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The Peyer’s Patch Microenvironment Suppresses T Cell Responses to Chemokines and Other Stimuli

Sirid-Aimée Kellermann and Leslie M. McEvoy

Immunosurveillance of mucosal sites presents immune cells with challenges not encountered in the periphery. T cells in the gut must distinguish enteric pathogens from innocuous non-self Ag derived from food or commensal bacteria. The mechanisms that regulate T cells in the gut remain incompletely understood. We assessed the effect of the Peyer’s patch microenvironment on T cell responses to chemokines. Chemokines are believed to play an important role during T cell priming by facilitating T cell migration into and within lymphoid tissues as well as T cell encounter and interaction with APCs. We found a profound suppression of chemokine-stimulated T cell chemotaxis and actin polymerization in Peyer’s patch relative to lymph node. Chemokine hyporesponsiveness is imposed upon T cells within hours of their entry into Peyer’s patches and is reversed following their removal. Suppression was not restricted to chemokine stimulation, as T cell responses to Con A and PMA were also suppressed. The global nature of this defect is further underscored by an impairment in calcium mobilization. Evidence indicates that a soluble factor contributes to this hyporesponsiveness, and comparison of Peyer’s patches and lymph nodes revealed striking differences in their chemokine and cytokine constitution, indicating a marked Th2 bias in the Peyer’s patches. The role of the Th2 microenvironment in mediating suppression is suggested by the ability of *Nippostrongylus brasiliensis* to elicit hyporesponsiveness in lymph node T cells. The suppressive milieu encountered by T cells in Peyer’s patches may be critical for discouraging undesired immune responses and promoting tolerance. *The Journal of Immunology*, 2001, 167: 682–690.
HeJ, IL-6 knockout, and TCRδ knockout mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Green fluorescent protein–transgenic B10.D2 mice were obtained from S. Lira (Schering-Plough Research Institute, Kenilworth, NJ) and were bred in our facility. IL-4, IL-10, IL-12, IFN-γ, and inducible NO synthase knockout mice were kindly provided by R. Coffman. IL-10 knockout mice with inflammatory bowel disease were provided by D. Rennick.

Abs and reagents
All Abs and conjugated streptavidin second-step staining reagents were purchased from BD PharMingen (San Diego, CA) except as noted. Anti-L-selectin and α4 integrin were purified from ascites from mice injected, respectively, with the MEL-14 or PS/2 hybridomas (both from American Type Culture Collection, Manassas, VA). Anti-mouse CXCR3 Ab (IgG2a) was used as hybridoma supernatant. As a control, a rat IgG2a isotype-matched Ab was diluted in hybridoza culture medium. KJ1-26 (34) was used as a biotinylated purified Ab. All murine chemokines were obtained from R&D Systems (Minneapolis, MN). MIP-3β, biotinylated using the EZ-Link TFP-PEO-biotin (Pierce, Rockford, IL), was provided by J. Abrams and N. Nguyen and was used at 0.1–1.0 μg/10^6 cells. Control biotinylated cytochrome c was purchased from Sigma (St. Louis, MO). Apoptotic cells were assessed by flow cytometry using the ApoAlert Anxin-5 FITC kit (Clontech Laboratories, Palo Alto, CA).

Chemoattractant assays
Chemotaxis was conducted essentially as previously described (22). When freshly isolated cells were tested, cell suspensions obtained from mechanically disrupted tissue were washed once, resuspended in buffer containing 50 μg/ml gentamicin sulfinate (BioWhittaker, Walkersville, MD), and assayed. PBLs were isolated using Histopaque-1083 (Sigma) and washed three times in PBS containing BSA. For in vitro recovery assays, cells were washed once, then resuspended at 10^7/ml in RPMI 1640 containing 1% low endotoxin BSA (Sigma) and 50 μg/ml gentamicin sulfinate. Incubation was either on ice or at 37°C for the times indicated. The cells were washed twice before assessing chemotaxis. Flow cytometric analysis of the input and transmigrated cells was conducted using a FACSCalibur (BD Biosciences, San Jose, CA).

