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Expression of Functional Selectin Ligands on Th Cells Is Differentially Regulated by IL-12 and IL-4[1]

Yaw-Chyn Lim,* Lori Henault,* Amy J. Wagers,† Geoffrey S. Kansas,† Francis W. Luscinskas,* and Andrew H. Lichtman[2]*

Immune responses may be qualitatively distinct depending on whether Th1 or Th2 cells predominate at the site of Ag exposure. T cell subset-specific expression of ligands for vascular selectins may underlie the distinct patterns of recruitment of Th1 or Th2 cells to peripheral inflammatory sites. Here we examine the regulation of selectin ligand expression during murine T helper cell differentiation. Large numbers of Th1 cells interacted with E- and P-selectin under defined flow conditions, while few Th2 and no naive T cells interacted. Th1 cells also expressed more fucosyltransferase VII mRNA than naive or Th2 cells. IL-12 induced expression of P-selectin ligands on Ag-activated naive T cells, even in the presence of IL-4, and on established Th2 cells restimulated in the presence of IL-12 and IFN-γ. In contrast, Ag stimulation alone induced only E-selectin ligand. Interestingly, restimulation of established Th2 cells in the presence of IL-12 and IFN-γ induced expression of P-selectin ligands but not E-selectin ligands; IFN-γ alone did not enhance expression of either selectin ligand. In summary, functional P- and E-selectin ligands are expressed on most Th1 cells, few Th2 cells, but not naive T cells. Furthermore, selectin ligand expression is regulated by the cytokine milieu during T cell differentiation. IL-12 induces P-selectin ligand, while IL-4 plays a dominant role in down-regulating E-selectin ligand. ***The Journal of Immunology*****, 1999, 162: 3193–3201.**

T-helper 1 and Th2 cells are subsets of effector $CD4^+$ T cells that are best characterized by the effector cytokines they secrete. Th1 cells produce IFN-γ and TNF-β and play an important role in protective cell-mediated immune response as well as pathologic delayed type hypersensitivity (DTH)[3] in the setting of autoimmunity and allograft rejection (1–4). Th2 cells produce IL-4, IL-5, and IL-13 and participate in protective responses to helminthic infections, as well as pathologic allergic responses (1–4). In addition, IL-4 and IL-10 produced by Th2 cells down-regulate the effector phase of Th1 responses (1). Th0 cells are a heterogeneous group of effector T cells that produce cytokines typical of both Th1 and Th2 cells (2, 4). Helper T cell subset differentiation has been extensively studied in vitro, often with T cells from TCR transgenic mice (2, 3, 5). These studies indicate that Th1 and Th2 populations arise from naive T cells after at least one round of Ag stimulation, and the exogenous cytokine milieu has a profound influence on which pathway of differentiation is operative. IL-12 drives naive T cell differentiation toward the Th1 phenotype, although autocrine IFN-γ participates early on during the process. IL-4 drives Th2 differentiation. Studies on the stability of the effector T cell phenotypes have shown that Th1 cells retain the ability to transduce IL-4 signals and can be redifferentiated toward a Th2 phenotype by secondary Ag stimulation in the presence of IL-4 (6, 7). Th2 cells are refractory to IL-12-induced redifferentiation toward a Th1 phenotype because the β_2 chain of the IL-12 receptor is down-regulated during Th2 differentiation. However, treatment of Th2 cells with IFN-γ restores the expression of the β_2 chain of the IL-12 receptor and the ability to be redifferentiated to a Th1 phenotype (8).

Our knowledge of T lymphocyte recirculation is largely based on indirect studies of the phenotype of T cells in lymph nodes and lymphatics, using imperfect surface markers to distinguish naive, recently activated, and memory T cells. One widely accepted model of T lymphocyte recirculation suggests that naive T cells circulate from lymph node to lymph node, but they have little capacity to home to peripheral inflammatory sites (9–12). Although the idea that naive T cells are excluded from entry into nonlymphoid tissues has been challenged (13–18, 19), there is little evidence showing that they are recruited in significant numbers to inflammatory sites (20). Therefore, the main location for the differentiation of naive T cells into Th1 or Th2 effector cells is likely to be in the lymph node where naive T cells are activated by their cognate Ags. Very little is known about subset phenotypes of memory T cells, but it is possible that memory Th1 and Th2 cells may also be reactivated in draining lymph nodes. Therefore, the utility of subset Th1 or Th2 polarization must rely in part on selective recruitment of only Th1 or Th2 cells to an inflammatory site at a particular time (21).

The selective recruitment of a particular subset of T helper cells may depend on the type of inflammatory response, the expression of adhesion molecules on the vascular endothelium, and the expression of complementary functional ligand/activated receptor on the T cells. Extravasation of effector and/or memory T cells into inflamed tissue is mediated by a series of adhesive interactions between specific ligands expressed on the T cell surface with their

*Vascular Research Division, Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115; and †Department of Microbiology-Immunology, Northwestern Medical School, Chicago, IL 60611

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[2] Address correspondence and reprint requests to Dr. Andrew H. Lichtman, Department of Pathology, Brigham and Women's Hospital, 221 Longwood Avenue, Boston, MA 02115-5814. E-mail address: alichtman@rics.bwh.harvard.edu

[3] Abbreviations used in this paper: DTH, delayed type hypersensitivity; PSGL-1, P-selectin glycoprotein ligand-1; FucT-VII, fucosyltransferase VII; PE, phycoerythrin; DHFR, dihydrofolate reductase; RT, reverse transcriptase; C2GnT, core 2 *N*-acetylglucosaminyltransferase.

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respective adhesion molecule expressed on the vascular endothelium. Previous studies have indicated that selectins are involved in T cell recruitment to peripheral inflammatory sites. For example, L-selectin appears to be involved in recruitment of alloreactive T cells into skin grafts in mice (22). We have shown that in contrast to naive T cells, previously activated or memory T cells express functional PSGL-1 (23). Recent studies in mice, in fact, suggest that adoptively transferred Th1 cells are preferentially recruited to cutaneous DTH reactions and to arthritic joints (24) compared with Th2 cells. In addition, in vitro differentiated Th1 but not Th2 cells have been shown to bind to a soluble P-selectin fusion protein (25). To date, however, there has been no assessment of selectin ligand expression on T cell subsets using physiologically relevant flow assays. Furthermore, the factors that determine expression of functional selectin ligands during the process of effector T cell differentiation are not known. Since exogenous cytokines such as IL-12 and IL-4 direct T helper subset differentiation, it is reasonable to hypothesize that these same cytokines influence selectin ligand expression. These cytokines may regulate the expression of enzymes such as fucosyltransferase VII (FucT-VII) required for the synthesis of selectin ligands by blood leukocytes (26–28).

