells are anergic, but after TCR triggering, these cells are able to inhibit immune responses both in vitro and in vivo (5, 8, 9). In vitro, the suppression seems to be mediated via cell contact-dependent mechanisms (5, 10–12), whereas in vivo studies suggest that cytokines such as IL-10 and TGF-β might play a role (13, 14). Other subsets of murine CD4$^+$ regulatory T cells, such as the Th3 cells generated during low-dose oral tolerance regimens and the in vitro-generated Tr1 cells, also seem to act via secretion of regulatory cytokines (15, 16). Although the field of regulatory cells has been focused on CD4$^+$ T cells, regulatory CD8$^+$ T cells have been described in oral tolerance (17, 18), and recently a population of human regulatory cells, characterized by their CD8$^+$CD28$^-$ phenotype, has also been described (19, 20).

In the context of the mucosal immune system, regulatory cells have primarily been demonstrated in rodents. Both the above-mentioned Tr1 cells and the CD4$^+$CD25$^+$ T cells from normal mice have been shown to be capable of preventing the development of experimental colitis in SCID mice (14, 16, 21). Recently, it has also been demonstrated that CD4$^+$ T cells with regulatory activity can be isolated from the intestinal lamina propria (LP) of normal mice (22, 23). The induction of low-dose oral tolerance mediated by the above-mentioned Th3 cells is another example of the generation of regulatory cells in the intestinal mucosa.

Little is known about the mechanisms by which regulatory cells are generated in vivo in the mucosa. It has been suggested that immature dendritic cells located in the subepithelial dome region of the Peyer's patch are able to induce regulatory T cells (24, 25). However, intestinal epithelial cells (IECs) may also play an important role in the generation of regulatory cells, because these cells are able to process and present luminal Ags to T cells in the LP (26–28). Using an allogeneic in vitro coculture system, we have previously demonstrated that interactions between IECs and peripheral blood T cells (PBTs) leads to the preferential expansion of CD8$^+$CD28$^-$ T cells with regulatory activity (27, 29). We have recently shown that a subset of the expanding CD8$^+$ T cells is characterized by the biased usage of the TCR Vβ5.1 chain.[4] The

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[3] Abbreviations used in this paper: IBD, inflammatory bowel disease; LP, lamina propria; IEC, intestinal epithelial cell; UC, ulcerative colitis; CD, Crohn's disease; LPMC, LP mononuclear cell; PWM, pokeweed mitogen; PBT, peripheral blood T cell.

[4] M. Allez., J. Brimnes, L. Shao, L. Dotan, A. Nakazawa, and L. Mayer. Mucosal expression of CD8$^+$ T regulatory cells by CD1d and a CEA family member. *Submitted for publication.*

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expansion of these regulatory cells is dependent on the expression of the nonclassical MHC class I molecule, CD1d, and the carcinoembryonic Ag subfamily member, gp180, on the surface of the IEC (29–32). We have termed these cells TrE cells. In contrast to normal IECs, IECs from patients with IBD have an aberrant expression of gp180 and cannot expand these regulatory $CD8^+$ T cells in vitro (33) (I. Dotan, manuscript in preparation). Taken together, these data suggest that defects, which interfere with the normal IEC-mediated generation or expansion of regulatory $CD8^+$ TrE cells, may play a role in the pathogenesis of IBD.

In the present work, we have investigated whether such regulatory $CD8^+$ TrE cells are present in vivo in the LP. Our results show that $CD8^+$ T cells with regulatory activity are present in the LP of healthy individuals, but not in the LP of patients with IBD, suggesting that these cells—activated by IECs—might play a central role in maintaining controlled inflammation in the intestinal mucosa.

Materials and Methods

Patients and tissues

Surgical specimens from patients undergoing bowel resection for cancer or IBD at the Mount Sinai Medical Center were used as the source for IECs and LP mononuclear cells (LPMCs). Colonic tissue was always used. The group termed "normals" consisted of patients undergoing bowel resection for cancer, noncancerous polyps, or diverticulitis. In this group, cells were always isolated from normal tissue >10 cm from the tumor. The IBD patient group consisted of both ulcerative colitis (UC) and Crohn's disease (CD) patients with moderate-to-severe colitis. The characteristics of these patients are listed in Table I.

Isolation of IECs and LPMCs

IECs were isolated as described earlier (27). In brief, colon specimens were washed extensively in PBS, followed by stripping of the mucosa from the underlying submucosa. The mucosa was cut into small pieces and treated with 1 mM DTT (Sigma-Aldrich) for 15 min to remove mucus. The tissue was then washed in PBS and incubated twice for 30 min in medium containing 3 mg/ml Dispase II (Roche Diagnostics) at 37°C, 5% CO_2. The supernatant (released IECs) was collected and washed in medium twice. The viability of the isolated IECs was >95%.

LPMCs were isolated from the tissue remaining after the dispase treatments. The tissue was incubated for 1 h at 37°C in medium containing 1 mg/ml collagenase (clostridiopeptidase A) and 5 μg/ml DNase I (both Sigma-Aldrich). The supernatant was collected and centrifuged on a Percoll (Amersham Biosciences) density gradient and the LPMCs were harvested from the 40–60% interface. Viability was >90%.

LP $CD4^+$ and $CD8^+$ T cells were purified after culturing the isolated LPMC with 20 ng/ml IL-15 overnight followed by staining with allophycocyanin-conjugated anti-CD3, PE-conjugated anti-CD4, and FITC-conjugated anti-CD8 mAbs (all BD Pharmingen). The cells were then isolated by sorting using a MoFlo Sorter (DakoCytomation). Purity of the sorted cells was >98%.