Assessment of F-actin
Cells were washed once, resuspended in prewarmed assay buffer (50% DMEM, 50% DMEM without bicarbonate or HEPES, 3% low endotoxin BSA), and equilibrated at 37°C for 15 min. The cells were then stimulated by addition of 2× chemokine, phorbol ester, or Con A, or buffer control. At the indicated times, 50-μl aliquots were removed and mixed immediately with ice-cold 50 μl of 4% paraformaldehyde in round-bottom 96-well plates. Cells were fixed for at least 20 min after the last aliquot had been taken, then pelleted and stained first for cell surface markers and subsequently for F-actin. F-actin staining was achieved by incubating the cells for 30 min at room temperature in 0.4% saponin (Sigma) in PBS containing 3.3 nM BODIPY-FL-phallacidin (Molecular Probes, Eugene, OR). Cells were washed twice and analyzed by FACS.

Calcium flux assays
CD4+ CD45RBhigh T cells freshly isolated from PP and LN cell suspensions were sorted to >98% purity. Sorted cells were labeled with 1 μM fluo-3 (Molecular Probes) and 0.04% Fluronic-F127 (Molecular Probes) in HBSS for 30 min at 37°C. Cells were pelleted and plated in black 96-well plates with clear bottoms (Costar, Cambridge, MA) precoated with CellTak (BD Labware, Bedford, MA), and the plate was centrifuged to adhere the cells. Mobilization of calcium in response to 10 μg/ml SLC or 2.5 μM ionomycin (Sigma) was assessed using a FLIPR fluorescence imaging plate reader system (Molecular Devices, Sunnyvale, CA). Data are presented as percentage of maximum (ionomycin-induced) fluorescence averaged from at least three wells of each cell population.

Adoptive transfer
Cells were isolated from peripheral LN (PLN) of C57BL/6 mice, washed once, and transferred i.v. to SJL.B6 mice (10–15 × 10^6 cells/recipient). At various times following transfer, mice were sacrificed and cells were isolated from PLN. Chemotaxis assays were conducted, and the input and transmigrated cells were stained with mAbs specific for Ly5.1, CD4, and CD8. The percentage of responding transferred cells (Ly5.1+) and host cells (Ly5.1−) was then assessed by FACS as described above.

Fli3L expansion of DC
Recombinant human Flt3L was purified as an Ig fusion protein (FL) from Escherichia coli and was kindly provided by S. Menon and Schering-Plough Research Institute (Union, NJ). Mice received daily i.p. injections of 10 μg (4.3 × 10^7 U) FL for 9 days before sacrifice. Cell suspensions obtained from homogenized, collagenase-digested tissues were stained with Abs for CD11c and MHC class II and double-positive cells were sorted to >95% purity.

TaqMan analysis of mRNA expression
RNA from whole PLN and LN or sorted cell suspensions was extracted using RNA STAT60 (Tel-Test, Friendswood, TX) or the StrataPrep total RNA kit (Stratagene, Cedar Creek, TX) according to the manufacturers’ protocols. RNA was treated with DNase I (Boehringer Mannheim, Mannheim, Germany) and reverse transcribed with oligo(dT18) (Life Technologies, Gaithersburg, MD) and random hexamer primers (Promega, Madison, WI) using standard protocols. cDNA was diluted to a final concentration of 10 ng/μl. cDNA was analyzed for the expression of murine chemokine receptor and cytokine genes as indicated in the text by the fluorogenic 5′ nucleic acid (N) polymerase chain reaction (PCR) assay (35) using a Perkin-Elmer Applied Biosystems Prism 7700 Sequence Detection System (Perkin-Elmer, Foster City, CA). Briefly, 10 μl cDNA (100 ng) was amplified in the presence of 12.5 μl TaqMan universal master mix (Perkin-Elmer), 0.625 μl gene-specific TaqMan probe, 0.5 μl gene-specific forward and reverse primers, and 0.5 μl water. 18S RNA-specific TaqMan probe and forward and reverse primers served as an internal positive control. Samples underwent the following stages: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; and stage 3, 95°C for 15 s followed by 60°C for 1 min. Stage 3 was repeated 40 times. Gene-specific PCR products were measured using an Applied Biosystems Prism 5700 Sequence Detection System (Perkin-Elmer).

In vivo blockade of lymphocyte entry into LN and PP
BALB/c mice were injected i.v. with 20 × 10^6 splenocytes from D10.110 donors. Two hours later, they received a single i.v. injection of Mel-14 and PS/2 (100 μg each). One group of mice received no mAbs and was immediately sacrificed. The remaining mice were sacrificed at various times after mAb injection, the PP and superficial inguinal LN harvested, and cell suspensions stained for KJ1-26 and CD4+ donor cells. At least three mice were measured at each time point.

