P-Selectin Can Support Both Th1 and Th2 Lymphocyte Rolling in the Intestinal Microvasculature

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Lymphocyte localization to inflammatory sites is paramount for developing and maintaining an immune response. Rolling is the first step in recruitment, but our knowledge of its mechanisms in Th1 and Th2 CD4⁺ lymphocytes is incomplete. Whereas initial studies suggested that Th1 but not Th2 lymphocytes used P-selectin for recruitment, more recent studies have proposed that both subtypes bind selectins. We used intravital microscopy to demonstrate *in vivo* that polarized Th1 and Th2 lymphocytes both use P-selectin to roll and adhere to cytokine [tumor necrosis factor (TNF)- α or interleukin (IL)-4]-activated intestinal microvasculature. The majority of Th1 lymphocyte flux in TNF- α - and IL-4-treated animals was P-selectin-dependent. Th1 lymphocytes also interacted with E-selectin to control rolling velocity after TNF- α stimulation. Th2 lymphocytes, which make IL-4 but not interferon- γ , bound P-selectin *ex vivo*, with more than 95% rolling on P-selectin *in vivo*. Both Th1 and Th2 lymphocytes regulated rolling velocity by interacting with α_4 -integrin. Furthermore, in a model of spontaneous intestinal inflammation (ie, IL-10-deficient mice), both Th1 and Th2 lymphocytes rolled, adhered, and ultimately emigrated into the local microenvironment. These results suggest that both Th1 and Th2 lymphocytes use P-selectin in the initial rolling step *in vivo* in response to a global activator of the vasculature (TNF), a subtle inducer of P-selectin (IL-

4), and pathological inflammation (IL-10-deficient mice). (*Am J Pathol* 2005, 167:1647–1660)

An immune response is primarily regulated by cytokines differentially produced by two distinct CD4⁺ T helper (Th) lymphocytes, Th1 and Th2 cells. Moreover, the imbalance between Th1 and Th2 immunity has been well described as critical for the outcome of various diseases.^{1,2} Th1 effector lymphocytes promote a Th1 cytokine milieu [eg, tumor necrosis factor (TNF)- α , interferon (IFN)- γ , and interleukin (IL)-1 β], are involved in cellular immunity and are usually observed in clinical cases of chronic inflammation and have been implicated in the development of Crohn's disease, multiple sclerosis, and arthritis.^{3–6} By contrast, Th2 effector lymphocytes initiate a Th2 cytokine milieu (eg, IL-4, IL-5, and IL-10), promote humoral immunity, and are associated with allergic reactions to environmental antigens such as asthma, ulcerative colitis, and other diseases.^{2,3} However, the mechanism(s) by which Th1 and Th2 lymphocytes are recruited to sites of inflammation continue to be an important area of research.

The current paradigm of general leukocyte recruitment suggests that cells are recruited via a series of interdependent and closely regulated adhesive interactions between the circulating leukocytes and the vascular endo-

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thelium. Circulating leukocytes first tether to and roll along the endothelium via selectins (eg, P-selectin and E-selectin).^{7,8} Although more recently, a number of additional molecules including α_4 -integrin, CD44, and VAP-1 have also been demonstrated to induce rolling. If appropriate signals (chemokines) are encountered, leukocytes then firmly adhere to the vessel wall via integrins before transmigrating into the surrounding tissue.^{9–11} Clearly, the initial tethering and rolling events will regulate which cell type is recruited to the endothelial interface to encounter chemokines. Alternatively, if all leukocytes bind selectins then their selection into tissues may be regulated further downstream, presumably by chemokines. It is also possible that there is no real selection process at any of these levels and both cell types enter the tissue.

The importance of selectins for the initial tethering and rolling for Th1 and Th2 lymphocytes *in vivo* remains somewhat controversial because our knowledge of the process is not complete. For example, initial *in vitro* studies suggested that Th1 but not Th2 lymphocytes used P-selectin for recruitment. For example, Austrup and colleagues,^{12–15} have reported that Th1-polarized cells infiltrate skin and joints via P-selectin. By contrast, Th2-polarized cells lacked adequate expression of P-selectin ligand/s and must therefore use some other molecule(s) to infiltrate sites of inflammation.¹³ More recently however, isolation from lymphoid tissues of already polarized T cells revealed that Th1 and Th2 lymphocytes expressed similar levels of P-selectin ligands as assessed by flow cytometric binding of P-selectin IgM fusion protein.^{16,17} However, a demonstration that the Th2 cells actually used P-selectin was not shown. The limitation seems to be related to the fact that *in vitro*, Th2 cells do not express P-selectin whereas *in vivo* simply adding P-selectin antibody to an animal does not necessarily reveal what cell-type is the target of P-selectin inhibition. What is needed is an ability to produce P-selectin-expressing Th2 cells *in vitro* to assess the importance of P-selectin-dependent recruitment *in vivo*.

In this study we have successfully polarized CD4⁺ T cells toward either cells that produced IFN- γ and not IL-4 (Th1) or cells that produced IL-4 but not IFN- γ (Th2) but have the ability to bind P-selectin. We then used intravital microscopy to investigate two key issues in Th1/Th2 trafficking in the microvasculature. Firstly, we examined whether the number of Th1 and Th2 lymphocyte-endothelial interactions differ in intestinal microvasculature in response to a potent global stimulus such as TNF- α that induces P-selectin, E-selectin, and other adhesion molecules or a more subtle stimulus IL-4 known to primarily induce P-selectin but not E-selectin. To stimulate the intestinal vasculature the cytokines had to be administered intravascularly which induces rolling, adhesion, but not emigration. Therefore, in a second series of experiments, we determined what mechanisms were used by Th1 and Th2 lymphocytes to roll in the vasculature of a well-described mouse model of irritable bowel disease (IBD), the IL-10-deficient mouse, and whether both cell types would actually emigrate into the tissues.