IEC:LPL coculture and cytokine stimulation of LPLs

LPMCs were labeled with CFSE and cocultured with gamma-irradiated IECs (3000 rad). Both LPMC and IEC were adjusted to 1×10^6 cells/ml and cocultured in serum-free AIM-V medium containing 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen Life Technologies) for 5–8 days at 37°C, 5% CO_2. At different time points, aliquots of the coculture were harvested, stained for surface markers, and analyzed for proliferation by flow cytometry. No surviving IECs were detected at 48 h or later.

For cytokine stimulation, IL-2 (100 U/ml), IL-4 (100 ng/ml), IL-7 (10 ng/ml), IL-10 (10 ng/ml), or IL-15 (20 ng/ml) (all R&D Systems) were added to the CFSE-labeled LPMCs at day 1 of the culture, and aliquots of the culture were analyzed as described above. These concentrations were determined to be optimal in preliminary studies.

Flow cytometry

Cells were resuspended in PBS and stained with Abs recognizing different cell surface markers: FITC-, PE-, PerCP-, or allophycocyanin-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD28, anti-CD45RA, and anti-CD45RO Abs were all from BD Pharmingen. Abs recognizing 17 different Vβ families of the TCR were from Immunotech (Beckman-Coulter). Anti-CD101 (18F7) and CD103 (28C12) Abs were a kind gift from Drs. M. Brenner and G. Russell (Brigham and Women's Hospital, Boston, MA). Specificity of the Abs was confirmed by isotype-matched control Abs. After staining, cells were washed in PBS, and surface staining was analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). Intracellular staining for perforin was done by staining the cells for surface markers (CD3, CD4, CD8) followed by fixation of the cells in 3.5% formaldehyde solution. The cells were then permeabilized by 0.2% saponin for 15 min and stained for intracellular perforin using a PE-conjugated anti-perforin Ab (BD Pharmingen). The cells were then washed in 0.2% saponin and PBS before flow-cytometric analysis.

Pokeweed mitogen (PWM) regulatory assay

PBMC were adjusted to 1×10^6 cells/ml in culture medium (RPMI 1640 containing 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine; all Invitrogen Life Technologies) and stimulated with 1% PWM. To evaluate the regulatory potential of different cell populations,

Table I. *Patient characteristics*[a]

Age/Sex	Diagnosis and Disease Location	Medications
65/M	Right colon polyp noncancer	None
72/F	Right colon cancer with local metastasis	None
28/M	Sigmoid diverticulitis with local abscess	Ciprofloxacin and metronidazole
80/M	Ilecolic resection for cecal polyp/benign	None
68/F	Right colon cancer with local metastasis	None
66/M	Left colon polyp with carcinoma in situ and local metastasis	None
94/F	Villous adenoma, right colon	None
19/M	Severe pancolitis (UC)	i.v. steroids/5-ASA
61/M	Severe pancolitis (UC)	6-Mercapto-purine and 5-ASA
32/M	Severe pancolitis (UC)	i.v. steroids/5-ASA
39/M[b]	Severe pancolitis (UC)	i.v. steroids/5-ASA
32/M	Moderate UC pancolitis with adenocarcinoma	5-ASA
40/M	Left-sided UC moderate	Cortenema/5-ASA/6-MP
44/M	Crohn's fibrostenosis of ileum and obstruction	None
23/F	Ilieitis—10 cm/intractable to therapy	6-MP/5-ASA
76/F	Crohn's recurrent ileocolic anastamosis	Ciprofloxacin
38/M	Fistulizing Crohn's (ileocolonic) with mesenteric abscess	6-MP/Cipro/5-ASA
42/F	Fistulizing Crohn's (ileocolonic) with perianal disease	Infliximab/Cipro/5-ASA
28/F	Fistulizing Crohn's with perianal disease	Infliximab/Cipro/5-ASA/6-MP

[a] Abbreviations: M, Male; F, female; 5-ASA, 5-asathioprin; 6-MP, 6-mercaptopurine; Cipro, ciprofloxacin.
[b] The one UC patient whose LP $CD8^+$ T cells suppressed in coculture.

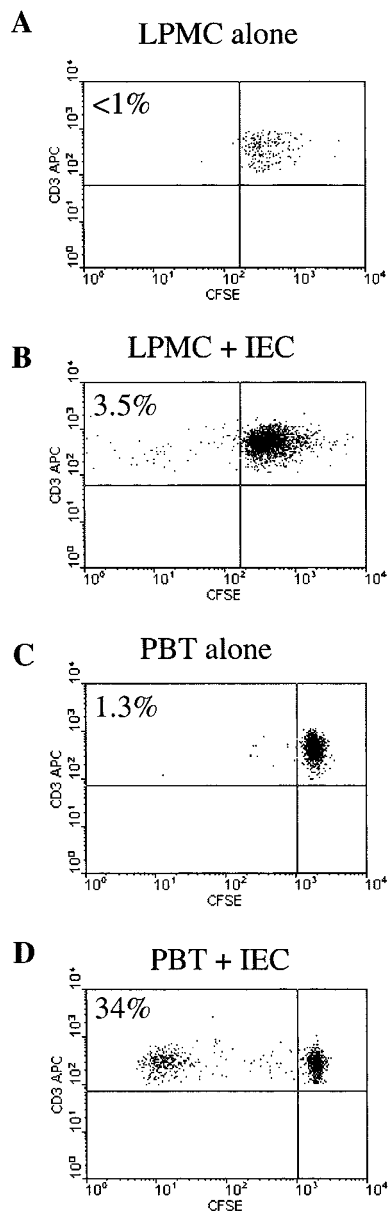

FIGURE 1. LP T cells are only marginally activated by IECs. T cells from either LP or peripheral blood were labeled with CFSE before coculture with irradiated IECs. At day 7, cells were harvested and stained with an anti-CD3 Ab. The analysis was gated on CD3$^+$ cells, and the percentages of proliferating cells (CFSE low) are shown in the *upper left panel*. *A*, LPMCs alone. *B*, LPMCs cocultured with IECs. *C*, PBTs alone. *D*, PBTs cocultured with IECs. These data are representative of four independent experiments.