Analysis of Nippostrongylus brasiliensis-infected mice
N. brasiliensis were infected to 2500 nematodes/ml and mice were infected s.c. with 500 nematodes. Eight to 13 days after infection, PLN and PP were removed from infected mice or age-matched controls, and the cells were tested for their responses to PMA, Con A, or chemokine in actin polymerization assays as described above.

Statistical analysis
Statistical significance was determined using the two-tailed Student’s t test.

Results
PP T cells exhibit a reduced response to chemokines
Because of the importance of SLC and MIP-3β in naive T cell trafficking into and microlocalization within lymphoid tissues necessary for T cell priming, we surveyed T cells from PP and other tissues for their responsiveness to these chemokines. Compared with naive T cells obtained from blood, peripheral or mesenteric LN, spleen, or the cecal lymphoid patch, T cells from PP consistently exhibited reduced chemotaxis to SLC as well as MIP-3β (Fig. 1A). This was not due to a shift in the dose-response curve (Fig. 1B). The phenomenon was general in several respects. First, not only naive, but also T cells with an effector/memory phenotype (defined as CD44high or L-selectinlow), were affected (data not shown). In addition, β2 integrin-high T cells from IP were less responsive than their PP counterparts (data not shown). Second, the observation extended to every mouse strain investigated (including C57BL/6, BALB/c, SJL, C3H/HeJ, and B10.D2) and was independent of gender as well as age. Finally, the response to not only SLC and MIP-3β, but also other chemokines including stromal-derived factor (SDF)-1α and IFN-γ-inducible protein (IP)-10 was reduced (Fig. 1C). We also observed that the response of

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B cells was impaired (Fig. 1B). It is interesting to note that the profound reduction in chemotaxis was not a general characteristic of all mucosal T cells, since T cells from mesenteric LN or the cecal patch (Fig. 1A) were not affected.

**FIGURE 1.** The migration of freshly isolated T cells from PP is inhibited compared with T cells from other sites. A. Cell suspensions from blood (BL), PLN, mesenteric LN (MLN), spleen (SPL), cecal patches (CP), and PP were tested in transwell chemotaxis assays. The percentage of naive (CD44low) CD4+ (upper panel) and CD8+ (lower panel) T cells that migrated spontaneously through 5-μm transwells without chemokine (gray areas) or in response to 0.1 μM SLC (black areas) is shown. **p**, degree of statistical significance in the increased chemotaxis of T cells from each tissue compared with PP. B, The response of naive CD4+ (upper panel), CD8+ (middle panel) T cells from LN (○, ●), and PP (□, □) was tested using a range of SLC and MIP-3β concentrations in a transwell chemotaxis assay setup. The response of CD19+ B cells to BLC was also assessed. C, Chemotactic response of CD4+ (upper panel) and CD8+ (lower panel) T cells to 0.1 μM SLC, 0.01 μM SDF-1α, or 0.5 μM IP-10. The response of naive T cells is shown for SLC and SDF-1α and of memory T cells in the case of IP-10. Spontaneous migration in the absence of chemokine (filled areas) and in response to each chemokine (open areas) is shown. The data obtained with T cells in A–C are the average of three wells ± SD and represent at least three experiments. ****, p < 0.0001; ***, p < 0.0005; ***, p ≤ 0.001; **, p ≤ 0.005; *, p ≤ 0.01.

**FIGURE 2.** Actin polymerization is impaired in PP T cells. Freshly isolated lymphocytes from LN and PP were stimulated with 5 nM SLC (6Ckine) or MIP-3β or 10 nM SDF-1α for the indicated times. F-actin levels in CD44low CD4+ and CD8+ were assessed by BODIPY-phallacidin staining of permeabilized cells and presented as mean fluorescence intensity (MFI) above background levels. Each point is the mean of three measurements ± SD. Data are representative of at least five experiments. ***, p ≤ 0.005; *, p ≤ 0.05.