Materials and Methods

Reagents and Mice

RB40.34 (anti-P-selectin), R1-2 (anti- α_4 -integrin), GAME-46 (anti- β_2 -integrin), 11B11 (anti-IL-4), XMG1.2 (anti-IFN- γ), JES5-16E3 (anti-IL-10), and isotype control antibodies were purchased from BD Biosciences (Mississauga, Canada). RME-1 (anti-E-selectin) was a kind gift from Dr. A. Issekutz (Halifax, Canada). TNF- α and IL-4 were purchased from R&D Systems (Minneapolis, MN). BALB/c (Charles River Laboratories, Montreal, Canada), DO11.10 (gift from Dr. C. Weaver, Birmingham, AL), 129svev and IL-10-deficient mice (bred in house) were maintained in the viral antigen-free double-barrier unit at the University of Calgary. BALB/c and DO11.10 mice were used once they weighed between 20 and 25 g and were between 6 to 10 weeks of age, and the 129svev and IL-10-deficient mice were used between 10 to 13 weeks of age. All experimental procedures were approved by the Animal Care Committee of the University of Calgary and conform to the guidelines established by the Canadian Council for Animal Care.

Lymphocyte Purification and Characterization of OVA-Specific Th1 and Th2 Cells

CD4⁺ T cells were isolated from pooled spleens of DO11.10 mice using mouse CD4 (L3T4) Dynabeads per the manufacturer's protocol (DynaL Biotech Inc., Brown Deer, WI). The resulting CD4⁺-enriched cells were stained with fluorescein isothiocyanate-conjugated KJ1-26 (anti-DO11.10 TCR)¹⁸ and phycoerythrin (PE)-conjugated anti-CD4 and analyzed by flow cytometry to determine purity of the population. Total events ($n = 10,000$) were acquired on a Becton Dickinson FACScan (Mountain View, CA), gating on cells with forward and side scatter properties of lymphocytes, and were analyzed using CELLQuest software. Isolations routinely resulted in greater than 90% purity of CD4⁺ cells.

Th1 and Th2 cells were generated *in vitro* from antigen-naïve CD4⁺ T cells as described previously.¹⁹ Briefly, purified CD4⁺ T cells were cultured at a ratio of 1:5 (for Th1) and 1:25 (for Th2) with irradiated BALB/c splenocytes and 5 μ g/ml OVA peptide 323-339. The addition of 50 U/ml of IL-12 (R&D Systems) and 10 μ g/ml of anti-IL-4 (11B11)²⁰ was used to generate Th1 cells, whereas 1000 U/ml of IL-4 (R&D Systems) and 10 μ g/ml of anti-IL-12 (C17.8)²¹ were used to generate Th2 cells.²² To ensure that the majority of *ex vivo* polarized cells were of a Th2 phenotype that bound P-selectin, the cells went through a second 7-day period of culture, as above. Recovered cells were washed and transferred on day 7 (for Th1) and day 14 (for Th2) at 10^7 cells/recipient mouse. Although initially, we also compared Th1 cells after two cycles of cytokines and Th2 cells after a single cycle of cytokines and observed similar trends to those described in the results, ultimately we settled on the above-described protocol as it generated the best polarized P-selectin-expressing Th1 and Th2 cells (see companion paper by

Mangan and colleagues²³ in this issue). A separate aliquot of 5×10^6 cells was restimulated for 6 hours with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml; Sigma Chemical Co., St. Louis, MO) and ionomycin (750 ng/ml; Sigma Chemical Co.) for analysis of cytokine expression. Intracellular cytokine staining was used to assess cytokine expression with the Cytofix/Cytoperm Plus with GolgiPlug Kit (BD BioSciences) following the manufacturer's instructions. Permeabilized cells were incubated with PE-conjugated anti-IL-4 (11B11, BD BioSciences), anti-IL-10 (JES5-16E3, BD BioSciences), or anti-IFN- γ (XMG1.2, BD BioSciences) and analyzed with a BD Biosciences FACScan. Unstimulated cells and a PE-labeled control monoclonal antibody (mAb) of the same isotype were used as controls in all experiments.

Functional P-selectin and E-selectin ligand expression was determined by P- and E-selectin binding using a murine P- or E-selectin/human IgM chimeric Ab (a gift from J. Lowe, University of Michigan, Ann Arbor, MI), followed by staining with biotin-goat anti-human IgM (1:100 dilution, Sigma Chemical Co.) and streptavidin-PE (1:50 dilution, BD Biosciences). Parallel staining with selectin-Ig in the presence of ethylenediamine tetraacetic acid served as controls.

For real-time polymerase chain reaction (PCR) quantitation of FucTIV and FucTVII cDNA, primers and TaqMan probes were designed using Primer Express software (Applied Biosystems, Foster City, CA), and had the following sequences: FucTIV: forward primer GCTGGTAC-TACGCGTGTTCGA, reverse primer CTACGGTCTCCAGGGCTTTG, and TaqMan probe ACCAGGAGGGAG-CAGTGACGCTAACTG (5'FAM, 3'TAMRA); FucTVII: forward primer TGAACCTACAGTTCAAGGGTACCA, reverse primer AAACCAAATTACCATGAATGTTGCT, and TaqMan probe CACCAGGAGGCTGCGGGCC (5'FAM, 3'TAMRA). All oligos were synthesized by Sigma Genosys, probes were purified by high performance liquid chromatography.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from 1×10^7 stimulated T cells using the RNeasy mini kit with on-column DNase digestion (Qiagen, Valencia, CA). RNA was quantified by absorption at 260 nm with a Biomate 3 spectrophotometer (ThermoSpectronic, Burlington, ON, Canada). Total RNA (1.5 μ g) was reverse transcribed using the RevertAid H minus first strand cDNA synthesis kit (Fermentas, Burlington, ON, Canada) as detailed in the manufacturer's guidelines.

TaqMan Real-Time PCR Assay

Real-time PCR was performed on the ABI Prism 7000 sequence detection system (Applied Biosystems). TaqMan PCR was performed in a total volume of 25 μ l containing $1 \times$ TaqMan Universal Master Mix (Applied Biosystems), 900 nmol/L each primer, 100 nmol/L probe, and 75 ng cDNA. Default thermocycler conditions were used: stage 1, 50°C for 2 minutes for one cycle; stage 2,

95°C for 10 minutes for one cycle; and stage 3, 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. Each PCR was performed in duplicate, and each sample was analyzed in parallel for FucTIV, FucTVII, as well as GAPDH to normalize for inefficiencies in cDNA synthesis and RNA input amounts. Controls were included for all PCRs to exclude PCR amplification of contaminating genomic DNA and to ensure that amplification was not due to contamination of other components within the PCR mix. Gene expression assays were validated by analysis of a standard curve to demonstrate that PCR efficiency was within 10% for all primer-probe sets. Gene expression data were subsequently calculated by the delta Ct method using the following formula: $dCT = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}$, where Ct is the cycle number at which PCR amplification crosses threshold.