sorted cells were added at varying concentrations (1:1 to 1:5 PBMC:LP or PB intact or CD8$^+$ T cells) into the culture of PWM-stimulated PBMCs. A ratio of 1:2 resulted in optimal suppression in all experiments and allowed us to use greater number of LPLs. After 7 days of culture, the supernatant was harvested, and the levels of total Ig were analyzed by ELISA. In some cases, the potential regulatory cells were separated from the PWM-stimulated PBMCs by a Transwell membrane with 0.4-μm pores (Costar). Percent suppression or enhancement of Ig secretion was determined by dividing the Ig secretion of PWM-stimulated PBMC by the Ig secretion of PBMC into which the T cell subpopulation was added and multiplying this by 100. Percent suppression of Ig secretion was used due to the inherent variability in Ig secretion by PWM-stimulated PBMC from donor to donor. The range of Ig secretion was 0.1–0.4 μg/ml in unstimulated cultures and 1.5–14 μg/ml in PWM-stimulated cultures. The range of fold increase in Ig secretion (stimulated/nonstimulated) was 6- to 26-fold.

Ig ELISA

Nunc Maxisorp 96-well plates were coated overnight at 4°C with anti-human Ig (BioSource) diluted 1/400 in 0.1 M carbonate buffer. Plates were washed and blocked with ELISA buffer (PBS/0.05% Tween 20) containing 1% BSA (Sigma-Aldrich) for 2 h at room temperature. Samples and standards were diluted in ELISA buffer, added to the plate and incubated for 1–2 h at room temperature, followed by incubation with alkaline phosphatase-conjugated anti-human κ and λ L chain Abs diluted 1/1000 in ELISA buffer for 1 h. The plates were washed, *p*-nitrophenyl phosphate substrate was added, and plates were read at 405 nm.

Statistical analysis

Differences in Ig production in the PWM regulatory assay were compared using Student's *t* test.

Results

Stimulation of LPMC by IECs

Using an allogeneic in vitro mixed coculture system consisting of IECs and PBTs (IEC:PBT), we have previously shown that IECs are able to induce PBTs to proliferate (27, 29). In the present work, we investigated whether LPMCs can be similarly activated by autologous or allogeneic IECs in an IEC:LPL coculture system. As seen in Fig. 1, *A* and *B*, only a small fraction of CD3$^+$ T cells from the LP was induced to proliferate in IEC:LPMC cocultures. In four independent experiments, the proliferating (CFSE^{low}) CD3$^+$ LP T cells represented only 2–5% of all T cells after 7 days of coculture. No difference in the proliferation of LPLs was observed between cocultures using allogeneic vs autologous IECs as stimulator cells. The limited number of cells seen in Fig. 1*A* reflects the spontaneous apoptosis of LPL cultures in medium alone (gating on live cells only). The small fraction of proliferating cells observed in the LPMC:IEC cocultures contrast the much higher fraction of proliferating T cells seen in IEC:PBT cocultures (Fig. 1, *C* and *D*). In such cultures, we have previously demonstrated that 5–35% of PBTs are proliferating at day 7. Cells proliferating in these normal IEC:PBT cocultures were predominantly CD8$^+$ T cells (29). However, this was not true for the IEC:LPMC cocultures, in which a similar percentage of CD4$^+$ and CD8$^+$ T cells expanded.

Table II. *Proliferation of LP T cells upon stimulation by cytokines (n = 4)*

Cytokine	Percentage of CD3$^+$ LP T Cells Proliferating (mean % CFSE^{low} (range))	Fold Increase in CD8$^+$ T Cell Proliferation over IL-15 Alone (range)	Proliferation of CD4$^+$ or CD8$^+$ T Cells
IL-2	20% (15–25)		Variable
IL-4	12% (11–14)		Variable
IL-7	17% (10–21)		Variable
IL-10	10% (7–14)		CD4>CD8
IL-15	22% (5–39)		CD8>CD4
IL-2/IL-7/IL-15		1.48 (1.01–1.89)	CD8>CD4
IL-4/IL-10/IL-15		1.51 (1–1.88)	CD8>CD4

Stimulation of LPMC by cytokines

Because the failure of LPLs to become activated by IECs (presumed restimulation) may reflect an anergic state of the LPL, we attempted to rescue the cells with cytokines. Several different cytokines, including IL-2, IL-4, IL-7, IL-10, and IL-15, were able to induce proliferation of LP T cells in vitro. As shown in Table II, IL-2, IL-7, and IL-15 were most effective, leading to proliferation of 20, 17, and 22% of the LP T cells, respectively. However, only stimulation by IL-15 led to the preferential expansion of $CD8^+$ LP T cells, because the proportion of proliferating $CD8^+$ LP T cells was between 1.3 and 2 times higher than the proliferating $CD4^+$ LP T cells. In contrast, IL-10 preferentially expanded $CD4^+$ LP T cells, whereas the results for IL-2, IL-4, and IL-7 were more variable. Thus, the failure to restimulate LPL with IECs may indeed reflect an anergized state. The cytokine microenvironment in the LP is quite complex. We therefore further assessed the effects of combinations of cytokines on LPL proliferation. As seen in Table II, neither the combination of IL-2, IL-7, and IL-15, nor IL-4, IL-10, and IL-15, enhanced proliferation of $CD8^+$ LPL significantly above that seen with IL-15 alone (1.5-fold each). In contrast, there was a modest inconsistent increase in $CD4^+$ T cells when either IL-2 or IL-4 were added in the cultures (data not shown).