In this study, we have used an in vitro flow chamber simulating flow conditions found in postcapillary venules and live time videomicroscopy to study the interactions of mouse Th1 and Th2 cells with E- and P-selectin. In addition, we examined the regulation of functional selectin ligand expression on these DO.11 TCR transgenic mouse T cell subsets following TCR Ag-specific stimulation (OVA), using defined in vitro cytokine-driven differentiation strategies.

Materials and Methods

Mice

The DO.11 TCR transgenic mice (29) were bred and maintained in approved animal housing facilities at the Longwood Medical Research Center, Boston, MA. The animals were used at 4 to 6 wk of age and were killed with carbon dioxide as approved by the panel on Euthanasia of the American Veterinary Association.

Preparation of T cells and APCs

DO.11 T cells express a transgenic Ag receptor specific for OVA peptide (323–339) plus I-A^d. Lymph nodes and spleens were removed from DO.11 mice after euthanasia, and cell suspensions were made by passing the tissues through wire mesh. CD4$^+$ T cells were purified by positive selection as previously described (30) using CD4$^+$-coated Dynal beads and Detachabead reagent (Dynal, Lake Success, NY) according to manufacturer's instructions. Greater than 95% of the cells were CD4$^+$ as assessed by flow cytometry using FITC-conjugated anti-mouse CD4$^+$ Abs. The CD4-negative fraction of the DO.11 spleen and lymph node suspensions or whole spleen cell suspensions from BALB/c mice were treated with mitomycin C for 30 min at 37°C and used as APCs for DO.11 T cells.

Reagents

Murine recombinant IL-12 was a gift from Genetics Institute (Cambridge, MA). Recombinant murine IL-4 was obtained from the culture supernatant of the I3XL6 cell line, obtained from Dr. Abul Abbas (Brigham and Women's Hospital, Boston, MA) which constitutively expresses a stably transfected murine IL-4 gene. The IL-4 concentration was determined by ELISA against a National Institutes of Health standard and used as a dilution of the supernatant. Alternatively, recombinant murine IL-4 was purchased from Genzyme (Cambridge, MA). Murine recombinant IFN-γ and IL-10 were purchased from PharMingen (San Diego, CA). The hybridoma cell line producing anti-IL-4 (clone 11B11) was obtained from the American Type Culture Collection (Manassas, VA).

Dr. Raymond Camphausen (Genetics Institute, Cambridge, MA) generously provided human E- and P-selectin human IgG chimeric proteins (31, 32). Murine E- and P-selectin human IgM chimeric proteins contained the lectin, epidermal growth factor, and two complement-regulatory protein-like domains of mouse E- and P-selectin (27). The chimeras were purified by anti-IgM affinity chromatography from supernatants of COS cells transfected with expression constructs generously provided by Dr. John Lowe (Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI).

PMA, ionomycin, brefeldin A, and saponin were all purchased from Sigma (St. Louis, MO).

In vitro differentiation of CD4$^+$ naive cells

Freshly isolated CD4$^+$ DO.11 T cells are mostly naive cells, which produce little detectable IL-4 or IFN-γ on Ag activation (5). These naive cells were differentiated into effector T cells as previously described (5). Briefly, the naive T cells were suspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, and 100 U/ml streptomycin and plated out in 2 ml polystyrene culture wells at a cell density of 2.5×10^5/well. APCs (2.5×10^6/well) and OVA peptide at a final concentration of 1 μg/ml were added to each well. For Th1 differentiation, recombinant murine IL-12 (10 ng/ml final concentration) plus neutralizing anti-IL-4 mAb (11B11 hybridoma supernatant, 25% v/v) were added to individual wells. For Th2 differentiation, murine recombinant IL-4 (1000 U/ml) was added to individual wells. After 2 days, the cultures were split 1:2, and the cells were further diluted with fresh culture medium containing recombinant murine IL-2 (10 U/ml final concentration). After an additional 4 days in culture, the cells were harvested and centrifuged through a Ficoll density gradient to remove dead APCs and cell debris. Cells were tested immediately in flow assays. The phenotype of the differentiated cells was tested by assaying culture supernatants for IL-4 and IFN-γ in response to restimulation with OVA peptide and APCs as described (33). Briefly, T cells were cultured in microwells (5×10^4/well) with OVA peptide (1 μg/ml) and APCs (5×10^5/well). After 24 h, supernatants from each well were collected and assayed for IFN-γ and IL-4 by ELISA using reagents from PharMingen. Cytokine production by Th1 and Th2 populations was also assessed by intracytoplasmic staining, as described below. In some experiments, the primary cultures were supplemented with various combinations of murine recombinant cytokines, including: no added cytokines; IL-4 (1000 U/ml) plus IL-12 (10 ng/ml); IL-12 plus IL-10 (50 U/ml); and IFN-γ (500 U/ml).

In other experiments, previously differentiated Th1 and Th2 populations were restimulated in bulk in the presence of exogenous cytokines and Ab reagents to modify the differentiated phenotype. Thus Th1 cells were restimulated with OVA peptide and APCs in the presence of IL-4, and Th2 cells were restimulated with OVA and APCs in the presence of IL-12, IFN-γ (30 U/ml), and anti-IL-4. These secondary differentiation cultures were conducted in the same manner as the primary cultures.

Indirect immunofluorescence and FACS analysis

For single-color analysis of selectin binding, CD4$^+$ naive cells, Th1 cells, or Th2 cells (5×10^5 cells/100 μl) were incubated with murine P- or E-selectin human IgM chimeras (1 μg/100 μl) for 20 min on ice, washed in RPMI containing 5% FCS, and then incubated with phycoerythrin (PE)-conjugated goat anti-human IgM for 20 min on ice. In parallel experiments, T cell populations were incubated with human P- or E-selectin-human IgG chimeras and stained with PE-labeled anti-human IgG.