Quantification of P-Selectin Expression

Expression of P-selectin was determined as a measure of endothelial activation using a modified dual-radiolabeled Ab technique.^{24,25} The Abs RB40.34 (against P-selectin) and A110-1 (a rat IgG₁, λ isotype control) were labeled with either ¹²⁵I (RB40.34) or ¹³¹I (A110-1) using the Iodogen method as previously described.^{24,25} A110-1 was used to control for nonspecific binding in the murine system.

To determine quantitative P-selectin expression, animals were injected intravenously with a mixture of 10 μ g of ¹²⁵I-RB40.34, and a variable dose of ¹³¹I-A110-1 calculated to achieve a total injected ¹³¹I activity of 400,000 to 600,000 cpm (total volume, 200 μ l). The Abs were allowed to circulate for 5 minutes, then the animals were treated with heparin. A blood sample was obtained from the carotid artery catheter, and the mice were exsanguinated. The lungs, heart, liver, mesentery, small intestine, large intestine, muscle, skin, and stomach were harvested and weighed. ¹³¹I and ¹²⁵I were measured in plasma and tissue samples. P-selectin expression was calculated per g of tissue, by subtracting the accumulated activity of the nonspecific Ab (¹³¹I-A110-1) from the accumulated activity of the P-selectin Ab (¹²⁵I-RB40.34). P-selectin data were represented as the percentage of the injected dose of Ab per g of tissue. It has been previously demonstrated that this approach provides reliable quantitative values of adhesion molecule expression, and that radiolabeled binding Ab can be displaced specifically with sufficient amounts of unlabeled Ab. The technique is sufficiently sensitive that basal levels of P-selectin can be detected in wild-type mice relative to P-selectin-deficient mice.^{24–26}

Intravital Microscopy in the Intestine

Mice were prepared for surgery using isoflurane as an inhalation anesthetic as previously described.²⁷ For intravital microscopy briefly, the jugular vein was cannulated for the administration of fluorescently labeled cells and antibodies. A segment of the small intestine is exteriorized through an abdominal incision and draped over a

viewing pedestal and the exposed tissue is suffused with bicarbonate-buffered saline (pH 7.4, temperature 37°C) to avoid temperature changes and drying out. Mice are allowed to stabilize for 15 minutes after surgery. The intestinal microcirculation is visualized by autofluorescence before experiments for identification of postcapillary venules. Endogenous leukocytes were visualized by intravenous injection of rhodamine 6G. In a separate series of experiments prelabeled subsets of T lymphocytes were injected into the jugular vein and their rolling and adhesion were visualized in the intestine. Lymphocytes were labeled with rhodamine 6G ($25 \mu\text{g}/10^7$ cells, Sigma Chemical Co.) for 5 minutes at room temperature before extensive washing and intravenous injection. This permits visualization of lymphocytes in the vascular bed but does not affect leukocyte-endothelial cell interactions in a nonspecific way. Rhodamine 6G-associated fluorescence is visualized by epi-illumination at 510 to 560 nm, using a 590-nm emission filter. A microscope (Optiphot-2; Nikon Inc., Mississauga, Canada) with $\times 25$ lens (water immersion, Leitz Wetzlar L25/0.35) and a $\times 10$ eyepiece was used. A silicon-intensified fluorescence camera (model C-2400-08; Hamamatsu Photonics, Hamamatsu City, Japan) mounted on the microscope projects the image onto a monitor. The image was recorded using a video camera (Panasonic-Digital 5100) and a video recorder (Panasonic NV8950) as previously described by our laboratory.^{26,28} The microcirculation of the lamina propria can be assessed from the serosal side in the closed part of the intestine by changing the depth of focus of the microscope.²⁹

Five randomly selected postcapillary venules with a diameter between 20 and 30 μm were observed throughout the 30- to 45-minute time span during each experiment to obtain a true representation of an overall response. All experiments were recorded for later analysis and scored in a blinded manner. Rolling leukocytes were defined as those cells moving at a velocity less than that of erythrocytes within a given vessel. Leukocytes were considered adherent if they remained stationary for 30 seconds or longer.

Experimental Protocol

TNF- α ($25 \mu\text{g}/\text{kg}$) or IL-4 ($100 \text{ ng}/\text{mouse}$) was administered intraperitoneally to BALB/c mice before analysis.^{25,30} This is a model we have used previously to determine the molecular mechanisms underlying rolling of leukocytes on endothelium *in vivo* in any organ microcirculation. It should however be noted that no leukocyte emigration takes place and no pathology occurs. Antibodies to the following adhesion molecules P-selectin ($20 \mu\text{g}$), E-selectin ($100 \mu\text{g}$), α_4 -integrin ($70 \mu\text{g}$), or β_2 -integrin ($30 \mu\text{g}$) in a final volume of 200 μl of sterile saline were administered intravenously. Preliminary experiments and previous studies have shown that 5 minutes is required for optimal inhibition of rolling by P- and E-selectin, 20 minutes is required for optimal inhibition of rolling by α_4 - and β_2 -integrin, and that isotype-matched Ab has no effect on leukocyte recruitment.^{8,31} For exper-

iments requiring pretreatment, antibodies were administered at the same concentrations and pretreatment times as described above.