Normal LP contain LP $CD8^+$ T cells with regulatory potential

If LP $CD8^+$ T cells are the result of interactions of precursor $CD8^+$ regulatory T cells with Ag presented by IECs, we should be able to define these cells in vivo. Previous studies have demonstrated that intact LPLs only provided help for T and B cell functions (34, 35). We assessed the regulatory potential of different cell populations isolated from the LP by adding the cells of interest into a culture of PBMCs

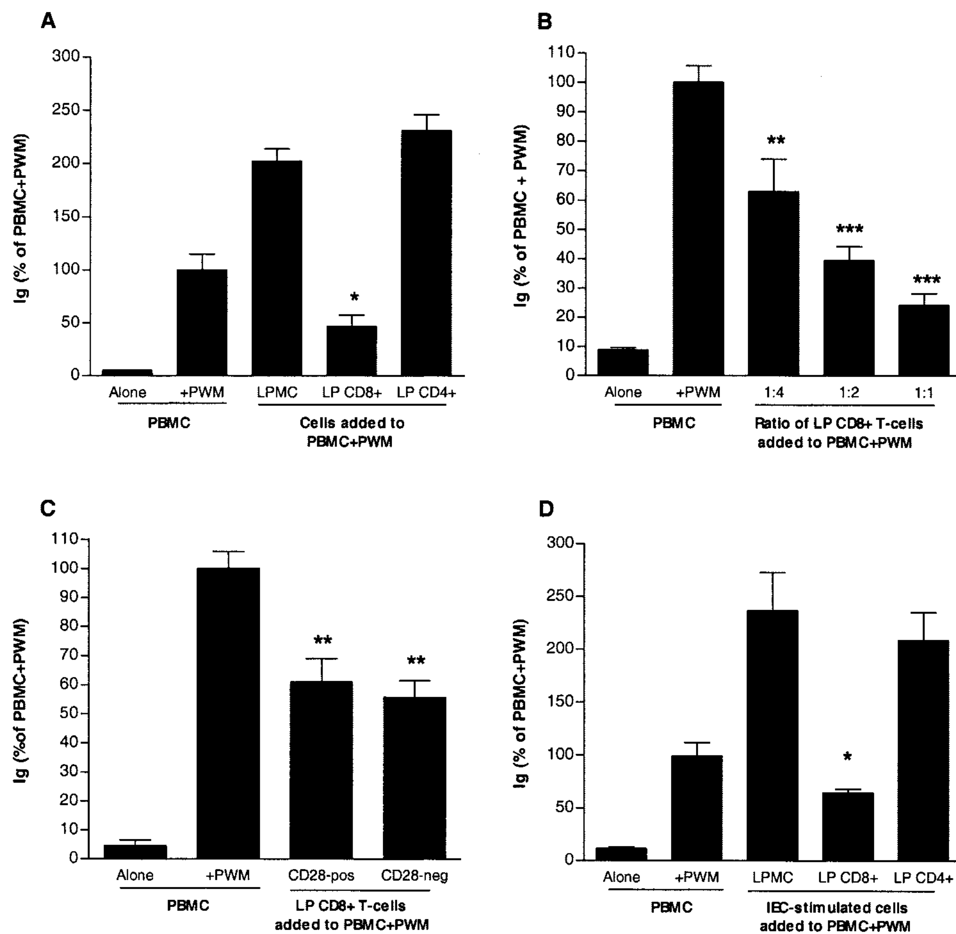

FIGURE 2. *A*, Suppressive activity of freshly isolated LPMCs, LP $CD4^+$ and $CD8^+$ T cells. PBMCs were either left alone or stimulated by PWM for 7 days, followed by analysis of the supernatant for the presence of secreted Igs by ELISA. The suppressive activity of different cell populations from the LP was evaluated by adding 5×10^4 of the cells of interest into a culture of 1×10^5 PWM-stimulated PBMCs. PBMCs + PWM was set as 100%. Each bar represents the mean of triplicate cultures. *, $p < 0.05$ vs PWM-stimulated PBMCs. These data are representative of six independent experiments. *B*, Suppression mediated by regulatory LP $CD8^+$ T cells is dose dependent. PBMCs were either left alone or stimulated by PWM. LP $CD8^+$ T cells were added to 1×10^5 PWM-stimulated PBMCs at different ratios at the start of the culture. Each bar represents the mean of quadruplicate cultures. **, $p < 0.05$; and ***, $p < 0.001$ vs PWM-stimulated PBMCs. These data are representative of three independent experiments. *C*, Suppressive potential of CD28-positive and CD28-negative LP $CD8^+$ T cells. PBMCs were either left alone or stimulated by PWM. 3×10^4 CD28-positive or CD28-negative LP $CD8^+$ T cells were added to 1×10^5 PWM-stimulated PBMCs at the start of the culture. After 7 days of culture, the supernatant was harvested and analyzed for the presence of Igs. Each bar represents the mean of triplicate cultures. **, $p < 0.01$ vs PWM-stimulated PBMCs. These data are representative of three independent experiments. *D*, Suppressive activity of LPMCs, LP $CD4^+$ and $CD8^+$ T cells after coculture with IECs. LPMCs were cocultured for 7 days with normal IECs. After coculture, LPMCs were either added directly to cultures of PWM-stimulated PBMCs or sorted into $CD4^+$ or $CD8^+$ T cell populations before addition into the stimulated PBMCs. PBMCs were either left alone or stimulated by PWM. The following cell populations were added into the culture of 1×10^5 PWM-stimulated PBMCs: 5×10^4 unfractionated LPMC; 5×10^4 LP $CD8^+$ T cells; 5×10^4 LP $CD4^+$ T cells. After 7 days of culture the supernatant was harvested and analyzed for the presence of Igs. Each bar represents mean of triplicate cultures. *, $p < 0.05$ vs PWM-stimulated PBMCs. These data are representative of four independent experiments.

stimulated by PWM. This system has been used as a standard assay to assess regulatory activity by our group as well as by others (27, 29, 34). In this assay, stimulation of PBMC by PWM led to a 10- to 20-fold increase in Ig production compared with unstimulated PBMC, as seen in Fig. 2*A*. The addition of unfractionated LPMCs into these cultures did not result in the reduction of Ig production by the PBMCs. Rather, as previously described, these cells provided help, leading to ~2-fold increase in Ig production.

