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Difference in Th1 and Th17 Lymphocyte Adhesion to Endothelium

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T cell subset-specific migration to inflammatory sites is tightly regulated and involves interaction of the T cells with the endothelium. Th17 cells often appear at different inflammatory sites than Th1 cells, or both subsets appear at the same sites but at different times. Differences in T cell subset adhesion to endothelium may contribute to subset-specific migratory behavior, but this possibility has not been well studied. We examined the adhesion of mouse Th17 cells to endothelial adhesion molecules and endothelium under flow in vitro and to microvessels in vivo and we characterized their migratory phenotype by flow cytometry and quantitative RT-PCR.

More Th17 than Th1 cells interacted with E-selectin. Fewer Th17 than Th1 cells bound to TNFα-activated E-selectin–deficient endothelial cells, and intravital microscopy studies demonstrated that Th17 cells engage in more rolling interactions with TNFα–treated microvessels than Th1 cells in wild-type mice but not in E-selectin–deficient mice. Th17 adhesion to ICAM-1 was dependent on integrin activation by CCL20, the ligand for CCR6, which is highly expressed by Th17 cells. In an air pouch model of inflammation, CCL20 triggered recruitment of Th17 but not Th1 cells. These data provide evidence that E-selectin– and ICAM-1–dependent adhesion of Th17 and Th1 cells with endothelium are quantitatively different. The Journal of Immunology, 2012, 188: 000–000.

Different subsets of CD4+ Th cells play distinct roles in protective immunity and immune-mediated diseases (1). Th1 cells secrete IFN-γ, protect against intracellular infections, and are implicated in the pathogenesis of granulomatous responses to infections and in various organ-specific autoimmune diseases. Th2 cells secrete IL-4, -5, and -13 and protect against helminthes, and Th2 responses to environmental Ags underlie the pathogenesis of allergic diseases. In addition to the distinct cytokines produced by Th1 and Th2 cells, these two subsets have different migratory properties. Th17 cells, which were discovered more recently (2, 3), secrete IL-17A and IL-17-F, IL-22, and IL-21 (2, 4, 5). Th17 cells participate in several human immune/inflammatory diseases, such as multiple sclerosis, inflammatory bowel disease, rheumatoid arthritis, and psoriasis, as well as in different animal models of autoimmune/inflammatory diseases (3, 5–8). The critical roles of Th17 cells during the immune response against extracellular bacteria and fungi have also been demonstrated (9).

The selective recruitment of a particular Th subset is tightly regulated and depends on the type of inflammatory response and the expression of adhesion molecules on both the vascular endothelium and T cells (10, 11). Adhesive interactions between specific ligands expressed on the T cell surface with their respective adhesion molecule expressed on the vascular endothelium, in combination with appropriate chemokines, are believed to underlie selective Th subset recruitment. Interestingly, Th1 and Th2 cells have different migratory phenotypes. In general, Th1 cells express more functional E- and P-selectin ligands (12–14) and have higher levels of the chemokine receptors CXCR3 and CCR5 compared with Th2 cells. In contrast, Th2 cells may express more CCR3, CCR4, and CCR8 (15). These differences are believed to support different patterns of migration of Th1 and Th2 cells. In contrast, the mechanisms underlying recruitment of Th17 cells remain less well characterized.

Emerging data on the dominance of Th17 versus Th1 cells in certain organ-specific autoimmune diseases raise the question of whether selective migration of Th17 cells occurs. Flow cytometry analyses have shown that human Th17 cells are CCR6+CCR4+ or CCR2+CCR5−, in contrast with Th1 cells, which are CCR6+ CXCX3+ or CCR2+CCR5+ (15, 16). In mice, E-selectin ligand–positive CD4+ T cells found in inflamed skin included Th1 and Th17 cells, both of which were CCR4+ (17), suggesting that migration of Th1 and Th17 into some tissues may share the same mechanisms. Also, in the mouse, CCR6 is reported to be a marker of Th17 cells and necessary for their migration into the gut (18) and the CNS in the setting of experimental autoimmune encephalomyelitis (19). In contrast to studies of chemokine receptor expression, to date, there has been no assessment of adhesion molecule expression and function on murine Th17 cells under physiologically relevant flow conditions.

In the current study, we have used an in vitro flow chamber that simulates flow conditions in postcapillary venules with live time videomicroscopy and confocal intravital microscopy to examine the interactions of mouse Th17 cells with immobilized recombiant endothelial adhesion molecules, with mouse heart endothelial cells (MHEC) in culture, and with microvessels in inflamed cremaster muscle in vivo. We report significant differences between...
Th1 and Th17 cells with respect to E-selectin rolling and ICAM-1 chemokine-dependent arrest on activated endothelium both in vitro and in vivo and examine recruitment of in vivo-generated Th17 versus Th1 cells using an air pouch model of inflammation.