Isolation of Intestine-Infiltrating Lymphocytes

The isolation of intestine-infiltrating lymphocytes was modified from that previously described.³² Briefly, IL-10-deficient mice were anesthetized and injected intravenously with 5×10^7 carboxy-fluorescein succinimidylester (CFSE)-labeled ($5 \mu\text{mol}/\text{L}$ for 10 minutes at room temperature; Molecular Probes, Invitrogen Canada Inc., Burlington, Canada) Th1 or Th2 cells. After 24 hours the mice were again anesthetized and perfused via the left ventricle after clipping the right atrium. The perfused small and large bowel were obtained from the mice and minced in digestive medium containing 0.05% collagenase and 0.002% DNase I. After gentle agitation at 37°C for 60 minutes, the digest was passed through a 40- μm nylon mesh and then washed twice with phosphate-buffered saline (PBS). Cells were then subjected to density gradient centrifugation on Lympholyte-M (Cedarlane Laboratories, Hornby, Canada) to isolate the lymphocyte population. Intestinal lymphocytes were resuspended in PBS, and viability was determined by trypan blue exclusion. CD4⁺ T cells were isolated from the intestines using mouse CD4 (L3T4) Dynabeads per the manufacturer's protocol (DynaL Biotech). The resulting CD4⁺-enriched cells were analyzed by flow cytometry to determine CFSE-T-cell infiltration into the intestinal interstitium. No less than 25,000 total events were acquired on a Becton Dickinson FACScan, gating on cells with forward and side scatter properties of lymphocytes, and were analyzed using CELLQuest software.

Statistical Analysis

All results are expressed as mean $\pm$ SE (SEM). An unpaired Student's *t*-test was used for comparisons between 2 means with a Welch's correction where necessary, and analysis of variance followed by Newman-Keul posthoc test was used for comparisons between more than 2 means. Statistical significance was set at $P < 0.05$.

Results

Characterization of Th1 and Th2 Lymphocytes

To confirm the polarization of the CD4⁺ splenocytes into Th1 and Th2 lymphocytes we assessed their production of cytokines after ionomycin and PMA stimulation. By intracellular cytokine staining we observed that $\sim 75 \pm 6\%$ of the activated Th1 lymphocytes produced the Th1 cytokine IFN- γ and less than 1% of these cells produced the Th2 (IL-4 and IL-10) cytokines. By contrast, the majority of the stimulated Th2 lymphocytes produced IL-4 and less than 1% produced IFN- γ (Figure 1). These observations were identical to those presented in more detail by Mangan and colleagues.²³

Figure 1. Cytokine production by PMA/ionomycin-treated Th1 and Th2 cells. CD4⁺ lymphocytes were polarized to a Th1 or Th2 phenotype and subsequently treated with PMA and ionomycin to investigate their cytokine profile. Intracellular staining of IFN- γ or IL-4 production was assessed by flow cytometric analysis. Data are expressed as the arithmetic mean $\pm$ SEM of five separate polarizations.

Induction of Vascular Inflammation after TNF- α and IL-4 Treatment

To directly examine the activation of the intestinal endothelium, we investigated the expression of P-selectin in response to TNF- α and IL-4. As shown in Figure 2, only very low levels of P-selectin were detected in the small intestine of control mice. After 5 hours of TNF- α treatment (25 μ g/kg, i.p.) we observed a profound increase in P-selectin expression by intestinal endothelium. In the IL-4-treated mice (100 ng/mouse, i.p.), P-selectin expression was also significantly increased when compared to control values. It should be noted that, TNF- α induced a 10-fold increase in P-selectin expression over that observed by IL-4 (Figure 2).

Figure 2. Increased P-selectin expression in the small intestine of TNF- α and IL-4-treated mice. Radiolabeled P-selectin antibody was administered to untreated animals (control) and animals treated with TNF- α or IL-4. Data are expressed as the arithmetic mean $\pm$ SEM of four animals per group. * P < 0.05 relative to control; ** P < 0.05 relative to TNF- α treatment group.

Th1 Lymphocyte Rolling and Adhesion Is Increased in the Intestine of TNF- α - and IL-4-Treated Mice

We used intravital microscopy to observe *in vivo* naïve lymphocyte endothelial interactions in the intestine of untreated and TNF- α - or IL-4-treated mice. Without polarization to either Th1 or Th2, antigen-naïve CD4⁺ lymphocytes do not roll along the endothelium of control or cytokine-treated mice (data not shown). As shown in Figure 3, polarized Th1 cells will roll on the endothelium of untreated mice and within 5 hours of TNF- α or IL-4 administration this rolling flux increased significantly. In the postcapillary venules of TNF- α -treated mice we observed more than a twofold increase in Th1 lymphocytes rolling per minute compared to controls and a threefold increase in the IL-4-treated mice (Figure 3A). We next determined the velocity with which these cells rolled. Figure 3B shows that Th1 lymphocytes in the intestinal vasculature of TNF- α -treated mice rolled significantly slower than that in control mice. The velocity of the Th1 lymphocytes in the intestine of IL-4-treated mice was equivalent to that observed in controls. A reduction in rolling velocity allows for leukocytes to accumulate within the intestinal microvessels. We calculated the number of rolling cells per 100- μ m length (flux/rolling velocity) and as shown in Figure 3C, we observed 1.3 ± 0.5 rolling Th1 cells per 100 μ m in the control mice. By contrast, we observed a more than threefold and twofold increase in rolling Th1 cells per 100 μ m in the TNF- α - and IL-4-treated mice, respectively. In the control animals we observed very few cells adhering to the postcapillary venules and a tenfold and fivefold increase in adhesion for the TNF- α - and IL-4-treated mice, respectively (Figure 3D).

Th2 Lymphocyte Rolling and Adhesion Is Increased in the Intestines of TNF- α - and IL-4-Treated Mice

We next investigated the Th2 lymphocyte endothelial interactions in the postcapillary venules of the intestine of control, TNF- α -treated, and IL-4 treated mice. Compared to the control group, in the TNF- α - and IL-4-treated mice we observed a 30% and 50% increase in Th2 lymphocytes rolling flux (Figure 4A). Figure 4B shows that Th2 lymphocytes in the intestinal vasculature of unstimulated mice rolled at a velocity of 57 ± 8 μ m/second. In TNF- α - as well as IL-4-treated mice Th2 cells rolled $\sim$ 35% slower than that of the control group (Figure 4B). From these data we calculated the number of rolling cells per 100- μ m length (flux/rolling velocity). As shown in Figure 4C, we observed 1.6 ± 0.5 rolling Th2 cells per 100 μ m in the control mice. By contrast, we observed a more than twofold increase in rolling Th2 cells per 100 μ m in both the TNF- α - and IL-4-treated mice. In the intestinal vasculature of unstimulated mice we observed very minimal adhesion of Th2