We then investigated the regulatory potential of LPMC subpopulations. By surface staining and flow-cytometric sorting, LP CD4$^+$ and CD8$^+$ T cells were isolated to a purity of >95%. Fig. 2*A* shows that LP CD4$^+$ T cells, which constituted around two-thirds of the CD3$^+$ LP T cells, were also unable to suppress the production of Ig by the PWM-stimulated PBMCs. In contrast, purified LP CD8$^+$ T cells, did indeed down-regulate the PWM-induced Ig production of PBMCs (Fig. 2*A*). In six independent experiments, the suppression ranged from 31 to 80%, when 5×10^4 LP CD8$^+$ T cells were added to 1×10^5 PWM-stimulated PBMCs. Furthermore, the suppression mediated by the LP CD8$^+$ T cells, seemed to be dose dependent, because addition of increasing numbers of cells led to increased suppression (Fig. 2*B*). The LP CD8$^+$ T cells mediating the suppression were CD101$^+$, CD103$^+$, and CD45RO$^+$ (data not shown).

We have previously observed that in vitro stimulation of PBTs by IECs leads to an increase in the proportion of CD28-negative CD8$^+$ T cells among the proliferating T cells compared with the nonproliferating T cells (29). Furthermore, other groups have described a CD8$^+$CD28$^+$ regulatory T cell population. As determined by flow cytometry, 62–75% of the LP CD8$^+$ T cells were CD28$^+$. However, as seen in Fig. 2*C*, in the case of T cells from the LP, there was no significant difference in the regulatory activity of CD28-positive vs CD28-negative LP CD8$^+$ T cells.

We further investigated whether LP T cells purified after coculture with IECs were able to suppress Ig secretion. As seen in Fig. 1*D*, coculture with IECs for 7 days did not change the regulatory activity of any of the cell populations: LP CD8$^+$ T cells still suppressed the Ig production after coculture with IECs, whereas none of the other cell populations were capable of mediating suppression. Similar results were obtained regardless of whether the LPMCs were cocultured with IECs in the presence or absence of IL-15. These results demonstrate that CD8$^+$ T cells with suppressive activity are present in the LP, and that this activity is not further altered by subsequent coculture with IECs in vitro.

LP CD8$^+$ T cells with regulatory activity are confined to the LP

To investigate whether the observed inhibitory activity of the LP CD8$^+$ T cells was confined to the LP, we compared the suppression mediated by LP CD8$^+$ T cells to that of CD8$^+$ T cells from peripheral blood. As seen in Fig. 3, CD8$^+$ T cells isolated from peripheral blood are not able to down-regulate Ig production by PWM-stimulated PBMC, indicating that the regulatory activity is unique to LP CD8$^+$ T cells. Because we cultured the LP CD8$^+$ T cells in IL-15 overnight before sorting to gain re-expression of surface CD8, we also assessed the regulatory potential of CD8$^+$ T cells with and without IL-15 treatment. Although the IL-15 treatment increased the yield of CD8$^+$ T cells after sorting due to the higher expression of CD8, this treatment did not alter the regulatory activity of LP or PB CD8$^+$ T cells (Fig. 3).

Mechanisms of regulation

We have previously observed that in vitro-generated TrE cells mediate their suppressive effect by a contact-dependent mechanism (29). To investigate the mechanisms by which the LP CD8$^+$ T cells mediate suppression, we separated these cells from the PWM-stimulated PBMCs by use of a Transwell culture system. As shown in Fig. 4, prevention of cell-cell contact between PBMCs and CD8$^+$ T cells, almost completely abolished the suppression mediated by the latter cell population. Although a trend toward suppression was seen in the absence of cell-cell contact, this was not significant, indicating that the dominant mechanism of suppression requires contact. Next, we examined whether the LP CD8$^+$ T cells mediated their suppression by killing their targets by perforin. However, as determined by intracellular staining and flow cytometry, only a small percentage (<7%) of the LP CD8$^+$ T cells stained positive for perforin (data not shown), indicating that the cell contact-dependent mechanism of suppression was not likely to be mediated by cytotoxicity.

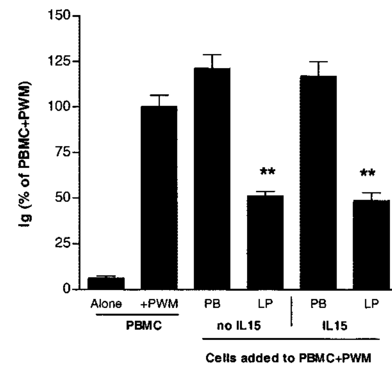

FIGURE 3. The suppressive activity of CD8$^+$ T cells is confined to the LP and not altered by IL-15. LPMC and PBMC were incubated overnight in AIM-V medium alone or in medium containing 20 ng/ml IL-15 before purification of CD8$^+$ T cells by flow-cytometric sorting. The suppressive activity of the purified cells was evaluated by adding 50,000 CD8$^+$ T cells from either peripheral blood or LP to 100,000 PWM-stimulated PBMCs. As depicted in Fig. 2, unstimulated PBMCs and PWM-stimulated PBMC are represented. After 7 days of culture, the supernatant was analyzed for Ig by ELISA. Each column represents the mean of quadruplicate cultures. **, $p < 0.01$ vs PWM-stimulated PBMCs. These data are representative of three independent experiments.