Materials and Methods

Reagents

Recombinant mouse IL-23, E-selectin, P-selectin, VCAM-1, ICAM-1, and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) Fr-chimeras and anti-C-Cr6-PE were from R&D Systems (Minneapolis, MN). Recombinant mouse IL-12, IL-2, IL-6, TNF-α, and recombinant human TGF-β were purchased from BioLegend (San Diego, CA). Also from BioLegend were blocking Abs to the following mouse molecules: IL-4 (clone 1B11), IFN-γ (clone XMG 1.2), IL-2 (clone JES6-1A12), CD4 (clone GK 1.5), CD3 (clone145-2C11), CD28 (clone 37.51), IL-17A (clone 2C11-18H10.1), CD43 activation-associated glycoform (clone 1B11), and Thy 1.1-Alexa 488 (CD90.1). Abs to CXC4R, CD11a, PSGL-1, ICAM-2, and PECAM-1 were purchased from BD Pharmingen (San Jose, CA) and stromal cell-derived factor-1a (SDF-1α), TNF-α, and carrier-free CCL20 from PepProTec (Rocky Hill, NJ). PMA and ionomycin were from Sigma-Aldrich (St. Louis, MO). Vibrant CFSE, CMRA, and Alexa-680 cell tracker stains were from Invitrogen (Carlsbad, CA). Collagenase was from Worthington (Lakewood, NJ).

Mice

All mice used were bred in the pathogen-free facility at Harvard Medical School New Research Building in accordance with the guidelines of the Committee of Animal Research at the Harvard Medical School and the National Institutes of Health animal research guidelines. C57BL6/wild-type (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). Strain-matched E-selectin-deficient mice (E-sel−/−) (20) were purchased from The Jackson Laboratory or obtained from Dr. Richard Hynes (Massachusetts Institute of Technology, Cambridge, MA). These animals have been backcrossed onto the C57BL6 background >10 generations. IL-17−/−RFP (21) and Ifng/Thvl.1 BAC-In (22) transgenic cytokine reporter mice were obtained from Dr. Cheng Dong (MD Anderson, Houston, TX) and Dr. Casey Weaver (University of Alabama, Birmingham, AL), respectively. Mice were sacrificed at 7–12 wk of age for harvest of naive T cells and 8–12 d of age for harvest of heart endothelial cells.

Preparation of naive and effector T cells

CD4+ cells were isolated from spleen and lymph node cell suspensions of WT, IL-17−/−RFP, or Ifng/Thvl.1 BAC-In mice using negative selection by immunomagnetic beads (Invitrogen). Th1 cells were derived from the naive T cells by anti-CD3 and anti-CD28 stimulation in the presence of IL-12 and IFN-γ, as previously described (23). To achieve Th17 differentiation, naive T cells were stimulated with anti-CD3 in the presence of human TGF-β (5 ng/ml), mouse IL-6 (30 ng/ml), mouse IL-23 (20 ng/ml) plus anti-IFN-γ (10 μg/ml), anti-IL-4 (10 μg/ml), and anti-IL-2 (10 μg/ml) mAb. On day 3, Th1 and Th17 cultures were diluted 1:1 with fresh medium containing IL-2 (25 U/ml) and IL-23 (20 ng/ml), respectively. Cells were harvested on day 5 and immediately used in experiments. Th1 cells derived from Ifng/Thvl.1 BAC-In mice and Th17 cells from IL-17−/−RFP mice were sorted by flow cytometry to prepare 100% reporter-expressing cells before being used in the adhesion experiments.

Isolation of endogenous Th17 cells and expansion ex vivo

WT mice were immunized with MOG peptide as described (24). Briefly, mice were immunized s.c., sacrificed at day 8 postimmunization, and the inflamed lymph nodes harvested. Cells were further cultured ex vivo with MOG peptide (1 μg/ml) and anti-CD3 (clone 145-2C11), anti-CD28 (clone 37.51), IL-17A (clone 2C11-18H10.1), CD43 activation-associated glycoform (clone 1B11), and Thy 1.1-Alexa 488 (CD90.1). Abs to CXC4R, CD11a, PSGL-1, ICAM-2, and PECAM-1 were purchased from BD Pharmingen (San Jose, CA) and stromal cell-derived factor-1a (SDF-1α), TNF-α, and carrier-free CCL20 from PepProTec (Rocky Hill, NJ). PMA and ionomycin were from Sigma-Aldrich (St. Louis, MO). Vibrant CFSE, CMRA, and Alexa-680 cell tracker stains were from Invitrogen (Carlsbad, CA). Collagenase was from Worthington (Lakewood, NJ).

Intravitral microscopy studies of the mouse cremaster muscle microcirculation were performed as described (27, 28). Mouse recombinant TNF-α (0.5 μg in 200 μl saline) was injected intrascrotally 1.5 h prior to cremaster exteriorization. Mice were anesthetized, and a microcatheter was introduced into the right femoral artery to enable retrograde injection of fluorescently labeled Th1 and Th17 cells (28). Transmitted light and fluorescent cremaster imaging was performed with an Olympus FV1000 confocal intravitral microscope using a 20× water immersion objective (Olympus). Fluorescence imaging was done sequentially at 473 and 635 nm to reduce the potential for channel cross talk. The centerline RBC velocity (Vcl) in each venule was measured in real time with an optical Doppler velocimeter (Texas A&M, College Station, TX), and Vcl was used to determine the wall shear rate and critical velocity (Vcrit) (28). CFSE-labeled Th1 and Alexa 680-labeled Th17 cells were suspended at 33 × 107 cells/ml, and small boluses (3 × 105 of each cell type) of a mixture of both cells were injected retrograde into the femoral artery catheter to visualize their adhesion in the postcapillary venules. Microvessel images were analyzed off-line using Imaris software (Imaris, South Windsor, CT).