Figure 3. Effect of TNF- α and IL-4 treatment on Th1 lymphocyte trafficking in the small intestine. Animals were treated with saline (control), TNF- α , or IL-4 for 5 hours before Th1 cell injection. Th1 rolling flux (A), Th1 rolling velocity (B), the number of Th1 cells rolling per 100 μ m (C), and Th1 adhesion (D) in postcapillary venules of the small intestine were determined by intravital microscopy. Data are expressed as the arithmetic mean $\pm$ SEM of at least five animals per group. * P < 0.05 relative to control group; # P < 0.05 relative to TNF- α treatment group.

cells with a 10-fold increase in adhesion of Th2 cells in TNF- α -treated mice and a fivefold increase in IL-4-treated mice (Figure 4D). Taken together these results suggest that Th2 lymphocytes can roll in the intestinal vasculature when an inflammatory response is induced by either TNF- α or IL-4. Interestingly, these results also suggest that Th2 cells interact to a greater degree with TNF- α -activated intestinal endothelium as opposed to IL-4-activated endothelium, which closely correlated with increased P-selectin expression.

A Dominant Role for P-Selectin in Th1 Lymphocyte Rolling on the Intestines of TNF- α - and IL-4-Treated Mice

To determine the mechanisms by which Th1 lymphocytes roll in the intestinal microvasculature of TNF- α - and IL-4-treated mice, intravital microscopy was used to directly observe the effects of adhesion molecule blockade on Th1 cell recruitment. Because many *in vitro* studies implicate P-selectin, E-selectin, and α_4 -integrin as possible mediators of leukocyte recruitment we proceeded to examine their role *in vivo*.^{7,33,34} After baseline rolling was determined in the TNF- α - and IL-4-treated mice, antibodies to P-selectin, E-selectin, or α_4 -integrin were administered intravenously and lymphocyte rolling was observed 5 to 20 minutes later. As shown in Figure 5A, with the addition of a blocking antibody to P-selectin Th1 cell

rolling in TNF- α -treated mice was completely abolished. With the addition of blocking antibodies to either E-selectin or α_4 -integrin no change in rolling flux was observed (Figure 5A). By contrast, both E-selectin and α_4 -integrin play a role in controlling the velocity of Th1 cells in TNF- α -treated mice (Figure 5B). Notably, the velocity with which the Th1 cells rolled after administration of Ab to either E-selectin or α_4 -integrin increased to a level observed in the control mice (Figure 5B). The addition of an isotype control antibody did not alter Th1 rolling flux or velocity (Figure 5, A and B).

In a similar set of experiments we investigated the mechanisms by which Th1 lymphocytes roll in the intestine of IL-4-treated mice. We observed that blocking P-selectin completely inhibited all Th1 cells from rolling (Figure 5C). Neither E-selectin nor α_4 -integrin affected Th1 rolling flux (Figure 5C). Blocking α_4 -integrin had a slight but significant effect on Th1 cell velocity in the IL-4 microenvironment (49 ± 5 versus 69 ± 7 μ m/second, respectively; Figure 5D). The addition of an isotype control antibody did not alter Th1 rolling flux or velocity (Figure 5, C and D). Taken together these results suggest that P-selectin has a major role in mediating Th1 lymphocyte rolling in the intestinal vasculature of TNF- α - as well as IL-4-treated mice whereas a role for α_4 -integrin is minor for Th1 cells. In addition, these results identify a role for E-selectin in mediating Th1 cell-rolling velocity in TNF- α -treated but not IL-4-treated mice.

Figure 4. Effect of TNF- α and IL-4 treatment on Th2 lymphocyte trafficking in the small intestine. Animals were treated with saline (control), TNF- α , or IL-4 for 5 hours before Th1 cell injection. Th2 rolling flux (A), Th2 rolling velocity (B), the number of Th2 cells rolling per 100 μ m (C), and Th2 adhesion (D) in postcapillary venules of the small intestine were determined by intravital microscopy. Data are expressed as the arithmetic mean $\pm$ SEM of at least five animals per group. * $P < 0.05$ relative to control group; ** $P < 0.05$ relative to TNF- α treatment group.

P-Selectin and α_4 -Integrin Mediate Th2 Lymphocyte Rolling along the Intestinal Microvasculature of TNF- α - and IL-4-Treated Mice

We next investigated the mechanisms by which Th2 cells rolled in the intestinal vasculature of TNF- α - and IL-4-treated mice. As shown in Figure 6A, blocking P-selectin completely abolished all Th2 lymphocyte rolling in the intestine of TNF- α -treated mice. The administration of antibodies to either E-selectin or α_4 -integrin did not reduce Th2 rolling flux in these mice (Figure 6A). Blocking E-selectin had no effect on the velocity of Th2 cells in TNF- α -treated mice (Figure 6B). By contrast, blocking α_4 -integrin significantly increased Th2 rolling velocity to control levels (Figure 6B). The administration of isotype control antibodies did not alter Th2 rolling flux or velocity (Figure 6, A and B).

In a separate series of experiments, we investigated the adhesion molecules used for Th2 cell rolling in the intestine of IL-4-treated mice. As shown in Figure 6C, blocking P-selectin also completely abolished Th2 lymphocyte rolling in these mice. The administration of an antibody to α_4 -integrin had no effect on Th2 cell rolling flux but significantly increased Th2 lymphocyte rolling velocity to control levels (Figure 6D). The addition of antibodies to either E-selectin or an isotype control did not affect Th2 rolling flux or velocity in these mice (Figure 6, C and D). Taken together these results

suggest that P-selectin and α_4 -integrin, but not E-selectin, mediate Th2 lymphocyte rolling in the intestine of TNF- α - as well as IL-4-treated mice.