LP CD8$^+$ T cells from patients with IBD do not have regulatory activity

Because IBD is believed to be the result of a breakdown in the regulatory mechanisms of the mucosal immune system, we investigated whether the CD8$^+$ T cells from LP of IBD patients had lost

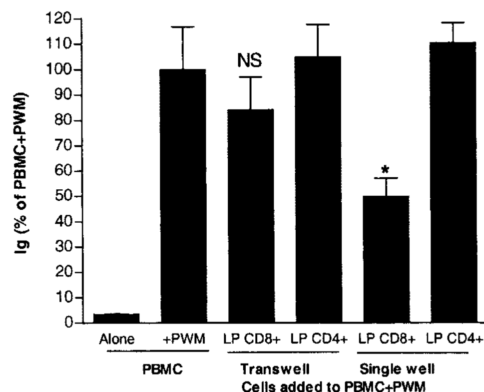

FIGURE 4. LP CD8$^+$ T cells need cell contact to mediate suppression. PBMCs were either left alone or stimulated with 1% PWM for 7 days, followed by the analysis of the supernatant for the presence of secreted Igs by ELISA. A total of 5×10^4 LP CD8$^+$ T cells or CD4$^+$ T cells were added to 1×10^5 PWM-stimulated PBMCs either physically separated by a Transwell or in contact with the PBMCs (single well). Each bar represents the mean of triplicate cultures. *, $p < 0.05$ vs PWM-stimulated PBMCs. These data are representative of three independent experiments.

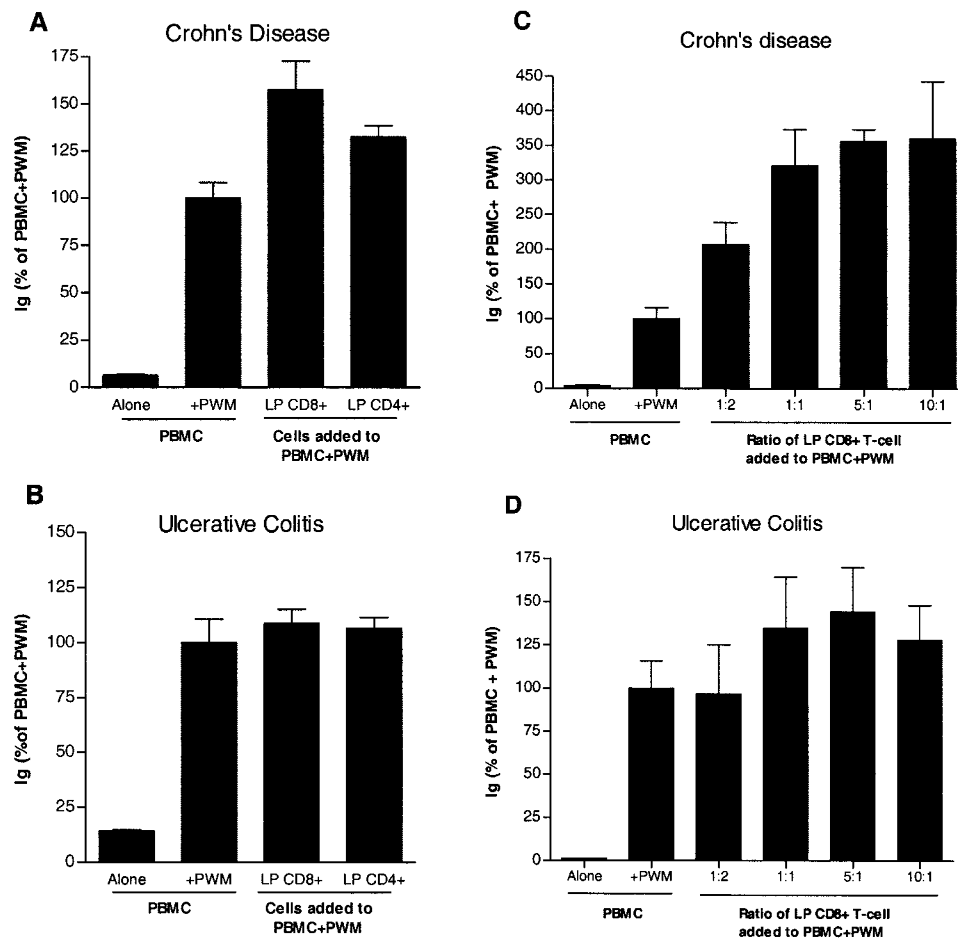

FIGURE 5. Suppressive activity of LP CD4$^+$ and CD8$^+$ T cells from IBD patients. *A*, Representative example of the suppressive activity of CD4$^+$ and CD8$^+$ LP T cells from CD patients ($n = 4$). PBMC were either left alone or stimulated with 1% PWM. A total of 5×10^4 LP CD4$^+$ or CD8$^+$ T cells were added to 1×10^5 PWM-stimulated PBMC. Supernatants were collected after 7 days of culture and analyzed for Ig levels by ELISA. Each column represents the mean of quadruplicate cultures. In six of six CD specimens, no suppression was observed. *B*, Representative example of the suppressive activity of CD4$^+$ and CD8$^+$ LP T cells from UC patients ($n = 6$). PBMC were either left alone or stimulated with 1% PWM. A total of 5×10^4 LP CD4$^+$ or CD8$^+$ T cells were added to 1×10^5 PWM-stimulated PBMC. Supernatants were collected after 7 days of culture and analyzed for Ig secretion by ELISA. Each column represents the mean of quadruplicate cultures. In five of six UC specimens, no suppressive activity was observed. *C* and *D*, Representative examples (one CD and one UC) of dose titration study of CD8$^+$ LPL cells from IBD patients (CD = 2; UC = 2). Addition of 5×10^4–10×10^5 LP CD8$^+$ T cells to 1×10^5 PWM-stimulated PBMC in triplicate microwell cultures not only failed to mediate suppression but in some cases actually enhanced Ig secretion in these cocultures.

their regulatory activity. LP CD4$^+$ and CD8$^+$ T cells were purified from noninvolved or mildly involved segments of resection specimens from patients with either UC or CD, and these were then added into cultures of PWM-stimulated PBMC as described above. As shown in Fig. 5*A*, neither CD4$^+$ nor CD8$^+$ LP T cells from patients with CD were able to suppress the PWM-induced Ig production of PBMC. The absence of CD8$^+$ T cells with regulatory activity was observed in six of six specimens from patients with CD. Similarly, in five of six specimens from patients with UC, CD8$^+$ T cells with regulatory activity were absent (Fig. 5*B*). These findings did not correlate with disease activity, site of disease, or medication use (Table I).