Two-color analysis of simultaneous P- and E-selectin binding was performed using the murine E-selectin-human IgM chimera and human P-selectin-human IgG chimera, followed by a PE-anti-human IgM Ab (Southern Biotechnology Associates, Birmingham, AL) and FITC-anti-human IgG Ab (Caltag Laboratories, Burlingame, CA). Two-color analysis of selectin binding and CD25 or CD44 expression was performed using the murine E-selectin-human IgM or human P-selectin-human IgG chimeras followed by FITC-conjugated anti-human Ig and PE-conjugated anti-CD44 or anti-CD25 (PharMingen). The stained cells were subsequently washed, fixed in 1% formaldehyde, and analyzed with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). Analysis was performed on 10^4 cells for each condition.

For intracytoplasmic staining of IFN-γ and IL-4, we adapted a previously described method (34). Briefly, Th1 or Th2 cells were activated with PMA (20 ng/ml) and ionomycin (2.5 μM) for 2 h and then treated with brefeldin A (20 μg/ml) for an additional 3 h. The cells were then fixed in 4% paraformaldehyde and washed once in PBS/BSA (1%) followed by a second wash in PBS/BSA with 0.5% saponin and 0.1% sodium azide. The permeabilized cells were stained with FITC-conjugated anti-IFN-γ, or PE-conjugated anti-IL-4, or their respective fluorochrome-conjugated isotype controls (all from PharMingen) in the presence of 0.5% saponin for 45 min. The cells were then washed with PBS/BSA with 0.5% saponin and 0.1% sodium azide followed by PBS/BSA to reseal the plasma membranes. These cytokine-stained cells were then stained for E- or P-selectin binding and analyzed by two-color flow cytometry as described above.

Table I. *Cytokine expression by DO.11 Th1 and Th2 cells*[a]

	IFN-γ (U/ml)	IL-4 (U/ml)
Th1	1190 ± 376	85 ± 134
Th2	159 ± 155	627 ± 429

[a] Th1 and Th2 populations were derived in vitro and restimulated with Ag, and 24-h culture supernatants were analyzed for IFN-γ and IL-4 by ELISA, as described in *Materials and Methods*. Data represent the mean ± SD of nine separate experiments using cells that were also used in flow chamber studies presented in this article. The differences in the mean values for each cytokine between Th1 and Th2 cells are statistically significant ($p < 0.0001$).

Measurement of Th cell interactions with P-selectin and E-selectin chimera under defined flow conditions

Th cell interactions with P-selectin- and E-selectin-coated slides under defined laminar flow were studied in a parallel plate flow chamber as previously described (35). After 6 days in culture, the Th cells were resuspended in Dulbecco's PBS containing 0.1% (v/v) human serum albumin and 20 mM HEPES, pH 7.4, at 37°C (5×10^5/ml) and perfused over P-selectin- and E-selectin-coated coverslips. Human P-selectin-Ig or E-selectin-Ig fusion protein (10 μg/ml) was captured on glass coverslips using goat $F(ab')_2$ anti-human Fc Ab as previously described (35). This concentration was found to be saturating by performing dose-response adhesion assays. T cell interactions with selectins were recorded using a ×20 phase contrast objective and a videomicroscopy and VCR system. T cells were drawn through the chamber at decreasing flow rates for 2.5 min each, i.e., 1.3 ml/min (2.1 dynes/cm^2), 0.94 ml/min (1.5 dynes/cm^2), 0.78 ml/min (1.2 dynes/cm^2), 0.52 ml/min (0.8 dyne/cm^2), and 0.26 ml/min (0.4 dyne/cm^2). T cell accumulation was determined after the initial minute of each flow rate by counting the number of cells in four different fields as previously described (35). The instantaneous rolling velocities of Th1 or Th2 subsets on the P-selectin and E-selectin substrates were measured every 0.2 s over a time period of 4 to 6 s using a customized image analysis program (OPTIMAS Bioscan, Edmonds, WA).

Semiquantitative RT-PCR analysis of glycosyltransferases

Determinations were made of the relative levels of RNA encoding core 2 *N*-acetylglucosaminyltransferase (C2GnT), α-3-fucosyltransferase VII (FucT-VII), and dihydrofolate reductase (DHFR). Isolation of RNA, reverse transcriptase reactions (RT), and PCR reactions were performed as previously described for human cells (36). As a negative control, RT was omitted from otherwise identical RT reactions. Primers were as follows: murine FucT-VII sense 5′-acc cta cgg tcg ctt gga gcc tct-3′, antisense 5′-caa gca aag aag cca cga taa cga-3′; murine C2GnT sense 5′-ttt tcw ggc agt gcc tac ttc gtg gtc-3′, antisense 5′-atg ctc atc caa aca ctg gat ggc aaa-3′; murine DHFR sense 5′-cca caa cct ctt cag tgg aag gta aac aga-3′, antisense 5′-ttg gca aga aaa tga gct cct cgt gg-3′. PCR reactions were conducted for 32, 26, and 26 cycles for FucT-VII, C2GnT, and DHFR, respectively, which we previously established were below plateau phase for these genes. PCR reactions were run out on 1.2% gels, transferred to nitrocellulose, and Southern blotted with probes specific to these three genes.

Statistical analysis

All results were expressed as the mean ± SEM unless otherwise stated. Statistical analyses by ANOVA followed by paired *t* test were performed using Microsoft Excel 5.0 (Microsoft, Redmond, WA) and were considered statistically significant at $p \leq 0.05$.

Results

Phenotype of in vitro differentiated DO.11 effector T cell populations

After naive T cells are stimulated with Ag in the presence of IL-12 and anti-IL-4 in primary cultures, they display a Th1 phenotype, producing abundant IFN-γ and little IL-4 after restimulation. Conversely, after Ag stimulation in the presence of IL-4 in primary cultures, the cells display a Th2 phenotype, producing abundant IL-4 and little IFN-γ after restimulation (Table I). These results are consistent with numerous reports on DO.11 T cell subset differentiation (8, 37, 38), and they establish the validity of correlating the adhesion properties of these bulk populations of T cells described below, with Th1 and Th2 phenotypes.