P-Selectin Binds Both Th1 and Th2 Lymphocytes Whereas E-Selectin Only Binds Th1 Cells

To confirm the expression of functional ligands to P- and E-selectin on our Th1 and Th2 lymphocytes we used chimeric fusion proteins and flow cytometric analysis. Although a more comprehensive listing of these results can be found in the companion paper by Mangan and colleagues,²³ Table 1 shows that both Th1 and Th2 lymphocytes bound to the P-selectin fusion protein. These observations were confirmed by real-time PCR investigation of fucosyltransferase mRNA levels. As shown in Table 1, comparable levels of FucTIV and FucTVII were present in both Th1- as well as Th2-polarized cells. Interestingly, there was a clear difference in the ability of Th1 and Th2 lymphocytes to bind to an E-selectin fusion protein suggesting minimal expression of an E-selectin ligand by the Th2 cells (Table 1). Using flow cytometry, we confirmed that on stimulation Th1 and Th2 cells that produced IFN- γ and IL-4 also bound P-selectin, respectively (Table 1).²³

Figure 5. Effects of adhesion molecule blockade on Th1 rolling in TNF- α - and IL-4-treated mice. Animals were treated with TNF- α or IL-4 and after Th1 injection and basal levels of rolling recorded, blocking antibodies to P-selectin, E-selectin, α_4 -integrin, and isotype controls were administered. **A** and **C**: Effect of antibody administration on Th1 rolling flux. **B** and **D**: Effect of antibody administration on Th1 rolling velocity. Data are expressed as the arithmetic mean $\pm$ SEM of at least four animals per group. * P < 0.05 relative to TNF- α or IL-4 treatment alone; ND, not determined.

β_2 -Integrin Mediates Th1 and Th2 Lymphocyte Adhesion in TNF- α - and IL-4-Treated Mice

Integrins mediate firm adhesion.⁷ In this study, pre-treatment of Th1 and Th2 cells with α_4 -integrin antibodies did not abrogate adhesion in either microenvironment (data not shown). Because recent literature suggests an important role for CD18 (β_2 -integrin)^{35,36} we investigated it in Th1 and Th2 lymphocyte adhesion to the intestine of TNF- α - and IL-4-treated mice using intravital microscopy and blocking antibodies. As shown in Figure 7 (bars 1 and 2), the addition of a blocking β_2 -integrin antibody before the addition of Th1 lymphocytes to TNF- α -treated mice significantly reduced the adhesion of cells by 95%. Similarly, blocking β_2 -integrin inhibited Th1 cell adhesion in IL-4-treated mice by 95% (Figure 7, bars 3 and 4). We also observed a significant reduction in Th2 cell adhesion.

Figure 7 (bars 5 and 6) illustrates that Th2 cell adhesion in TNF- α -treated mice was reduced by nearly 100% with the addition of an antibody to β_2 -integrin as was Th2 adhesion in IL-4-treated mice (Figure 7, bars 7 and 8). These results suggest that β_2 -integrin plays a key role in Th1 and Th2 lymphocyte adhesion in the intestinal vasculature of TNF- α - and IL-4-treated mice. The addition of an isotype control antibody did not alter Th1 or Th2 cell adhesion (data not shown).

P-Selectin and α_4 -Integrin Mediate Th1 and Th2 Cell Rolling in IL-10-Deficient Mice

Using intravital microscopy to examine endogenous leukocyte trafficking in the small intestine we observed a significant increase in rolling and adherent leukocytes within the IL-10-deficient mice when compared to their

Figure 6. Effects of adhesion molecule blockade on Th2 rolling in TNF- α - and IL-4-treated mice. Animals were treated with TNF- α or IL-4 and after Th2 injection and basal levels of rolling recorded, blocking antibodies to P-selectin, E-selectin, α_4 -integrin, and isotype controls were administered. **A** and **C**: Effect of antibody administration on Th2 rolling flux. **B** and **D**: Effect of antibody administration on Th2 rolling velocity. Data are expressed as the arithmetic mean $\pm$ SEM of at least four animals per group. * $P < 0.05$ relative to TNF- α -treatment alone; ND, not determined.

controls (Table 2). Notably, this doubling in leukocyte rolling is unlikely to be due to the slight increase in circulating cells observed by us and others in the IL-10-deficient mice (data not shown).³⁶ To see how Th1 and Th2 cells rolled in the vasculature subjected to chronic inflammation, we examined lymphocyte rolling in the IL-10-deficient mice.³⁷ Using intravital microscopy we observed that, compared to controls, there was significant

fourfold increase in Th1 cell rolling flux and a twofold increase in Th2 cell rolling flux (Figure 8A). As observed in the cytokine-treated mice, we found a significant reduction in Th1, but not Th2 cell rolling velocity (Figure 8B) and an increase in Th1 as well as Th2 cell adhesion (Figure 8C).

In a similar set of experiments as those described above, we investigated the adhesive mechanisms by which Th1

Table 1. Analysis of P- and E-Selectin Ligand and FucT mRNA Expression on Th1 and Th2 Effector Populations

CD4 ⁺ lymphocytes	% Cells binding to P-selectin FP	% Cells binding to E-selectin FP	FucTVII (dCT)	FucTIV (dCT)
Th1	31.2 $\pm$ 1.5	33.0 $\pm$ 8.1	12.7 $\pm$ 0.1	13.3 $\pm$ 0.1
Th2	25.2 $\pm$ 1.8	3.5 $\pm$ 0.3*	13.7 $\pm$ 0.5	14.0 $\pm$ 0.8

Th1 or Th2 cells were polarized *ex vivo* and surface stained with P- or E-selectin human IgM chimera fusion proteins (FP) or assessed for FucTVII and IV mRNA expression using real-time PCR. Results are expressed as percentage of cells binding or threshold cycle normalized to GAPDH (dCT) $\pm$ SEM from three to five experiments. * $P < 0.05$ (versus Th1).

Figure 7. Effect of β_2 -integrin blockade on Th1 and Th2 adhesion in TNF- α and IL-4-treated mice. Animals were treated with TNF- α or IL-4 and before Th1 or Th2 injection a blocking antibody to β_2 -integrin was administered. Data are expressed as the arithmetic mean $\pm$ SEM of at least four animals per group. * $P < 0.05$ relative to TNF- α or IL-4-treatment alone.

and Th2 lymphocytes traffic in the intestine of IL-10-deficient mice during inflammation. As shown in Figure 9, A and B, blocking P-selectin completely inhibited all Th1 and Th2 cell rolling. As observed in the cytokine-treated mice, blocking α_4 -integrin had no effect on Th1 lymphocyte rolling flux but our observation that blocking α_4 -integrin significantly reduced Th2 lymphocyte rolling flux by ~40% was unique (Figure 9, A and B). The addition of an isotype control antibody had no effect (Figure 9).