The frequency of Vβ5.1$^+$CD8$^+$ T cells is decreased in IBD LP

In a separate study, we have analyzed the TCR repertoire of the proliferating CD8$^+$ T cells in IEC:PBT cocultures and observed an expansion of a subpopulation of the CD8$^+$ T cells expressing the TCR Vβ5.1 chain.[4] These cells possess regulatory activity, and their expansion is dependent on interaction with the gp180-CD1d complex expressed on the surface of IECs. We also observed that, when IECs from IBD patients are used in the IEC:PBT cocultures, the frequency of Vβ5.1$^+$ cells among the proliferating CD8$^+$ T cells was significantly decreased, most likely due to the aberrant expression of gp180 on IBD IECs.

In the present study, we investigated whether the frequency of the regulatory Vβ5.1$^+$CD8$^+$ T cells also was reduced in vivo in the LP of IBD patients. To this end, we analyzed the TCR repertoire of LP CD8$^+$ T cells from IBD patients and compared it to that of normal individuals. Consistent with our previous results in vitro, the frequency of Vβ5.1$^+$ cells among LP CD8$^+$ T cells from IBD patients (three UC; three CD) was significantly decreased ($p = 0.0018$) compared with LP CD8$^+$ T cells from normal individuals (Table III). The low frequency of Vβ5.1$^+$ cells among CD8$^+$ LP T cells in IBD patients did not appear to be related to nonspecific inflammation, because the frequency of Vβ5.1$^+$ cells among CD8$^+$ LPLs isolated from noninflamed areas of IBD specimens ($n = 3$) remained low. In contrast, the frequency of Vβ5.1$^+$ cells among CD8$^+$ LPLs isolated from non-IBD inflammatory controls (diverticulitis; $n = 3$) was higher and comparable to normal specimens (data

Table III. *Frequency of Vβ5.1$^+$ cells among LP CD8$^+$ T cells*

	IBD	Normal
In vivo (LP)	2.23 ± 1.04%* (n = 6)	4.72 ± 1.41% (n = 9)

*, p = 0.0018 vs normal.

not shown). This indicates that IBD IECs are less effective in expanding this regulatory population of CD8$^+$ T cells in vitro as well as in vivo.

Discussion

The mucosal immune system is characterized by a general tone of immunosuppression and tolerance. The importance of maintaining this tolerant state is illustrated in IBD, because this disease is believed to be the result of a breakdown of tolerance to resident enteric bacteria. Regulatory T cells play an important role in the maintenance of tolerance in the mucosal immune system (4, 21), and both CD4$^+$ and CD8$^+$ T cells can act as regulatory cells. However, it still remains unclear as to how these cells are generated. We have previously demonstrated that IECs are able to expand regulatory CD8$^+$ T cells in an allogeneic in vitro mixed IEC:PBT coculture system (27, 29), indicating that IECs may play a role in the generation of regulatory T cells. However, in order for these results to be relevant to the situation in vivo, one would expect to be able to isolate such cells from the normal intestinal LP. To address this, we have, in the present study, analyzed human LP lymphocytes for the presence of CD8$^+$ T cells with regulatory activity. Our results demonstrate that CD8$^+$ T cells with regulatory activity are indeed present in the LP of normal, healthy individuals. Other groups have also investigated the regulatory capacity of LP CD8$^+$ T cells, but with diverging results. Lee et al. (35) showed that LP CD8$^+$ T cells had both the capacity to help and to suppress the production of Ig in an indicator culture consisting of PWM-stimulated B and T cells. Whether the LP CD8$^+$ T cells helped or suppressed depended on the T:B ratio in the indicator culture. In contrast, James et al. (34) demonstrated that CD8-enriched LP T cells did not significantly suppress Ig production in a similar PWM assay. As we in the present study used highly purified LP CD8$^+$ T cells instead of just CD8-enriched LPMC, a difference in the purity of the LP CD8$^+$ T cells could explain the discrepancies between our study and the ones alluded to above. We specifically used the PWM assay to be consistent with these previous studies.

Furthermore, we demonstrate that, in contrast to LP CD8$^+$ T cells, neither unfractionated LPMCs nor purified LP CD4$^+$ T cells displayed any regulatory activity. Rather, these cells provided additional help resulting in increased Ig secretion by the PWM-stimulated PBMCs. The finding that unfractionated LPLs and CD4$^+$ LPLs provide help rather than suppression is in accordance with the above-mentioned studies (as well as others (36, 37)), in which it was shown that mononuclear cells from both peripheral blood and LP provided help for Ig production in a similar assay (34, 35).

If the CD8$^+$ T cells, at the numbers present in situ, are mediating suppression in vivo, why do we need to fractionate LPL (i.e., isolate CD8$^+$ T cells) to see suppression in vitro? The failure of unfractionated LPL to mediate suppression in vitro may reflect one of several possibilities. First, in the microenvironment in vivo there may be niches where CD8$^+$ T cells interact with either CD4$^+$ T cells or APCs. This would be lost following in vitro isolation. Second (related to the first possibility), there may be a need for cell contact, which may be limiting in in vitro cultures (we show that suppression is cell contact dependent). Third, this may still represent a numerical where the numbers of CD8$^+$ T cells are insufficient to mediate suppression of equivalent numbers of PBMC. This latter possibility could also explain the seemingly limited potency of CD8$^+$ regulatory T cells in in vitro cocultures.