**FIGURE 3.** Reduced chemokine responsiveness of PP T cells is not due to lower levels of chemokine receptor expression. A, TaqMan real-time quantitative PCR of RNA isolated from sorted naive (CD44low) or memory (CD44high) CD4+ T cells. Cells were sorted from PP or LN. Probes and primers specific for the indicated chemokine receptors were used. The data are presented as the fold difference in expression. See Materials and Methods for details. B, Biotinylated MIP-3β staining (bold lines) of fresh CD44low CD4+ LN and PP cells. Thin lines, Staining with control biotinylated cytochrome c. Stippled lines, Staining with conjugated streptavidin second step alone. C, Staining of LN (left panel) and PP (right panel) with a mAb specific for mouse CXCR3. Gating was on CD3+ cells. Ten percent of LN CD8+ (i.e., CD4+) and 13% of PP CD4+ cells were CXCR3+ with an MFI of 253 and 245, respectively; 49% of LN CD8+ and 45% of PP CD8+ cells were CXCR3+ with an MFI of 167 and 151, respectively. Staining with an isotype control mAb was negligible (data not shown). Density plots show cells gated for CD3 expression. D, CXCR3+ CD8+ cells gated as shown in C were assessed for their ability to polymerize actin in response to IP-10. ▲, LN cells; △, PP cells. Each point is the mean of three measurements ± SD.
Actin polymerization in response to chemokines is diminished in PP T cells

Chemokine-stimulated directed migration of T cells requires cell polarization, a process that involves cytoskeletal rearrangement and actin polymerization. Treating freshly isolated LN cells with chemokines led to very rapid and potent triggering of actin polymerization in T cells (Fig. 2, see also Fig. 3D). In contrast, T cells obtained from PP exhibited significantly reduced levels of actin polymerization (Figs. 2 and 3D), which was not due to a shift in kinetics (Fig. 2) nor in the dose-response curve (data not shown).

Chemokine receptor expression on PP T cells

Altered chemokine responsiveness by PP T cells could be explained by a reduction in chemokine receptor expression. We used real-time quantitative PCR to measure chemokine receptor RNA levels in T cells and found that CCR7 mRNA was more abundant in LN than in PP naive T cells (Fig. 3A). Lacking an Ab specific for murine CCR7, we assessed cell surface expression of CCR7 using a biotinylated form of murine MIP-3β. This chemokine was biologically active, as it stimulated migration as well as actin polymerization of T cells (data not shown). Biotinylated MIP-3β specifically stained freshly isolated T cells above the level of staining with control biotinylated cytochrome c (Fig. 3B). Comparison of MIP-3β binding by PLN and PP T cells showed that CCR7 levels were somewhat lower on PP T cells. Thus, the weaker response of PP T cells to SLC and MIP-3β might be due to decreased receptor expression or, alternatively, a reduction in receptor affinity.

This did not, however, appear to be the case for other chemokine receptors. For example, CXCR4 and CXCR3 mRNA levels were equivalent in T cells from either tissue (Fig. 3A). A recently developed Ab specific for mouse CXCR3 (E. Bowman, M. Hagen, T. Churakova, and L. McEvoy, manuscript in preparation) revealed that this chemokine receptor is similarly expressed on CD4+ and CD8+ T cells from PP and LN, and in fact the percentage of CD4+ T cells that are CXCR3+ is greater in PP than in LN (Fig. 3C). We compared actin polymerization in T cells from LN and PP stimulated with IP-10 (CXCL10), gating on CD8+ cells with equal levels of CXCR3 expression (Fig. 3C). The results showed that CXCR3+CD8+ PP T cells were less responsive than their LN counterparts (Fig. 3D). These results suggest that after entry into the PP, lymphocytes do not down-modulate surface levels of CXCR3; therefore, it appears that the PP microenvironment may affect lymphocyte responses to chemokines by disrupting the signals downstream of chemokine receptor ligation necessary for cytoskeletal reorganization and directed migration.

T cell hyporesponsiveness in PP is not limited to chemokine receptors

We next determined whether T cell responses to stimuli other than chemokines were also impaired in PP using actin polymerization as a readout. Both Con A and PMA stimulation led to consistently reduced levels of actin polymerization in PP T cells compared with LN T cells (Fig. 4). This suggests that in T cells that enter the PP, there is a general signaling defect which extends beyond chemokine receptors that minimally affects protein kinase C (PKC) activation. To determine whether signaling was globally affected, we assayed the ability of naive (CD45RBhigh) CD4+ T cells enriched from PP and LN to mobilize calcium in response to SLC. Fig. 5 illustrates that naive PP T cells exhibit decreased calcium flux compared with their LN counterparts. Chemotaxis can occur independently of calcium mobilization (36), thus reduced calcium flux may not be an underlying cause for the impaired chemotaxis of PP T cells. However, taken together, these data indicate that two intracellular processes elicited upon chemokine receptor ligation, PKC activation and calcium mobilization, are impaired in PP T cells.