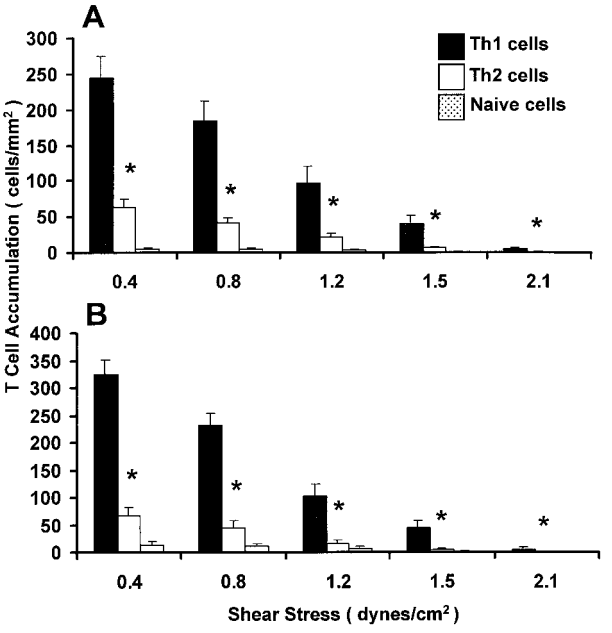

FIGURE 1. Accumulation of Th1, Th2, and CD4$^+$ naive cells on P- and E-selectin under flow. In vitro differentiated Th1 and Th2 cells were drawn across P-selectin (*A*)- and E-selectin (*B*)-coated coverslips within a flow chamber under decreasing levels of shear stress as described in *Materials and Methods*. The mean number of interacting Th1 cells (black bars), Th2 cells (open bars), and CD4$^+$ naive T cells (shaded bars) from four different fields were counted after 1 min of perfusion. Data are mean ± SEM from 9–11 experiments for P-selectin and 6–8 experiments for E-selectin. Data for CD4$^+$ naive T cells are representative of two different experiments. *, $p \leq 0.05$ as compared with Th1 cells.

Interactions of Th1 and Th2 cells with E- and P-selectin under flow conditions

The capability of the in vitro differentiated Th1 and Th2 cells and freshly isolated naive DO.11 T cells to initially attach to vascular selectins and roll under defined flow conditions was assessed using a parallel plate flow chamber system as described in *Materials and Methods*. The cells were initially drawn into the chamber at a flow rate (1.3 ml/min) that maintained an estimated shear stress of 2.1 dynes/cm^2 for 3 min. The flow rate was subsequently decreased stepwise every 2.5 min. Significantly more Th1 than Th2 cells accumulated and rolled on the P-selectin-coated surface at each level of wall shear stress (Fig. 1*A*). Similarly, significantly more Th1 cells interacted with E-selectin than Th2 cells under identical conditions (Fig. 1*B*). Similar to our previous report with human naive CD4$^+$ T cells (23), few if any naive mouse CD4$^+$ T cells interact with either E- or P-selectin under defined flow conditions (Fig. 1). This observed difference in selectin-binding capabilities between in vitro differentiated Th1 and Th2 cells is highly reproducible as the results in Fig. 1 are based on 9–11 experiments for P-selectin and 6–8 experiments for E-selectin. Moreover, these interactions are selectin mediated because the T cells did not interact with human IgG coated control coverslips under similar conditions, and treatment of the P-selectin and E-selectin coverslips