We show in this study that, in contrast to peripheral blood CD8$^+$ T cells, CD8$^+$ T cells from the LP can only be marginally activated by in vitro coculture with IECs. This is in agreement with the fact that LP T cells are difficult to activate in vitro through their TCR, because these cells are refractory to mitogenic and anti-CD3 stimulation, although they can be activated by stimulation through CD2 and CD28 (38–40). It is tempting to speculate that the LP CD8$^+$ T cells have already been stimulated by IECs in vivo leading to the generation of regulatory cells, which may be anergic and therefore refractory to a second stimulation by IECs. This is supported by results from our laboratory showing that regulatory CD8$^+$ T cells generated in vitro by interactions with IECs do not proliferate when they are restimulated by IECs a second time (data not shown). Furthermore, we show that the regulatory activity of LP CD8$^+$ T cells is not further altered by in vitro coculture with IECs. In line with this, several groups have demonstrated that T cells rendered anergic by stimulation with immature dendritic cells or in the presence of regulatory cytokines develop regulatory properties (16, 41, 42). Consistent with a potentially anergic state, several cytokines, including IL-2 and IL-15, were able to stimulate LP T cells to proliferate. However, only IL-15 consistently expanded CD8$^+$ T cells more than CD4$^+$ T cells. Because IECs have previously been demonstrated to produce IL-15 (43), this molecule may play a role in the initial activation of regulatory CD8$^+$ T cells in the LP.

The generation of regulatory cells in the intestine has mostly been studied by induction of oral tolerance. It is generally accepted that feeding a low dose of Ag leads to the generation of regulatory cells specific for that same Ag (44, 45). Several subsets of regulatory cells have been described in oral tolerance, including both CD4$^+$ and CD8$^+$ T cells (46–48), and most of these cells seem to mediate their suppression via secretion of regulatory cytokines such as IL-4, IL-10, and TGF-β. Contrary to the regulatory cells generated in oral tolerance, the regulatory LP CD8$^+$ T cells described in the present study mediate their suppression in a cell contact-dependent, but perforin-independent, manner. In this sense, these cells resemble both the CD4$^+$CD25$^+$ and the recently described CD28-negative CD8$^+$ regulatory T cells (19, 20). The latter cells are generated by repeated allogeneic stimulation, and are class I restricted in contrast to TrE cells, and they also mediate suppression by a cell contact-dependent mechanism. It was recently demonstrated that these cells act on APCs, rendering them tolerogenic (49).

Our results suggest that the CD8$^+$ T cells with regulatory activity present in the normal colonic LP may represent the in vivo counterpart of the in vitro-generated TrE cells that we have described earlier (29). Both populations express the surface molecules CD8, CD101, and CD103, and both are able to suppress the production of Ig by PWM-stimulated PBMCs in a cell contact-dependent, perforin-independent manner. The fact that the frequency of Vβ5.1$^+$CD8$^+$ T cells is increased both among the IEC-activated TrE cells in vitro as well as in normal LP compared with peripheral blood further underscores the similarities between these two cell populations. The only difference we observed between the regulatory CD8$^+$ T cells generated in vitro and those found in the LP, was that the in vitro-activated TrE cells have down-regulated CD28 compared with the nonactivated CD8$^+$ T cells (29), whereas roughly two-thirds of the LP CD8$^+$ T cells express CD28. Furthermore, when we compared the regulatory potential of CD28-negative to CD28-positive LP CD8$^+$ T cells, we did not see any

significant difference. Whether this is due to the fact that the in vitro regulatory TrE cells are generated by stimulation with allogeneic IECs is presently unknown.

Our results showing that $CD8^+$ T cells with regulatory activity could not be detected in the LP of patients with IBD underline the relevance of these cells in the maintenance of mucosal tolerance. These results are not in accordance with the results of James et al. (34), who found that LP $CD8^+$ T cells from both normal individuals and patients with IBD had a nonsignificant trend toward suppression in a PWM assay. Similar data for normal LPL were obtained in a primate model (50). $CD8^+$ suppressor inducer as well as suppressor cells were reduced (phenotypically) in the LP of patients with CD (51). Although not directly comparable, several studies have shown that peripheral blood $CD8^+$ T cells from CD patients possessed regulatory activity (52, 53).

The absence of regulatory cells in the LP of IBD patients, described in the present study, might be linked to the epithelial cell glycoprotein gp180. This molecule is a member of the carcinoembryonic Ag subfamily and expressed by all normal IECs (29, 31). The in vitro generation of TrE cells by IECs is dependent on both gp180 and the nonclassical class I molecule CD1d, because blocking Abs against these molecules prevents expansion of the TrE cells (29–32). In contrast to normal IECs, IECs from IBD patients have an aberrant expression of gp180 (54), and when such IECs are used as stimulators in the in vitro coculture system, potentially pathogenic, IFN-γ-producing $CD4^+$ T cells are expanded rather than the regulatory $CD8^+$ TrE cells (33) (I. Dotan, manuscript in preparation). The failure to expand this population was also observed in vivo, because the frequency of the $V\beta5.1^+CD8^+$ T cells were significantly reduced in the LP of IBD patients compared with normal individuals. Taken together, these data suggest the following scenario: In the normal LP, IECs generate or expand regulatory $CD8^+$ TrE cells via CD1d and gp180. These TrE cells contribute to the maintenance of mucosal tolerance and prevention of IBD. In patients with IBD, the absence of gp180 prevents the generation/expansion of TrE cells, and the increased expression of MHC class II on IECs allows for the expansion of potentially pathogenic $CD4^+$ T cells.

In summary, the results of the present study indicate that the IEC might play a central role in maintaining the normal state of tolerance in the mucosal immune system, due to their ability to generate/expand regulatory $CD8^+$ T cells. The defective expression of gp180 in IBD and the resulting absence of regulatory $CD8^+$ T cells may be an important contributor to the pathogenesis of IBD.

Disclosures

The authors have no financial conflict of interest.

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