Air pouch model of inflammation

Air pouches were created in the dorsal side of the back of WT and E-sel−/− mice as previously described (29). PBS, TNF-α (500 ng), or CCL20 (400 ng) were injected in the air pouch, and 20 h later, cells infiltrates were harvested by pouch lavage by repeated washes with PBS. Single-cell suspensions were permeabilized and stained for intracellular IL-17 or IFN-γ and analyzed by flow cytometry.

Statistical analysis

Data are expressed as the mean ± SD unless otherwise stated. Statistical analyses by Student t test or ANOVA followed by the Newman–Keuls post test were performed with GraphPad Prism software (GraphPad) and considered statistically significant at p < 0.05.

Results

Functional interactions of Th17 cells with endothelial cell adhesion molecules under flow conditions

Th17 and Th1 cells were generated in vitro from naive mouse CD4+ cells as described in Materials and Methods. Importantly, there were very few Th1 cells contaminating the Th17 cultures, or vice versa; Th17 and Th1 cells expressed ROR-γT and T-bet, re-
respectively, and Th17 cells also expressed IL-22 (Fig. 1A, 1B). In addition, we added anti–IL-2 neutralizing Ab to avoid the differentiation of regulatory T cells (<1% Foxp3+) under Th17-polarizing conditions (data not shown). Note that studies of T cell subsets in many laboratories use populations that do not uniformly produce the detectable defining cytokines by flow cytometric assays, and usually the signature cytokine-producing cells are <40% of the population (14, 18, 30, 31). This does not mean that only a minority of the cells has undergone the appropriate differentiation steps, but more likely reflects the nature of the assays, which cannot induce synchronized cytokine expression in all of the cells.

The capability of the in vitro-differentiated Th17 cells to initially attach to E- and P-selectins, ICAM-1, VCAM-1, or MadCAM-1 was assessed under defined flow conditions. Few naïve T cells accumulate on immobilized E- and P-selectin due to the lack of functional selectin ligands, whereas significant numbers of Th1 cells can adhere to both E- and P-selectin, as well as to ICAM-1 and VCAM-1 (32, 33). These properties make these two T cell subsets the appropriate positive and negative controls for comparison with Th17 cells. We found that Th17 cells avidly interacted with E-selectin, P-selectin, ICAM-1, VCAM-1, and MadCAM-1 under flow conditions (Fig. 2). Interestingly, significantly more Th17 than Th1 cells accumulated on immobilized E-selectin. These differences were sustained across the range of shear stresses tested (Fig. 2A, 2D). In contrast, there were no significant differences between Th17 and Th1 cell accumulation on P-selectin or VCAM-1 (Fig. 2B, 2C). Accumulation of T cells on ICAM-1 under flow conditions requires activation of the β2 integrins LFA-1 (αLβ2) and Mac-1 (αMβ2), and this can be induced by a variety of agents, including chemokines and PMA (34, 35). We did not observe any differences between binding of Th17 and Th1 cells to ICAM-1 upon PMA activation (Fig. 2E). Given the relevance of Th17 cells in intestinal barrier function, we examined the Th17 cell accumulation on immobilized MadCAM-1, which is expressed in the high endothelial venules of mesenteric lymph nodes, Peyer’s patches, and lamina propria (Fig. 2F). The total numbers of Th17 or Th1 cells that accumulated on MadCAM-1 were low, even under low stringency conditions of 0.5 dynes/cm². Binding was not enhanced upon T cell activation with PMA, and we found that similar numbers of Th17 and Th1 cells bound to MadCAM-1. We also generated Th17 and Th1 cells from IL-17 and IFN-γ reporter mice, respectively, and sorted them, and the uniform reporter-positive cell populations were used in flow chamber adhesion assays. Th17 cells obtained this way also showed enhanced adhesion to E-selectin as compared with Th1 cells (Fig. 2G), whereas no differences were observed on adhesion to P-selectin (Fig. 2H).

**Selectin ligand, integrin, and chemokine receptor phenotypic analysis of in vitro-differentiated Th17 cells**

To corroborate our findings of Th17 cell binding to E-selectin under flow conditions, we examined Th17 versus Th1 binding of E-selectin using a flow cytometric assay and soluble murine E-selectin–IgG chimera (14). We also analyzed the expression of glycoCD43, a known E-selectin ligand expressed by Th1 cells (23, 36), and the expression of PSGL-1, a ligand for both P-selectin and E-selectin. We found that Th17 cells bound significantly more E-selectin–Fc protein than did Th1 cells, and Th17 cells expressed more glycoCD43 than Th1 cells. However, the levels of PSGL-1 surface protein were not different (Fig. 3A, 3B).