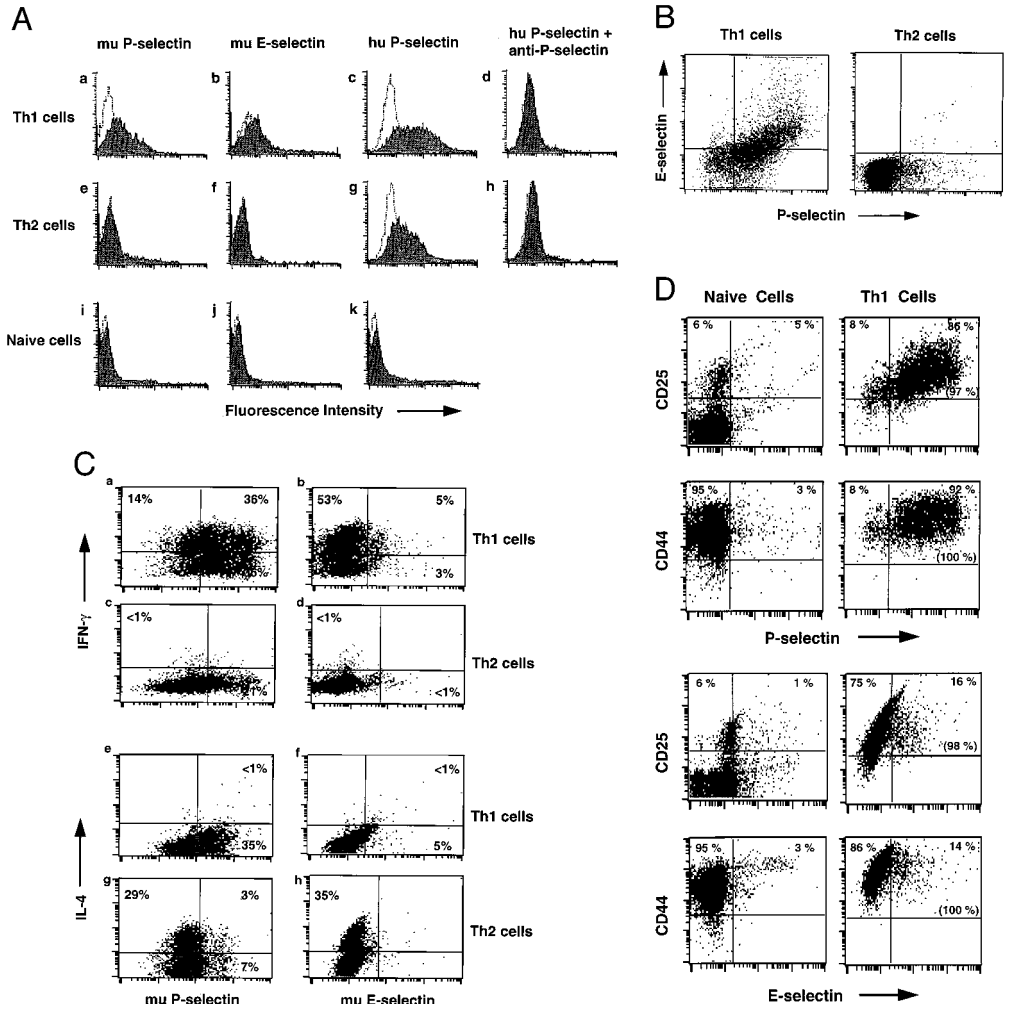

FIGURE 2. Flow cytometric analysis of selectin binding by Th1, Th2, and CD4$^+$ naive T cells. *A*, Th1, Th2, and naive T cells were stained with murine P-selectin-human IgM or E-selectin-human IgM fusion protein followed by PE-conjugated anti-human IgM Ab (27) or human P-selectin-human IgG fusion protein. The histograms show binding of murine (mu) P-selectin (*a, e, i*), murine (mu) E-selectin (*b, f, j*), and human (hu) P-selectin (*c, g, k*), by Th1 cells (*a, b, c*), Th2 cells (*e, f, g*), and naive T cells (*i, j, k*). Nonbinding human IgM was used as a control for the murine selectin-human IgM reagents, and human IgG was used as a control for the human P-selectin-human IgG reagent (dotted lines). The *x*-axis is a 4-decade log scale. The specificity of the observed binding of human P-selectin-human IgG to Th1 and Th2 cells was tested by addition of a blocking anti-P selectin Ab HDPG 2/3 (*d, h*). *B*, Two-color stains were performed on Th1 and Th2 populations, as described in *Materials and Methods*, to simultaneously detect both P- and E-selectin-binding cells. *C*, Two-color stains were performed, as described in *Materials and Methods*, to detect cytoplasmic expression of IFN-γ (*a–d*) or IL-4 (*e–f*) and surface binding of murine P-selectin (*a, c, e, g*) or murine E-selectin (*b, d, f, h*) by Th1 and Th2 cells, as indicated. The quadrant lines were set according to the fluorescence of samples incubated with isotype matched control Igs with the appropriate fluorochromes. *D*, Two-color stains were performed on naive and Th1 cells to simultaneously detect selectin-binding and expression of either CD25 or CD44, as indicated. The unbracketed numbers in the quadrants of the dot-plots represent the percent of total gated cells within that quadrant. The bracketed numbers represent the percent of total P- or E-selectin-positive cells that are also positive for CD25.

with blocking mAbs, HPDG 2/3 for P-selectin and 7A9 for E-selectin, completely abrogated T cell interactions (data not shown).

Interestingly, there was no significant difference in instantaneous rolling velocities between Th1 cells (6.11 ± 5.9 μm/s; n = 79) and Th2 cells (5.79 ± 6.04 μm/s; n = 39) on P-selectin at 0.8 dyne/cm^2. This suggests that the density of functional P-selectin ligands is approximately the same on rolling cells within Th1 and Th2 populations, but the frequency of T cells expressing the functional ligands is much higher in Th1 than in Th2 populations. This notion is supported by results obtained from flow cytometric analyses of P- and E-selectin binding (see Fig. 2, *C* and *D*, discussed below). In contrast, there was a significant difference in instantaneous rolling velocities on E-selectin at 0.8 dyne/cm^2 between Th1 cells (0.78 ± 1.76 μm/s; n = 69; $p < 0.05$) and Th2 cells (9.19 ± 9.31 μm/s; n = 74). This suggests that in comparison to Th1 cells, Th2 cells express lower density or lower avidity forms of E-selectin ligand(s).

Flow cytometric analysis of P- and E-selectin-binding by Th1 and Th2 cells

We performed flow cytometric studies using soluble murine selectin-IgM chimeras as ligands to determine whether the relative abilities of D0.11 Th1 and Th2 cells to roll on both P- and E-selectin in the flow adhesion assay correlates with binding of soluble ligand. This is a very relevant question because of a recent report that murine Th1 cells bind significantly more soluble P-selectin-Ig chimeric protein than do Th2 cells (25). We found that 50 ± 3.5% (n = 2) of Th1 cells had the capability of binding murine P-selectin-IgM in suspension (Fig. 2*Aa*). In contrast to Th1 cells, 15 ± 10% (n = 2) of Th2 cells bound murine P-selectin-IgM

chimera (Fig. 2*Ae*). Both the number of cells staining positive with the P-selectin reagents and the mean intensity of staining were higher for Th1 cells than for Th2 cells. Naive $CD4^+$ cells did not bind murine P-selectin-IgM (Fig. 2*Ai*). DO.11 Th1 cells also bound murine E-selectin-IgM, albeit the number of positively labeled cells were lower than that seen with P-selectin (Fig. 2*Ab*). Th2 or naive cells did not bind E-selectin (Fig. 2*A*, *f* and *j*). In parallel experiments, the capability of these murine T cell subsets to bind human P-selectin-IgG chimera was also examined. About 73% of the Th1 cells and 45% of Th2 cells bound human P-selectin-IgG (Fig. 2*A*, *c* and *g*). Although the amount of human P-selectin-IgG binding to both Th1 and Th2 cells was higher than binding of murine P-selectin-IgM, the specificity of the human reagent was confirmed with blocking anti-human P-selectin Ab (Fig. 2*A*, *d* and *h*). In contrast, naive T cells showed negligible binding of human P-selectin (Fig. 2*Ak*). No T cell binding of human E-selectin could be detected by flow cytometry, even though human E-selectin-IgG chimera supported Th1 subset rolling interactions under flow conditions (Fig. 1). These findings confirm those reported by Borges et al. (25) indicating that immunofluorescence analyses of the binding of soluble ligands can distinguish Th1 and Th2 populations. Nonetheless, the relative amounts of human P-selectin binding by Th1 and Th2 cells, as assessed by immunofluorescence, may not accurately reflect the quantitative differences in functional P-selectin ligand expression as assessed by flow chamber studies.

Two-color staining of T cells with both P- and E-selectin reagents was performed to determine whether the same or different cells bound each of these selectins (Fig. 2*B*). We found that the great majority of E-selectin binding Th1 cells also bind P-selectin, but, as we knew from our single-color analysis, there are more cells that bind soluble P-selectin than soluble E-selectin. Thus, many Th1 cells bind P-selectin but not E-selectin, and very few Th1 cells bind E-selectin but not P-selectin.