Our study to date used acute cytokine stimulation of mice and as such limited our investigation to Th1 and Th2 cell rolling and adhesion. We next used the chronic model of intestinal inflammation in the IL-10-deficient mice to examine the capability of our Th1 and Th2 cells to extravasate out of the intestinal vasculature and into the surrounding tissue. As shown in Figure 10, 24 hours after intravenous cell injection of CFSE-labeled cells, both Th1 and Th2 lymphocytes could be isolated from intestinal tissue. Data from experiments run in duplicate suggest that the number of CFSE-labeled Th1 and Th2 cells emigrating out from the vasculature are comparable between the two cell populations (data not shown). Taken together, these results suggest that our polarized Th1 and Th2 cells have the capability to emigrate out of the vasculature and that, at least in the inflamed intestinal tissue, both cells are perhaps recruited to a not so dissimilar extent.

Table 2. Analysis of Endogenous Leukocyte Trafficking in the Small Intestines of IL-10KO Mice

	Rolling flux (cells/minute)	Rolling velocity (μ m/second)	Leukocyte adhesion (cells/300 μ m)
129svev	29 $\pm$ 3	93 $\pm$ 7	0 $\pm$ 0
IL-10KO	55 $\pm$ 7*	48 $\pm$ 4*	3 $\pm$ 1*

Results are expressed as the mean $\pm$ SEM from three to four experiments. * $P < 0.05$ (versus 129svev).

Figure 8. Th1 and Th2 cell trafficking in IL-10-deficient mice. Th1 and Th2 cells were injected into control (129svev, open bars) or IL-10-deficient mice (IL-10KO, filled bars) and rolling flux (A), velocity (B), and adhesion (C) visualized in postcapillary venules of the small intestine by intravital microscopy. Data are expressed as the arithmetic mean $\pm$ SEM of at least four animals per group. * $P < 0.05$ relative to control group.

Discussion

The initial step in the recruitment of any leukocyte into a tissue is the tethering and rolling that occurs at the vas-

Figure 9. Effects of adhesion molecule blockade on Th1 and Th2 rolling in IL-10-deficient mice. After Th1 or Th2 injection and baseline levels of rolling recorded, blocking antibodies to P-selectin, α_4 -integrin, and isotype controls were administered. **A:** Effect of antibody administration on Th1 rolling flux. **B:** Effect of antibody administration on Th2 rolling flux. Data are expressed as the arithmetic mean $\pm$ SEM of at least four animals per group. * $P < 0.05$ relative to isotype controls.

cular interface. To better understand this initial step of the recruitment cascade of Th1 and Th2 cells we have investigated the mechanisms by which these lymphocytes roll in the intestinal vasculature of TNF- α - or IL-4-treated mice. Despite subtle differences, our results suggest that TNF- α - and IL-4-induced inflammatory microenvironments in the intestine are conducive to both Th1 and Th2 cell rolling and adhesion in the vasculature. Although it is not surprising that a global stimulus such as TNF- α can induce rolling and adhesion of both Th1 and Th2 cells it was somewhat more unexpected to see that a more selective cytokine such as IL-4 that expresses subtle amounts of P-selectin, can also cause P-selectin-dependent rolling *in vivo* of both Th1 and Th2 cells. Not only was this shown in cytokine-treated mice, but also in a well-described T-cell-driven mouse model of IBD and in the comprehensive *in vitro* studies presented in the companion paper.²³ Notably we also observed subtle increases

Figure 10. Flow cytometric analysis of Th1 and Th2 emigration from the intestinal vasculature of IL-10-deficient mice. CFSE-labeled T cells were injected intravenously into IL-10-deficient mice and after 24 hours CD4⁺ T-cell isolation from the intestine was performed on perfused mice. Mice were either not injected with CFSE-labeled T cells (**top**) or injected with CFSE-labeled Th1 (**middle**) or Th2 (**bottom**) cells. The presence of Th1 or Th2 cells was visualized using flow cytometric analysis. Data are representative of two experiments.

in Th2 over Th1 cell rolling under control conditions in both the control and IL-10-deficient mice. These differences were not observed *in vitro*²³ and may therefore reflect limited or absent effector substances, eg, granulocytes or platelets in the *in vitro* system.

Endothelial cell expression of adhesion molecules in response to stimulatory cytokines is critical for tissues to recruit lymphocytes during inflammation.³⁸ Lymphocytes flow untethered through the venules at high shear rates³⁹

so that their initial interactions with the blood vessel wall requires these specialized adhesive mechanisms permitting rapid association with vascular ligands. Previous studies have suggested that TNF- α and IL-4 differentially regulate the expression of adhesion molecules on endothelial cells. Within 5 hours of administration, TNF- α induces high expression of adhesion molecules such as P-selectin, E-selectin, VCAM-1, and ICAM-1 on intestinal endothelial cells both *in vitro* and *in vivo*.^{40,41} From *in vitro* data we know that human intestinal endothelial cells, human umbilical endothelial cells, and porcine aortic endothelial cells stimulated with IL-4 up-regulate P-selectin and VCAM-1 expression.^{40,42,43} There is also a small number of studies that suggest that IL-4 can induce ICAM-1 expression on endothelial cells, albeit to a lesser extent than TNF- α .^{40,44,45} Our study suggests that P-selectin expression is indeed induced by both TNF- α and IL-4, and that P-selectin can mediate lymphocyte rolling in these simple *in vivo* systems. Similarly, we demonstrated that P-selectin could also support lymphocyte rolling in the chronically inflamed intestine, namely that of IL-10-deficient mice. Interestingly, these observations may be specific for the intestinal vasculature as we have previously demonstrated that IL-4 does not up-regulate P-selectin in the cremaster muscle.²⁵