Recent reports have shown a different pattern for chemokine receptors expressed by Th17 and Th1 cells. In particular, CCR6 is more abundantly expressed by Th17 than Th1 cells (18, 19). We performed flow cytometric analysis, gating on either CD4+IL-17+ or CD4+IFN-γ+ cell populations to determine the levels of expression of CCR6, CXCR4, and LFA-1 (CD11a) (Fig. 3C, 3D). We found higher expression of CCR6 on Th17 cells, as previously described (18, 19), and additionally, we found similar levels of expression of CD11a (Fig. 3C) and CD8α and CD8β integrins (data not shown). We obtained similar results when we analyzed whole Th17 versus Th1 populations (i.e., not gated on IL-17 versus producing cells) or when we analyzed the percent positive cells for the indicated adhesion molecules within the gated or un gated IL-17+ and IFN-γ+ cells (Supplemental Fig. 1) as used in the in vitro and in vivo functional adhesion studies described below.

**Interactions of Th17 cells with ICAM-1 under flow conditions are dependent on chemokine activation via CCR6**

We also studied the interaction of Th17 cells with immobilized ICAM-1 under flow conditions. We found similar numbers of Th17 and Th1 cells adhered to ICAM-1 upon integrin activation by PMA. We next examined a more physiologically relevant stimulus, (i.e., chemokines presented on the endothelium that trigger rapid

![FIGURE 1. Phenotype of in vitro-differentiated T cell populations. Naive CD4+ T cells isolated from the spleen and lymph nodes of C57BL/6 mice were cultured under Th17 or Th1 cell induction conditions during 5 d, as described in Materials and Methods. A, Intracellular IFN-γ and IL-17A were detected in PMA plus ionomycin-stimulated Th cells by flow cytometry (see Materials and Methods). The dot plot data shown are representative of 12 separate cell preparations that were also used in flow chamber studies and adoptive transfer studies presented in this article. B, Th17 and Th1 cells that were differentiated for 5 d were analyzed by quantitative RT-PCR. Results were normalized to β-actin (Actb) gene expression, and relative gene expression levels are indicated. Data show the mean ± SD of four different experiments. **p < 0.001.](http://www.jimmunol.org/runner/fig1.cmp)
integrin activation) (34, 35). For this purpose, we measured the accumulation of Th17 cells on coverslips coated with ICAM-1 and coimmobilized with CXCL12 (SDF-1α) or CCL20 (MIP3α), ligands for the chemokine receptors CXCR4 and CCR6, which are expressed at low and high levels, respectively, in Th17 cells (37). Interestingly, the number of Th17 cells that accumulated on...
ICAM-1 in the presence of CCL20 was greater than that found in the presence of SDF-1α. Similar numbers of Th17 cells bound to ICAM-1 following integrin activation by either CCL20/CCR6 or PMA (Fig. 3E). In contrast, binding of Th1 cells to ICAM-1 was enhanced by both PMA and SDF1-α but not by CCL20 (Fig. 3F).

*Phenotypic analysis of adhesion molecules in Th17 cells induced in vivo*

The Th17 and Th1 cells used in the studies described above were derived in vitro by activation of naive T cells via TCR and co-stimulatory signals in the presence of a mixture of cytokines. To corroborate that the migratory phenotype of these T cells faithfully represents the phenotype of Th17 and Th1 primed in vivo, we used an immunization protocol known to generate Th17 and Th1 cells in experimental autoimmune encephalomyelitis (24) (Fig. 4A). In vivo-primed Th17 cells expressed significantly higher levels of E-selectin ligands as compared with the in vivo-primed Th1 cells, similar to in vitro-differentiated populations (compare Fig. 4B, 4C to Fig. 3A–D). The in vivo-primed Th17 and Th1 populations showed comparable expression levels of PSGL-1 and CD11a. Lastly, CCR6 levels on in vivo-primed Th17 cells were higher than on Th1 (Fig. 4C). Also, a higher percentage of Th17 versus Th1 cells were positive for CCR6 and E-selectin ligands, whereas a similar percentage of Th17 and Th1 cells were positive for integrins α4 and α4β7 and a higher percentage of Th1 cells expressed the chemokine receptor CXCR4 (Supplemental Fig. 2). Although the in vitro- and in vivo-generated Th17 cells showed slight differences in the total levels of expression of the various molecules, the relative expression of each molecule tested between subsets appeared to be comparable for both the in vitro- and in vivo-generated cells, thus validating the comparative results obtained with in vitro-generated cells.