We also performed intracytoplasmic staining for cytokines in combination with surface staining of the same cells with selectin ligands to determine the relative numbers of selectin-binding cells that produced IFN-γ or IL-4 (Fig. 2*C*). In the representative experiment shown, ~50% of the Th1 population produced IFN-γ (Fig. 2*C*, *a* and *b*), and <1% produced IL-4 (Fig. 2*C*, *e* and *f*). In contrast, ~1% of the Th2 population produced IFN-γ (Fig. 2*C*, *c* and *d*), and 33% produced IL-4 (Fig. 2C, *g* and *h*). About 72% of Th1 cells bound murine P-selectin-IgM, and 50% of these cells produced IFN-γ (Fig. 2*Ca*). About 8% of Th1 cells bound murine E-selectin-IgM, and 60% of these cells produced IFN-γ (Fig. 2*Cb*). Only 10% of Th2 cells bound murine P-selectin-IgM, and 30% of these cells produced IL-4 (Fig. 2*Cg*). As described above (Fig. 2*A*), very few Th2 cells bound E-selectin (Fig. 2*Ch*). Thus, the frequency of IFN-γ producing, P-selectin binding cells in the Th1 population was about 12 times greater than the frequency of IL-4-producing, P-selectin-binding cells in the Th2 population. Similarly, there was a much higher frequency of IFN-γ-producing, E-selectin-binding cells in the Th1 population compared with a negligible percentage of IL-4-producing, E-selectin-binding cells in the Th2 population.

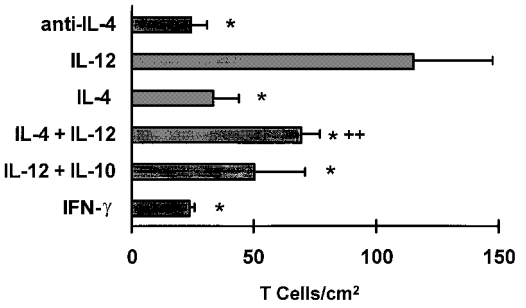

FIGURE 3. Influence of cytokine milieu on effector T cell capability to interact with P-selectin under flow. DO.11 naive $CD4^+$ T cells were stimulated with OVA peptide and APCs in the presence of anti-IL-4, IL-12 (+ anti-IL-4), IL-4, IL-4 + IL-12, IL-12 + IL-10, and IFN-γ. After 6 days in culture, the effector T cells were perfused over P-selectin-coated coverslips at 0.8 dyne/cm^2 as previously described. The mean number of interacting cells was determined from four fields. Data are mean ± SEM from three experiments. *, $p \leq 0.05$ as compared with the condition of IL-12 (+ anti-IL4); ++, $p < 0.05$ as compared with the condition of IL-4.

Further phenotypic analysis of selectin-binding Th1 and naive T cells was undertaken using the T cell activation markers CD25 and CD44 (Fig. 2*D*). The staining profiles indicate that the majority of P-selectin-binding Th1 cells (97%) are $CD25^+$ and Th1 cells express more CD44 than naive cells (mean channel fluorescence, 776 vs 253, respectively). These phenotypic features are typical of recently activated cells. Binding of soluble P- or E-selectin is largely limited to the $CD25^+$ Th1 cells.

IL-12 enhances expression of functional P-selectin ligand on T cells

The ability of Th1 but not Th2 cells to initially attach and roll on P-selectin under flow conditions suggests that one or more of the cytokines that direct T cell subset differentiation influences the expression of P-selectin ligand(s). To determine which cytokines are involved, flow adhesion assays were performed with DO.11 cells after 6 days of Ag stimulation, in the absence or presence of different combinations of added cytokines. As seen in Fig. 3, DO.11 T cells that were differentiated in the presence of anti-IL-4 mAb but without exogenous cytokines demonstrate a very low but consistent level of rolling interactions with P-selectin at 0.8 dyne/cm^2. These cells are Th1-like, producing little IL-4 and abundant IFN-γ (Table II and Fig. 2*C*). As described earlier, the presence of IL-12 significantly enhanced the differentiation toward a P-selectin-binding phenotype, while IL-4 had little effect (Fig. 3). Interestingly, T cells differentiated in the presence of both IL-4 and IL-12 exhibit an intermediate level of accumulation and rolling cells, significantly greater than the cells differentiated with anti-

Table II. *Cytokine production by DO.11 T cells after differentiated in the presence of various combinations of cytokines*[a]

Differentiation Conditions	Anti-IL-4	IL-12 + Anti-IL-4	IL-4	IL-4 + IL-12	IL-12 + Anti-IL-4 + IL-10	IFN-γ + Anti-IL-4
IFN-γ	707 ± 112	1002 ± 276	317 ± 112	472 ± 74	896 ± 51	182 ± 228
IL-4	37 ± 44	53 ± 84	445 ± 171	438 ± 186	87 ± 83	11 ± 5

[a] Freshly isolated DO.11 T cells were stimulated in vitro with Ag and APCs in the presence of the cytokine/Ab combinations indicated (Differentiation Conditions), as described in *Materials and Methods*. After 6 days of culture, the T cells were harvested, washed, and restimulated with Ag and 24-h supernatants were collected and analyzed for IL-4 and IFN-γ, as described in Table 1. $N = 2$ for all conditions.

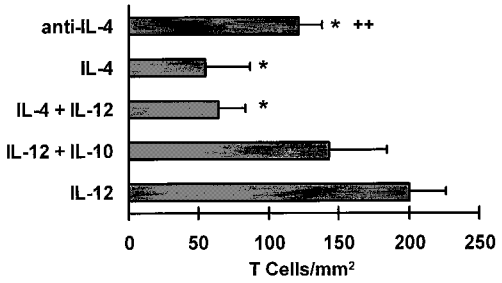

FIGURE 4. Influence of cytokine milieu on effector T cell capability to interact with E-selectin under flow. Effector T cells were differentiated from naive $CD4^+$ T cells in different combinations of cytokines and perfused over E-selectin coated coverslips at 0.8 dyne/cm^2 as described for Fig. 3. The mean number of interacting cells was determined in four fields. Data are mean ± SEM from three experiments. *, $p \leq 0.05$ as compared with the condition of IL-12 (+anti-IL4); ++, $p < 0.05$ as compared with the condition of IL-4.