The reduced response of PP T cells to chemokines is transient

The difference in the chemotactic responsiveness of naive T cells from PP and LN was surprising, based on elegant work that has...
shown that naive T cells are a homogeneous population that trafficks through all secondary lymphoid tissues without bias (reviewed in Refs. 3, 37, and 38). The reduced chemokine response of PP T cells could be due to selective trafficking of a heretofore unrecognized subset of naive T cells with inferior chemotactic capability to PP, or transient alteration of naive T cells after their entry into PP. To address these two possibilities, freshly isolated PP and LN cells were compared with those that were cultured for several hours. There was a temperature-dependent recovery of the chemotactic responsiveness of PP-derived T cells that was nearly complete by 3 h (Fig. 6). These results suggest a scenario in which naive T cells are transiently suppressed by the PP milieu, as opposed to one in which a subset of naive T cells exists that preferentially trafficks to PP, thus confirming earlier studies.

**T cell response to chemokines is determined by the local microenvironment**

The ability of in vitro incubation to reverse the impaired chemotaxis of PP-derived T cells suggested that the PP microenvironment transiently modulated T cell responsiveness. This was tested in vivo by adoptively transferring LN cells (i.e., lymphocytes that respond well to chemokines) from C57BL/6 mice into congenic SJL.B6 recipients. After 2–8 h, PLN and PP cells were assayed for their ability to migrate toward a chemokine gradient, and the cells that had migrated through the transwell were stained with a Ly5.1 mAb to distinguish transferred from resident lymphocytes. After as little as 2 h, transferred T cells that had migrated into PP exhibited a reduced chemotactic response compared with those that had entered PLN (Fig. 7). Thus, the local microenvironment of the PP can rapidly modulate T cell chemokine responses.

**The cellular, cytokine, and chemokine profiles of PP and LN are distinct**

It has long been recognized that PP and LN have contrasting cellular characteristics that predict unique microenvironments. Indeed, quantitative RT-PCR (TaqMan) revealed that LN and PP have distinct chemokine profiles. Several chemokines were more abundantly expressed in the LN, including SLC, MIP-3β, monocyte-derived chemokine (MDC) (CCL22), RANTES (CCL5), monokine induced by IFN-γ (Mig) (CXCL9), and IP-10 (CXCL10) (Fig. 8A). On the other hand, a small group of chemokines, MIP-3α (CCL20), thymus-expressed chemokine (TECK) (CCL25), and Vic (CCL28), were expressed 10–100-fold more in PP. Chemokine expression by DC was also analyzed, as this would impact on the ability of these cells to attract and interact with T cells. Mice were treated i.p. with an Ig fusion protein of human Flt3L (FL), a cytokine that expands DC populations in vivo (39). mRNA subsequently isolated from sorted FL-generated DC indicated that MIP-3β is expressed to a greater extent in LN DC. This was also the case for the CCR4 ligands MDC and thymus- and activated-regulated chemokine (CCL17) which, in humans, predominantly attract memory skin-homing T cells (40). TECK expression was similar in both populations of DC (Fig. 8B).

Parallel analysis of cytokine mRNA revealed that IL-2, IL-12, and IFN-γ were higher in LN, whereas IL-4 was expressed to a greater extent in PP (Fig. 8C). Because the higher proportion of B cells in the PP could create a dilution effect and to obtain a better approximation of the cytokines produced by cells in the T cell area of PP and LN, mRNA was prepared from PP and LN cell suspensions depleted of B cells (D). Chemokine and cytokine levels were assessed by real-time TaqMan quantitative PCR from reverse-transcribed, DNase-treated RNA. Data are shown as the fold difference in expression.
dramatically higher extent in PP (Fig. 8D). The potential contribution of IL-4 and IL-10, as well as IL-12 and IFN-γ, was further tested by analyzing mice genetically deficient in each of these cytokines. In each of these knockout strains, the differential responsiveness of LN and PP lymphocytes to chemokines or PMA cytokines was responsible for mediating suppression in the PP. We show that naive and memory T cells from PP exhibit a profound decrease in their chemokine responsiveness (data not shown). Thus, no single cytokine appears to be sufficient to explain the differences in LN and PP T cells.