**The interactions of Th17 cells with MHEC in vivo and with the cremaster microvasculature, in vivo, are E-selectin dependent**

Th17 and Th1 cells express several adhesion molecules including E-selectin, P-selectin, ICAM-1, and VCAM-1 (25). We therefore sought to corroborate the above findings by quantifying naive, Th17, and Th1 adhesion to TNF-α-activated MHEC in an in vitro flow chamber. Th17 and Th1 cells were labeled with different color fluorescent dyes, and both types of cells were combined together and drawn across the same MHEC monolayer to measure accumulation (Supplemental Video 1). In contrast to the predicted outcome, the number of Th17 cells accumulating on activated MHEC was lower than the number of Th1 cells (Fig. 5A). As expected, few naive cells adhered. Based on our data indicating both higher expression of E-selectin ligands and more accumulation on E-selectin by Th17 cells as compared with Th1 cells (Figs. 2, 3), we reasoned that in the absence of E-selectin, differences in Th17 versus Th1 binding to MHEC would be diminished. Therefore, we examined Th17 and Th1 cell binding to MHEC prepared from E-sel−/− mice. The lack of E-selectin resulted in decreased binding of both Th1 and Th17 cells; however, the decrease was significantly more pronounced with Th17 cells. The ratio ([Th17 accumulated on E-sel−/− MHEC/Th17 accumulated on WT] × 100) was 30%, indicating a 70% decrease in Th17 accumulation when E-selectin was absent, whereas the ratio for Th1 cells was 55%, indicating a 45% decrease in Th1 accumulation (Fig. 5B). This result confirms a higher dependence of Th17 cells on E-selectin in their interactions with MHEC compared with Th1 cells. Interactions of T cells with activated MHEC also depend on VCAM-1, P-selectin, and ICAM-1. Thus, a 90% reduction of both Th1 and Th17 binding to E-sel−/− activated endothelium was achieved by a combination of function-blocking mAbs to ICAM-1 on the endothelial cells and to α4 integrin on the T cells (data not shown). Based on these in vitro findings, we next evaluated the interactions of Th17 and Th1 cells with activated endothelium in vivo using intravital microscopy.

When mouse cremaster muscle is exposed to the mild trauma induced by surgical tissue preparation, leukocytes undergo rolling interactions on the vessel wall that are mediated by P-selectin and L-selectin (27). Tissue pre-exposure to TNF-α for 2 h additionally induces local E-selectin expression and further elevates leukocyte adhesion (27, 28). Hence, this model is ideal to study how Th17 interact with the vascular endothelium in vivo. Again, we chose to compare Th17 cells with Th1 cells, which have been previously...
described to interact with cremaster microvessels in vivo (30, 31, 38). Th17 and Th1 cells were labeled with different dyes and coinjected via the femoral artery after exteriorization of the TNF-α–stimulated cremaster muscle, thus enabling comparison of Th17 and Th1 interactions in the same vessels and competitive rolling analysis. The microvessel parameters for WT and E-sel−/− mice are listed in Table I. Th17 and Th1 cells both exhibited rolling interactions in WT and E-sel−/− mice. However, in WT mice, the Th17 cells interacted with a significantly higher frequency compared with the Th1 cells (3.68 ± 0.42 Th17 cells rolling/min versus 1.78 ± 0.25 Th1 cells rolling/min; p < 0.0148, and a rolling ratio Th17/Th1 of 2.3). In contrast, in E-sel−/− mice, the Th17/Th1 rolling ratio remained close to 1, and Th17 and Th1 cells from four independent preparations (33–44 Th17 or Th1-independent cells) were analyzed rolling on WT or E-sel−/− vessels. Data represent mean ± SD values of four independent experiments using independent T cell preparations, and the numbers of mice and vessels are indicated in Table I. *p < 0.05, **p < 0.01.

Table I. Microvessel parameters for WT and E-sel−/− mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of Mice</th>
<th>No. of Vessels</th>
<th>Vessel Diameter (μm)</th>
<th>Wall Shear Rate (s−1)</th>
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<tr>
<td>WT (C57BL/6)</td>
<td>8</td>
<td>28</td>
<td>32 ± 2.7</td>
<td>1181 ± 162</td>
</tr>
<tr>
<td>E-sel−/−</td>
<td>5</td>
<td>22</td>
<td>34.8 ± 2.6</td>
<td>1093 ± 112</td>
</tr>
</tbody>
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**FIGURE 5.** Th17 cell interactions with TNF-α–activated MHEC under flow conditions and with TNF-α–activated cremaster muscle microvasculature in vivo. A and B, Naive and in vitro-differentiated Th1 and Th17 cells were drawn across TNF-α–activated MHEC from C57/BL6 or E-sel−/− mice within a flow chamber at a level of shear stress of 0.8 dynes/cm2. Accumulation (A) was calculated as described in the Materials and Methods section. B is the ratio ([Th cells accumulated on E-sel−/− MHEC divided by Th accumulated on WT MHEC] × 100). Data show the mean ± SD values from three different experiments. C, A competitive assay was performed to compare Th17 and Th1 cells rolling on the microvasculature. Equal numbers of in vitro-differentiated Th17 and Th1 cells each labeled with a different fluorescent dye were coinjected into C57/BL6 or E-sel−/− recipient mice that had received TNF-α injection into the scrotal sac 2 h before the cells were transferred. Recordings of each vessel were analyzed for 60 s, and rolling T cells were identified as the visible cells passing through a plane perpendicular to the vessel axis. Rolling ratio represents the ratio Th17/Th1 cells that roll on each vessel. The number of Th17 and Th1 rollers per minute in several independent vessels studied are also shown (D). E, The rolling velocity of high-velocity cells (Vcell) was calculated to ensure that these cells qualified as rolling leukocytes, defined Vcell < critical velocity (Vcrit). Velocities of Th17 and Th1 cells from four independent preparations (33–44 Th17 or Th1-independent cells) were analyzed rolling on WT or E-sel−/− vessels. Data represent mean ± SD values of four independent experiments using independent T cell preparations, and the numbers of mice and vessels are indicated in Table I. *p < 0.05, **p < 0.01.