IL-4 and no added cytokines, hence implicating a role for IL-12 in inducing expression of functional P-selectin ligand (Fig. 3). The reduced number of interactions, as compared with IL-12-treated (Th1) cells, may be the result of the antagonizing effects of exogenous IL-4 on T cell responsiveness to IL-12. T cells differentiated in the presence of IL-4 plus IL-12 produced significant amounts of IL-4 and far less IFN-γ than Th1 cells (Table II). Similarly, the presence of exogenous IL-10 also can partially antagonize the inductive effects of IL-12 on P-selectin ligand expression and lead concomitantly to a moderate reduction in IFN-γ production by the differentiated cells.

In contrast to the observed effects of IL-12 on the induction of functional P-selectin ligand expression, exogenously added IFN-γ did not enhance P-selectin ligand expression. In fact, when IFN-γ alone was added to differentiation cultures, the resulting T cell population did not interact with P-selectin any more than Th2 cells (Fig. 3).

Capability of Th1 cells to initially attach and roll on E-selectin is independently regulated from that for P-selectin

In experiments performed in parallel to those described in Fig. 3, the capabilities of the different T-helper subsets to interact with E-selectin was also examined. Fig. 4 shows the number of T cells, differentiated in the presence of various cytokine combinations, that accumulate on E-selectin at 0.8 dyne/cm^2. Interestingly, cells differentiated in the presence of anti-IL-4 but in the absence of added cytokines, interacted in large numbers with E-selectin under flow. This is in contrast to their inability to interact with P-selectin under identical conditions (Fig. 3). However, the number of interacting cells after differentiation in the presence of IL-4 or IL-4 plus IL-12 is essentially the same, suggesting that IL-4 is dominant over IL-12 with respect to regulation of E-selectin ligand. After differentiation in the presence of IL-12 and IL-10, T cell interactions with E-selectin were no different from those of T cells differentiated in the presence of IL-12 alone. This suggests that IL-10 does not antagonize the effect of IL-12. This is in contrast to the decreased interactions with P-selectin caused by IL-10 (Fig. 3).

IL-12 can induce polarized Th2 cells to interact with P-selectin but not with E-selectin under defined flow conditions

To further examine whether T helper cell interactions with P-selectin and E-selectin can be modulated by the presence of IL-12, we investigated the effects of restimulating Th2-polarized cells in the presence of IL-12 (and IFN-γ). Fig. 5*A* shows the cytokine

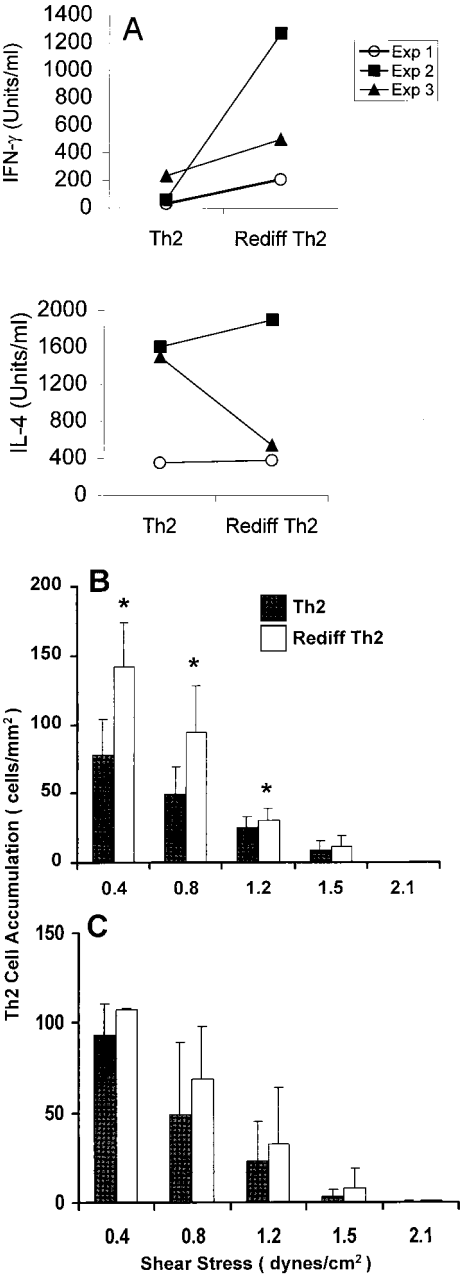

FIGURE 5. IL-12 induces polarized Th2 cells to interact with P-selectin but not E-selectin under flow. Th2 cells were restimulated in the presence of IL-12, IFN-γ plus anti-IL-4 for 6 days as described in *Materials and Methods*. The effector T cells were then analyzed for their ability to produce IFN-γ and IL-4 (*A*) and interact with P-selectin (*B*) or E-selectin (*C*) under flow. The number of interacting redifferentiated Th2 cells (open bars) was compared with the parent Th2 cells (dark bars) across the range of shear stress studied. Data are mean ± SEM from four experiments for *B* and two experiments for *C*. *, $p \leq 0.05$ as compared with parent Th2 cells.

production profile of DO.11 cells at two different differentiation time points: at 6 days following initial stimulation of naive $CD4^+$ cells with Ag and IL-4 (Th2 cells); and at 6 days after secondary Ag stimulation of the Th2 cells in the presence of IL-12, IFN-γ, plus anti-IL-4 mAb. IFN-γ was added to these cultures to ensure responsiveness to IL-12 by up-regulating the β_2 chain of the IL-12 receptor because Th2 cells do not constitutively express this receptor chain (39). As shown in Fig. 5, *B* and *C*, secondary restimulation of Th2 cells in the presence of IL-12 and IFN-γ (i.e.,