Our findings with Th1 cells and P-selectin support the recently described work by Haddad and colleagues¹⁴ who demonstrated that P-selectin mediates Th1 cell recruitment in an adoptive transfer model of intestinal inflammation. Our suggestion that P-selectin also regulates Th2 cell rolling *in vivo* is novel but in line with recent *in vivo* studies demonstrating that P-selectin ligand is found on CD4⁺ lymphocytes with a Th2 phenotype.¹⁷ Our work extends these observations to demonstrate that the Th2 P-selectin ligands interact in a functional manner with endothelial P-selectin *in vivo* in this study and predominantly via PSGL-1 *in vitro* in the companion paper.²³ Earlier studies reported differential expression of PSGL-1 on Th1 and Th2 cells and that only Th1 cells could efficiently enter inflamed sites in Th1-dominated models, such as sensitized skin or arthritic joints in a selectin-dependent manner.^{13,46} This varied expression of a P-selectin ligand on Th1 and Th2 cells is suggested to be due to the differential production of α 1,3-fucosyltransferase (FucT-VII),^{47,48} with Th2 cells expressing less FucT VII than Th1 cells⁴⁹ presumably due to a direct inhibitory effect on P-selectin ligand expression by IL-4. However, it appears from our study that with more prolonged exposure of T cells (14 days) to IL-4 and anti-IL-12, functional P-selectin ligand could be induced on IL-4/IL-10-producing T cells. Although it could be argued that our use of selectin chimeras containing IgM rather than the IgG (used by others) may have increased sensitivity for selectin ligand expression, we detected similar amounts of FucTIV and FucTVII mRNA as well as P-selectin ligand on both Th1 and Th2 cells and both cell types rolled abundantly on P-selectin. Clearly, this is not related to sensitivity.

Our observation of E-selectin mediating only Th1 rolling velocity in only TNF- α -treated animals follows the demonstration by us and others that Th1, but not Th2, cells express a functional ligand for E-selectin (Table

1);^{13,49} and TNF- α , but not IL-4, induces E-selectin expression on intestinal endothelial cells.^{40,41} However, once again it could be argued that much like the P-selectin discrepancy, the lack of E-selectin ligands on Th2 cells is related to *in vitro* cell culture. Indeed, Akdis and colleagues⁵⁰ have reported that IL-4 is an excellent suppressor of E-selectin ligand expression while IL-12 can induce E-selectin ligand expression in both Th1 as well as Th2 cells. Indeed, *in vivo* Teraki and Picker⁵¹ demonstrated that both Th1 and Th2 lymphocytes could express the E-selectin ligand CLA and both cell types could infiltrate skin. Although we could only study the role of E-selectin ligands on Th1 cells, our *in vivo* rolling data provide us with some interesting information. Although inhibition of E-selectin did not affect the flux of rolling Th1 cells, it significantly increased the velocity of rolling. It should be noted that increasing velocity of rolling has been demonstrated to diminish the opportunity for activation and firm adhesion of the rolling leukocytes.⁵² In addition, engagement of E-selectin has been reported to contribute significantly to subsequent adhesion.⁵³ Indeed, our E-selectin inhibition data led to some decrease in Th1 cell adhesion (data not shown) suggesting a significant contribution of this molecule to the recruitment paradigm.

Although our data do not show a significant role for α_4 -integrin in Th1 or Th2 cell rolling flux, we did see some increase in rolling velocity after α_4 -integrin inhibition. This is an observation previously described for eosinophils and CD8⁺ cells, but not CD4⁺ Th1 or Th2 lymphocytes.^{36,54} It is true that α_4 -integrin has been shown to inhibit inflammation in models of IBD and that our data suggest that α_4 -integrin antibody has minimal effects on Th1 cell rolling and adhesion in the intestinal vasculature in response to either TNF- α or IL4. This does not mean that α_4 -integrin is not important in the setting of IBD but perhaps suggests that α_4 -integrin antibodies may target other cell types aside from CD4 T cells. The other major integrin that mediates adhesion of leukocytes is the β_2 -integrin. Bernstein and co-workers⁵⁵ have previously demonstrated that there is altered expression of β_2 -integrins on CD4⁺ lamina propria lymphocytes in ulcerative colitis (UC) and Crohn's disease (CD) but whether β_2 -integrins served any major function in the recruitment of these cells has not been well characterized. In this current study we demonstrate that both Th1 and Th2 cells use β_2 -integrin to adhere to intestinal endothelium after exposure to TNF- α and IL-4. Although there is great interest in the targeting of $\alpha_4\beta_7$:MAdCAM-1 interactions in the treatment of IBD, our observations suggest some consideration should be given to the role of β_2 -integrin.^{34,56}

The final accumulation of leukocytes at a site of inflammation depends on both the nature of the locally secreted chemokines as well as the relative expression of the multitude of specific chemokine receptors. Many studies have focused on the fact that Th1 and Th2 cells express different chemokine receptors and respond to chemokines differently.^{57,58} For example, the chemokine receptors CCR5 and CXCR3 (for RANTES/MIP-1 β and IP-10/TARC/Mig, respectively) are preferentially expressed by

Th1 cells^{58,59} and CCR4, CCR8, and CCR3 to a lesser extent (for TARC/MDC, I-309 and eotaxin/MCP-3, respectively) are preferentially expressed by Th2 cells.^{58,60,61} Our data would suggest that exposure to the intestinal cytokine milieu in IL-10-deficient mice was insufficient to selectively induce just Th1 or Th2 lymphocyte adhesion and subsequent extravasation. These observations suggest that either Th1 and Th2 cells have chemokine receptors that overlap or that the expression of chemokines in these Th1 or Th2 microenvironments are not sufficiently selective to specifically recruit either Th1 or Th2 cells. Evidence supporting the former theory comes from Bonocchi and co-workers⁵⁸ who have shown that both Th1 and Th2 cells express an equivalent level of CXCR4 and CCR1 such that SDF-1 and RANTES/MIP-1 α /MCP-3 will recruit Th1 and Th2 cells indiscriminately *in vitro*. In the context of intestinal inflammation, studies have shown that Th1 and Th2 cells migrate in response to the chemokines MCP-1, MIP-1 α , and MIP-1 β , and that these are in fact up-regulated in IBD.^{58,62} A recent study by Iqbal and co-workers⁶³ demonstrated that the same stimulus could induce intestinal inflammation (of equivalent severity and time for development) by both Th1 and Th2 lymphocytes. Similarly, Xu and co-workers¹² demonstrated that both Th1 and Th2 cells are able to traffic to the retina in the initial stages of experimental autoimmune uveoretinitis. Taken together, these studies and our own suggest that the local microenvironment may not discriminate between Th1 or Th2 cells for CD4⁺ cell recruitment during an immune response and that similarities in the mechanisms for recruitment may exist.

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