Th17 slow rollers (velocity ≤10 μm/s) observed in the E-sel−/− mice (data not shown). These data show for the first time, to our knowledge, that Th17 cells roll on the microvasculature in vivo and confirm the in vivo relevance of our finding that Th17 cells express more functional E-selectin ligands than Th1 cells.

TNF-α promotes recruitment of Th17 and Th1 cells into the air pouch of WT and E-sel−/− mice

Leukocyte extravasation in an s.c. pouch is a well-characterized model used to examine leukocyte emigration in response to a specific stimulus (29, 39). To further explore the differences in Th17 and Th1 migration during inflammation in vivo, we generated an air pouch in the dorsal side of WT and E-sel−/− mice and locally injected TNF-α as an inflammatory stimulus or PBS as control. Twenty hours later, we harvested the air pouches and analyzed the cellular infiltrates by flow cytometry. The total cells harvested per pouch were significantly increased in TNF-α–treated mice compared with PBS-injected mice, and no quantitative differences were observed between WT and E-sel−/− mice (Fig. 6A). The number of cells recruited in PBS control mice was very low in both WT and E-sel−/− mice (Fig. 6A, Supplemental Fig. 3). The majority of the cell infiltrates were Gr1+ cells, as previously described in this model of inflammation (40) (data not shown). Interestingly, there was a component of cellular infiltrates that were CD3+ T cells, including Th17 cells and Th1 cells. However, no differences were observed in Th17 or Th1 cell recruitment between WT and E-sel−/− mice, indicating that E-selectin deficiency did not result in decreased recruitment of endogenous Th17 or Th1 cells (Fig. 6B–F). These data indicate that probably other selectins such as P-selectin overcome the lack of E-selectin and contribute to cell recruitment into the air pouch, similar to what
has been described in E-sel−/− mice in other models of inflammation (20, 41). More importantly, we can use this model to evaluate recruitment of Th subsets in response to other stimuli using TNF-α as a positive control of T cell recruitment.

The interactions of Th17 cells with MHEC in vitro are CCL20/CCR6 dependent, and CCL20 induces Th17 cell recruitment to the air pouch in vivo

Based on the different expression pattern of CCR6 in Th17 versus Th1 cells, and our finding that CCL20 can specifically trigger Th17 cell arrest on immobilized ICAM-1 under flow conditions (Fig. 3G), we next assessed the effect of CCL20 on Th17 cell accumulation on activated WT endothelium. Interestingly, we found that Th17 binding to MHEC was significantly enhanced in the presence of CCL20, whereas SDF-1α did not have any effect on Th17 accumulation (Fig. 7A). This is in agreement with a recent publication that shows that human Th17 cells can arrest on CCL20-treated HUVECs and mouse fibroblasts expressing ICAM-1 (42). Taken together, these results indicate that CCL20 binding to CCR6 in Th17 cells mediates firm adhesion to activated endothelium via ICAM-1. To test this in vivo, we next asked the question if CCL20 would trigger efficient recruitment of Th17 cells using the air pouch model. Other chemokines have been previously shown to recruit inflammatory cells in the air pouch; however, to our knowledge, none of these studies have addressed T cell recruitment (43–45). Local injection of CCL20 into the air pouch resulted in statistically significant increase of cell recruitment as compared with PBS control (Fig. 7B). The numbers of CD3+ recruited T cells into the CCL20-treated air pouch were significantly higher than the PBS control group and comparable to the TNF-α–treated mice (Fig. 7C). In response to TNF-α, we observed that comparable numbers of Th17 and Th1 cells were recruited into the air pouch. Strikingly, CCL20 injection in the air pouch resulted in specific recruitment of Th17 cells but not Th1 cells (Fig. 7D–G). Our data demonstrate that CCL20 cannot only induce Th17 adhesion to endothelium in vitro, but can also trigger recruitment of Th17 cells in vivo.

Discussion

In this study, we present a detailed description of the adhesion phenotype of murine Th17 cells and demonstrate that Th17 cells interact with activated endothelium differentially than Th1 or naive T cells in vitro and in vivo. We suggest these differences can contribute to temporal and spatial differences in recruitment of Th17 cells compared with other subsets during host defense and inflammation.