Although the analysis of mice lacking individual cytokines was unrevealing, we hypothesized that the complex interplay of Th2 cytokines was responsible for mediating suppression in the PP. We therefore used a well-established model in which mice were infected with N. brasiliensis to ask whether a systemic Th2 response would elicit a global suppression in the LN. This helminthic parasite invades through the skin, eventually takes up residency in the gut, and is expelled from the host in a Th2 cytokine-dependent process (41, 42). Eight to 11 days after s.c. infection, PP were visibly smaller, sometimes 24 h PP had become noticeably smaller, sometimes

The contribution of bacteria in PP was ruled out based on several pieces of evidence. First, T cells from cecal patches, which lack PP, are associated with M cells that can transcytose microorganisms, respond well to chemokines (Fig. 1A). Second, systemic administration of LPS did not alter the responsiveness of LN or PP T cells to PMA at time points that correspond to peak production of TNF-α, IL-6, and IFN-γ in LPS-treated monocytes (45, 46) (data not shown). Finally, the chemokine and PMA responsiveness of PP T cells from C3H/HeJ mice, which are LPS-resistant due to a mutation in Toll-like receptor 4 (47–49), are still reduced compared with LN T cells (data not shown).

Discussion

We show that naive and memory T cells from PP exhibit a profound decrease in their ability to migrate and polymerize actin in response to chemokines. In this respect, PP were unique among all tissues tested (including LN, spleen, blood, and cecal patch). The response to several CC and CXC chemokines, including SLC, MIP-3β, SDF-1α, IP-10, and Mig, was affected, as was the residence time of T cells in PP. This could be addressed by measuring the rate at which T cells exited PP compared with their exit from PLN. DO11.10-transgenic splenocytes were transferred into BALB/c recipients and allowed to traffic for 2 h. Then, the mice received blocking Abs specific for L-selectin and α4 integrins, adhesion molecules necessary for T cell entry from the blood into PP and LN (50–52). The number of DO11.10+ T cells remaining in PP and LN was monitored thereafter, and the rate of exit was calculated. We found that T cells exhibited reproducibly increased turnover in PP than in LN 2 h after administration of blocking Abs (Fig. 10), and by 24 h PP had become visibly smaller, sometimes to the point of being barely detectable. Although LN T cell numbers may have been augmented by memory T cells entering independently of L-selectin and α4 integrin via the afferent lymphatics, donor splenic T cells from unchallenged DO11.10 mice are >90% L-selectinhigh (data not shown); therefore, memory T cells do not constitute a significant contribution to the LN values. Similar experiments were conducted in which SJL.B6-congenic splenocytes were transferred into C57BL/6 recipients and tracked using a Ly5.1-specific mAb, with comparable results (data not shown).

T cell residence time is shorter in PP than in LN

FIGURE 9. T cell hyporesponsiveness is exacerbated by a Th2 response and unaffected by chronic inflammation. A, Fresh PP and LN naive T cells from N. brasiliensis-infected (Nippo) or uninfected (NT) C57BL/6 mice were tested 8–11 days after infection for their response to 10 μg/ml Con A or 5 ng/ml PMA. Data are the average ± SD of five to six mice per group. B, Fresh PP and LN naive T cells from IL-10 knockout mice with IBD (10KO) or age-matched wild-type C57BL6 healthy controls (WT) were tested for their ability to polymerize actin in response to 10 nM SDF-1α or 5 ng/ml PMA. Data are the average ± SD of five mice per group. NT, Not treated.

FIGURE 10. T cells turn over more rapidly in PP compared with LN. Splenocytes from DO11.10 donors were transferred i.v. into BALB/c mice. Two hours after transfer, mice received i.v. injections of 100 μg each of anti-L-selectin (MEL-14) and anti-α4 integrin (PS/2). Untreated mice were sacrificed to represent time = 0. PP and LN were harvested from mice at the time points indicated after administering mAb, and CD4+ KJ1-26+ T cells remaining in the LN and PP were quantitated. The number of transferred cells remaining in Ab-treated mice was compared with that of the untreated controls and expressed as a percent thereof cells remaining. Two to four mice were assayed per time point, and the results are expressed as an average ± SD. p = 0.02 at 2 h. The results are representative of three experiments.