The evidence supporting our conclusions is as follows. First, we have dissected the different known adhesion pathways by testing interactions of Th17 cells with endothelial cell–expressed adhesion molecules in isolation under flow conditions. Th17 cells have a high capacity to bind E-selectin, even higher than Th1 cells, which are well known to bind to E-selectin under flow conditions (14, 23). We saw greater Th17 versus Th1 binding to E-selectin under flow using either unsorted Th1 and Th17 cell preparations that typically do not show uniform IFN-γ or IL-17 expression or using sorted 100% positive cytokine reporter-expressing cells from IL-17RFP or Ifng/Thy1.1 BAC-In mice. The expression of high levels of E-selectin ligands, including glyco-CD43, on Th17 cells generated in vitro and in vivo is also demonstrated by our data (Figs. 3, 4). Second, using flow cytometry and quantitative PCR techniques, we have further characterized the level of expression of the integrin ligands for ICAM-1 and VCAM-1 and the expression of chemokine receptors expressed in Th17 cells. In agreement with previous reports (18, 19), we have found that Th17 cells express high levels of CCR6. However, for the first time, to our knowledge, we report that CCR6 interactions with its ligand CCL20 trigger robust Th17 arrest on immobilized ICAM-1. Third, these results were validated by analysis of Th17 cells generated in vivo by MOG immunization and show that these cells also express high levels of E-selectin ligands and CCR6. Fourth, we have corroborated our findings by studying Th17 interactions with TNF-α–activated endothelium using E-sel−/− or WT MHEC pretreated with CCL20 chemokine. Lastly, the interactions of Th17 cells with activated endothelium are different from other T cell subsets in vivo. Thus, by intravital microscopy, Th17 cells have a high capacity of rolling in cremaster microvessels in vivo, and this rolling is highly dependent on E-selectin. Indeed, we have established differences between Th17 and Th1 cells in competitive rolling experiments using WT and E-sel−/− mice. In addition, using an air pouch model of inflammation, we have demonstrated that Th17 cells are recruited into sites of acute inflammation differently than Th1 in response to CCL20. In summary, these findings demonstrate that Th17 cell interactions with activated endothelium are highly dependent on E-selectin due, in part, to the high levels of expression of E-selectin ligands and the ability to arrest on ICAM-1 in the presence of CCL20, and this is different from Th1 cells both in vitro and in vivo.

E-selectin is expressed at high density on the luminal plasma membrane of vascular endothelial cells at sites of inflammation (30,
and mediates the initial contact of T cells with activated endothelium through two different functional E-selectin ligands expressed on murine activated T cells, PSGL-1, and glycoCD43. PSGL-1 is the best-characterized selectin ligand on leukocytes that binds E-, P-, and L-selectins (47), and the glycosylated form of CD43, glycoCD43, binds E-selectin but not P-selectin (23, 36). Our prior studies with Th1 cells from PSGL-1–CD43 double-deficient mice predict the existence of other E-selectin ligands in activated T cells (23). From our results and recent work of other groups, it is clear that subsets of Th cells differ in their expression levels of functional E- and P-selectin ligands, although no previous studies have examined Th17 (10, 12–14, 23). Hence, we provide new information that demonstrates Th17 cells bind avidly to both P-selectin and E-selectin and express high levels of E-selectin ligands, PSGL-1, and glycoCD43. It is notable that Th17 cells have a greater capacity to bind E-selectin than Th1 cells, given the high levels of both E- and P-selectin ligands on Th1 cells and their ability to bind very avidly to both E- and P-selectins under flow conditions in vitro (10, 12–14, 23). The enhanced Th17 binding to E-selectin may be mediated in part by glycoCD43, but other E-selectin ligands may be involved, such as those detected by the E-selectin chimeric protein. The fact that E-selectin chimera staining is very high in Th17 cells is consistent with this, although the characterization of these new ligands would require additional biochemical analyses. Another E-selectin ligand candidate is CD44, which is expressed by both leukocytes and endothelium and contributes to T cell recruitment to sites of inflammation and immune reactions (30, 48, 49). Interestingly, CD44 expressed by Th1 and Th2 cells has been shown to contribute to rolling interactions on TNF-α–activated microvessels of the intestine (30, 48). Intravital microscopy experiments have previously shown that E-selectin function in mediating leukocyte rolling in TNF-α–stimulated vessels is largely redundant with that of P-selectin (20, 50, 51), and consequently, E-selectin–deficient mice have a subtle defect in leukocyte rolling, primarily in neutrophil low-velocity rolling (20, 50–52). We have found that this is also true for adoptively transferred Th17 and Th1 cells, which can also roll in the vasculature of E-selectin–deficient mice (Fig. 5). In contrast, the lack of E-selectin in MHEC did result in significantly reduced binding of both T cell subsets, and, more importantly, the defect in binding to MHEC was significantly more pronounced in Th17 cells. One likely explanation is that endothelium from heart tissue differs from cremaster tissue in cytokine-induced expression level of E- and P-selectin. Earlier studies support this explanation, and investigators did find significant heterogeneity in the expression of E- and P-selectin in the vasculature of organs following LPS administration (46). Furthermore, we have shown for the first time, to our knowledge, that Th17 cells do roll in microvasculature in vivo in a model that is highly dependent on E-selectin (27, 28), and these studies support the high tropism of Th17 cells for E-selectin. Consistent with this idea, adoptively transferred Th17 cells roll more slowly than Th1 cells. Moreover, the competitive rolling assays show that the rolling ratio of Th17/Th1 in WT mice is significantly higher than in E-selectin−/− mice, indicating that the enhanced rolling of Th17 versus Th1 is abolished in the absence of E-selectin. Taken to-
 altogether, these data support the conclusion that Th17 rolling in the cremaster vasculature is highly dependent on E-selectin.

In contrast to our competitive rolling studies using adoptively transferred cells and studying rolling interactions within minutes, our air pouch data indicate that both Th17 and Th1 cells are efficiently recruited into the air pouch in response to TNF-α in WT and also in E-sel−/− mice. One explanation for this is that in the air pouch model T cells are recruited into the air pouch after several hours, and, in that period of time, expression of P-selectin can overcome the absence of E-selectin, as it has been reported in other models of inflammation in which E-sel−/− mice are equally susceptible to inflammation (20, 41). Th17 cells have also been found to be increased in the inflamed lung tissue in E-sel−/− mice in an experimental model of scleroderma; however, this is a chronic inflammatory process with other vascular complications, and the increased presence of Th17 cells in the lung cannot only be attributed to Th17–endothelial cell interactions (53).

The regulation of T cell subsets recruitment into tissues represents an important point in the control of the timing, type, and progression of an inflammatory response. Sequential leukocyte–endothelial cell interactions mediated by different adhesion molecules play a prominent role during the inflammatory response, and the fact that these are different between Th17 and Th1 cells can result in new ways of controlling sequential recruitment of effector T cells during the immune response. Chemotaxis studies performed in transwell assays have shown that Th17 cells migrate toward CCL20, whereas Th1 cells do not, and this is abolished in CCR6−/− mice (19), indicating that CCL20–CCR6 interactions are critical in Th17 recruitment during inflammation. Our data provide an additional mechanistic explanation of how this recruitment may work. We demonstrate that CCL20 enhanced binding of Th17 cells to ICAM-1 and to activated endothelial cells under flow, and this step is essential for leukocyte transendothelial migration from the bloodstream into the inflamed tissue. In contrast, Th1 cells do not respond to CCL20, but do efficiently bind to ICAM-1 and activated endothelial cells in response to SDF-1α. A recent publication shows that human Th17 cells can arrest on CCL20-treated HUVECs and mouse fibroblasts expressing ICAM-1 (42). Our data using immobilized ICAM-1 and mouse Th17 cells could be extrapolated to mechanistically explain stable binding to endothelial cells is mediated by CCL20 activation of integrins. The presence of CCL20 in Peyers’ patches and intestinal lamina propria (18) may explain the high numbers of Th17 cells recruited during intestinal inflammation (54), and our findings would support this idea. An additional mechanism of how Th17 cells could be selectively recruited to the intestine could be by interacting with MadCAM-1. However, our data show that very few cells bound MadCAM-1 under low-stringency flow conditions, even in the presence of PMA activation or chemokines, and we did not observe differences between Th17 and Th1 cells (Supplemental Fig. 4). To our knowledge, there is no published data defining whether Th cell subsets can accumulate on MadCAM-1 under flow conditions or whether binding requires activation by chemokines. Abramson et al. (55) showed that human CD4+CD45RA+ T cells producing IFN-γ bind to immobilized MadCAM-1, although this was done under static conditions. Park et al. (3) also showed binding of T cells activated with anti-CD3 and anti-CD28 mAb and retinoic acid to immobilized MadCAM-1 under flow conditions; however, these were not polarized toward any specific Th cell subset. Given these data sets, we suggest two explanations. First, our in vitro assays are not sensitive enough to detect Th subset adhesion to MadCAM-1, or there is no difference in the ability of Th1 and Th17 to recognize MadCAM-1, and therefore, this pathway does not explain the Th17 dominance in parts of the intestine.

Despite the well-documented presence of Th17 cells in various inflammatory diseases that affect different organs (56, 57), very little is known about how Th17 cells, once primed in lymphoid organs, are recruited to these various inflamed tissues. Our results contribute to a better understanding of this process and shed some light in describing not only what pathways Th17 cells use to migrate, but also how these are different from Th1 cells. Our air pouch studies in WT mice show that a proinflammatory cytokine like TNF-α can induce recruitment of both Th17 and Th1 cells into the s.c. air pouch, and no differences are observed among the two subtypes, indicating that recruitment in this case is not highly dependent on E-selectin. However, we show evidence for the first time, to our knowledge, that a single injection of CCL20 can specifically recruit Th17 cells in vivo.

In summary, our results indicate that Th17 and Th1 cells use different adhesion mechanisms to migrate to tissues during inflammation, and these will be activated depending on the chemotactants and adhesion molecules presented by the vascular endothelium, which may differ depending on the disease, vascular bed, and presence of other inflammatory cells. Taken together, these factors can determine selective recruitment of Th17 cells and partly explain how and why Th17 and Th1 cells appear often at different sites of inflammation or at the same sites but at different times and open a window to develop T cell subset-specific therapeutic interventions based in their distinct migratory phenotypes.

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Disclosures
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References