Were the repressed effects observed in T cells in vitro reflected by differences in their behavior in vivo? We reasoned that reduced chemokine responsiveness may directly or indirectly affect the residence time of T cells in PP. This could be addressed by measuring the rate at which T cells exited PP compared with their exit from PLN. DO11.10-transgenic splenocytes were transferred into BALB/c recipients and allowed to traffic for 2 h. Then, the mice received blocking Abs specific for L-selectin and α4 integrins, adhesion molecules necessary for T cell entry from the blood into PP and LN (50–52). The number of DO11.10+ T cells remaining in PP and LN was monitored thereafter, and the rate of exit was calculated. We found that T cells exhibited reproducibly increased turnover in PP than in LN 2 h after administration of blocking Abs (Fig. 10), and by 24 h PP had become visibly smaller, sometimes to the point of being barely detectable. Although LN T cell numbers may have been augmented by memory T cells entering independently of L-selectin and α4 integrin via the afferent lymphatics, donor splenic T cells from unchallenged DO11.10 mice are >90% L-selectinhigh (data not shown); therefore, memory T cells do not constitute a significant contribution to the LN values. Similar experiments were conducted in which SJL.B6-congenic splenocytes were transferred into C57BL/6 recipients and tracked using a Ly5.1-specific mAb, with comparable results (data not shown).

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A suboptimal response to chemokines can be expected to profoundly influence T cell activities. This is especially important in the case of SLC, which has been shown to be critical in the initiation of the immune response, as it plays a key role in directing T cells and DC into T cell areas of lymphoid tissues and promoting T cell activation (28). We found that CCR7 expression is decreased in naive PP T cells at the mRNA level, and furthermore PP
T cells display a reduced ability to bind the CCR7 ligand MIP-3β. Therefore, reduced receptor expression and/or affinity may in part explain differential responsiveness to SLC; however, hyporesponsiveness cannot always be explained by differential expression of chemokine receptors. In contrast to CCR7, the mRNA levels of CXCR3, CXCR4, and CCR6 were similar in PP and LN T cells. Furthermore, a mAb specific for CXCR3 indicated that cell surface levels of this receptor are nearly identical on PP and LN T cells. Nevertheless, a comparison of PP and LN T cells expressing CXCR3 to the same degree revealed that PP T cells had a reduced capacity to respond to the CXCR3 ligands Mig and IP-10. Thus, mechanisms other than receptor down-modulation or reduced ligand affinity may be responsible for the chemokine hyporesponsiveness of PP T cells.

The reduced responsiveness was not restricted to chemokines, since several other key T cell activation pathways were compromised. Actin polymerization, an early event in T cell polarization necessary for migration and adhesion, was reduced in response to not only chemokines, but also Con A and PMA, pointing to a general signaling defect. The impaired PMA-induced response indicates that signals downstream of PKC activation are affected in PP T cells. Inhibition of PKC can block TCR-stimulated actin polymerization (53), although less is known about whether chemokine-stimulated actin polymerization in T cells is similarly sensitive to PKC inhibitors. The observation that PKC activation in response to PMA was compromised in PP T cells suggested a more global suppression, prompting an analysis of calcium mobilization, a central signaling event in T cell activation (54). Indeed, chemokine-induced calcium mobilization is also affected. Future work will address the role of PKC and other candidate molecules that are targeted in, or elicited, T cell hyporesponsiveness in PP.

Two possibilities could account for the differential responsiveness of T cells in PP and LN: selective recruitment of a subset of hyporesponsive naïve T cells to PP or a microenvironment-induced hyporesponsiveness. Several lines of evidence argue in favor of the latter model. First, previous studies have convincingly argued that naïve T cells do not have a predilection for any particular secondary lymphoid tissue (3). Second, the hyporesponsiveness of PP T cells is transient in that it could be relieved in vitro by several hours of incubation. Third, LN T cells transferred to a congenic recipient took on the chemotactic characteristics of the lymphoid tissue to which they migrated, and this effect was apparent within hours of transfer.

The cytokine/chemokine network plays a key role in orchestrating immune and inflammatory responses. We therefore investigated the relative expression of candidate soluble factors in PP and LN using real-time quantitative PCR. Analysis of a large panel of chemokines revealed striking differential expression of several chemokines, including SLC, MIP-3β, Mig, and MDC, which are more abundant in LN compared with PP (Fig. 8). This differential expression is of particular interest since one potential for reduced responsiveness is desensitization of the receptor in the presence of high concentrations of the ligand. The suppressed response of naïve PP T cells to SLC and MIP-3β, as well as IP-10 and Mig, is clearly not due to desensitization of the cognate receptors, since expression of these ligands at the mRNA level is lower in PP than in LN. The most striking observation was the dramatically higher expression levels of MIP-3α, TECK, and Vic in PP (Fig. 8). The receptors for these ligands are expressed on subsets of memory T cells rather than naïve T cells (7) and thus they cannot be directly responsible for the reduced chemokine responsiveness of naïve PP T cells. However, the tissue-selective recruitment of other leukocyte subsets by these chemokines may indirectly alter naïve T cell responsiveness by affecting the cellular makeup of these tissues.

The cytokine makeup of PP also contrasts with that of LN. Upon B cell depletion, IL-4 and IL-10 mRNA are expressed more highly in PP. On the other hand, IL-12 and IFN-γ are more abundant in LN. Therefore, the PP appears to be a generally “Th2-type” milieu while LN exposes T cells to a more “Th1-type” environment. Distinct populations of DC may contribute to this difference in cytokine levels, as in vitro priming using PP DC generates T cells that express higher levels of IL-4 and IL-10 and lower levels of IFN-γ compared with T cells primed in the presence of splenic DC (55, 56). This may be due to higher levels of IL-10 secreted by PP DC (56). IL-4 has been reported to affect the cytoskeleton in neutrophils (57), and T cells polarized in vitro toward a Th2 phenotype using IL-4 have a distinct morphology compared with Th1 cells that were generated with IL-12 and IFN-γ.

Although analysis of knockout mice did not reveal a role for the cytokines differentially expressed in PP and LN, the suppressive effect may be multifactorial. This has been suggested by a recent study by D’Amico et al. (58), who showed that IL-10, in the presence of inflammatory signals, converts the inflammatory chemokine receptors CCR1, CCR2, and CCR5 on DC or monocytes into “decoy” receptors that bind their cognate ligands but fail to signal. We therefore asked whether a systemic Th2 response had a suppressive effect on T cell responses and found that infection with N. brasiliensis suppressed LN T cell responses and exacerbated PP T cell hyporesponsiveness. Interestingly, a chronic Th1 response modeled in IL-10 knockout mice with IBD did not have the converse effect. In summary, our results suggest that a Th2-type response such as that elicited by a helminthic infection can dampen T cell stimulation, although the exact mechanism remains to be determined.

Together, these results suggest that the PP microenvironment has the unique ability to render naïve T cells hyporesponsive to several physiological stimuli, including chemokines, that are key to the initiation of an immune response. We propose that impaired chemokine-stimulated migration, actin polymerization, and possibly adhesion triggering has consequences on T cell activation. The impaired migration of T cells toward chemokine-expressing DC, along with the reduced expression of chemokines by PP DC, would severely compromise DC-T cell interactions. Such hyporesponsiveness might raise the threshold required for T cell activation that can only be overcome by specific circumstances, e.g., Ag presentation in the context of a pathogenic/microbial signal (however, normal gut flora or LPS appear to be insufficient), or an altered cytokine profile in which regulatory cytokines such as TGF-β and IL-10 are less dominant. The observed impairment in actin polymerization may compromise the formation of the immunological synapse with APC that is critical for T cell activation (59–62). Furthermore, our results show that T cells in the PP exit faster than those in the LN. A shorter retention time may serve to limit the exposure of T cells to APCs presenting nonpathogenic gut Ags that do not warrant an immune response.

LN and PP differ in numerous respects, from their anatomical location to their cellular makeup and microarchitecture. These are adaptations that the immune system has evolved to address the distinct situations encountered by the immune system in the periphery and the gut, respectively. In contrast to PLN, mucosal lymphoid tissues are continuously exposed to large amounts of externally derived Ag. The challenge of the mucosal immune system is to mount protective immune responses against enteric pathogens while avoiding responses to innocuous Ags derived from ingested food or commensal bacteria (i.e., oral tolerance) (33, 63, 64). Our results add to the growing understanding of the nature of immune
responses in the PP, underscore the importance of microenvironmental regulation of T cell function, and suggest potential means of manipulating the nature and intensity of an immune response.

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