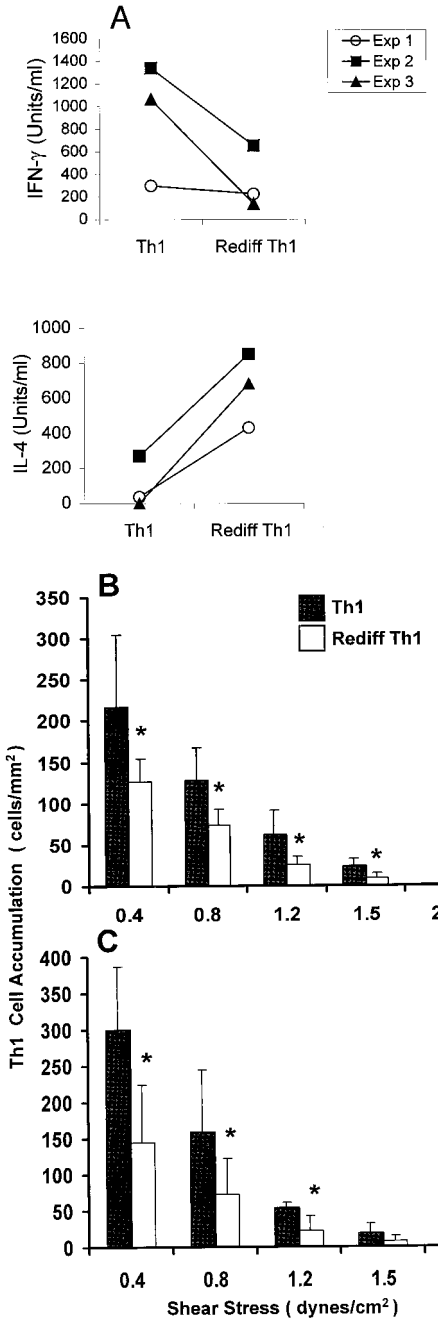

FIGURE 6. Th1 cells restimulated in IL-4 interact with P-selectin but not E-selectin under flow. Polarized Th1 cells were restimulated in the presence of IL-4 for 6 days and were analyzed for their ability to produce IFN-γ and IL-4 (*A*) and interact with P-selectin (*B*) or E-selectin (*C*) under flow as in Fig. 5. The number of interacting redifferentiated Th1 cells (open bars) was compared with the parent Th1 cells (dark bars) for the range of shear stress studied. Data are mean ± SEM from four experiments for *B* and two experiments for *C*. ∗, $p \leq 0.05$ as compared with parent Th1 cells.

Th1 polarizing conditions) resulted in a cell population with enhanced rolling/accumulation on P-selectin as compared with the parent Th2 population. This was accompanied by an enhanced capacity to secrete IFN-γ (Fig. 5*A*). In contrast, these same cells did not show enhanced ability to roll/accumulate on E-selectin (Fig. 5*C*). Conversely, when Th1 cells were restimulated with Ag in the presence of IL-4 (Th2 differentiation conditions), interactions with both P- and E-selectin decreased (Fig. 6, *B* and *C*). Furthermore, secondary restimulation of Th1 cells with IL-4 induced the capacity to produce IL-4 and reduced IFN-γ production (Fig. 6*A*).

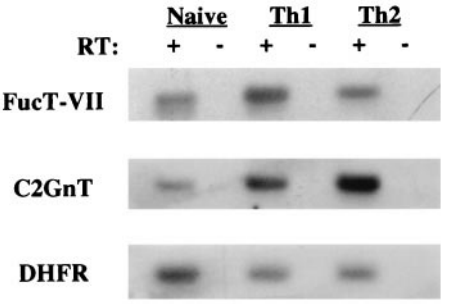

FIGURE 7. Th1 cells express more FucT-VII mRNA than naive or Th2 cells. RNA was prepared from freshly isolated naive DO.11 T cells and from cells after 6 days of culture in Th1 or Th2 differentiation conditions, as described in *Materials and Methods*. Semiquantitative RT-PCR was then performed, using primers specific for the *FucT-VII* and *C2GnT* genes and the "housekeeping gene" *DHFR*. The lanes labeled + and − indicate the inclusion or absence of reverse transcriptase, respectively, in the reaction mixture.

Expression of FucT-VII and C2GnT mRNA in naive, Th1, and Th2 cells

Semiquantitative RT-PCR analysis was conducted to determine the changes in the levels of mRNA for FucT-VII and C2GnT, enzymes that have previously been shown to be essential to selectin ligand biosynthesis (40) (Fig. 7). A low level of both FucT-VII and C2GnT mRNA was detected in naive cells, possibly due to contamination of naive cell preparations with monocyte/macrophages, which express high levels of these gene products. Th1 cells expressed much higher levels of FucT-VII mRNA than naive cells, whereas Th2 cells showed only a modest increase (compare FucT-VII levels with DHFR). In contrast, C2GnT levels increase equally in both Th1 and Th2 cells. Thus, FucT-VII mRNA levels correspond to the levels of adhesion to endothelial selectins demonstrated above.

Discussion

The data presented here show that during Ag-specific differentiation from naive T cells, murine $CD4^+$ Th1 cells acquire the ability to interact with selectins under levels of defined wall shear stress that approximate in vivo conditions in microvessels. In contrast, Th2 cells express much lower levels of functional selectin ligands. These findings establish that expression of physiologically functional selectin ligands is regulated in a T cell subset-specific manner. The acquisition of functional selectin ligands during transition from naive to effector phenotype is consistent with our previous studies of human $CD4^+$ T cells in which $CD45RA^+$ cells did not roll on E- or P-selectin but in vitro differentiated $CD45RO^+$ T cells did (23). The Th1-restricted expression of functional selectin ligands that support rolling interactions with vascular selectins under flow is also consistent with previous reports that murine Th1, but not Th2, cells bind soluble P-selectin (25) as assessed by indirect immunofluorescence and flow cytometry.

Differentiation of naive $CD4^+$ T cells to effector cells requires Ag stimulation by professional APCs. Furthermore, in vitro studies have established that cytokines added exogenously can drive effector T cell differentiation toward polar Th1 or Th2 phenotypes. In particular, IL-12 is required for Th1 differentiation, and IL-4 is required for Th2 differentiation. Hence, we stimulated naive DO.11 TCR transgenic $CD4^+$ T cells in the presence of different

culture conditions to address the effects of cytokines on synthesis of E- and P-selectin ligands. We have found that the addition of IL-12 to naive T cell differentiation cultures, whether or not IL-4 is present, will induce functional P-selectin ligands. In contrast, adding IFN-γ without IL-12 does not. Furthermore, when Th2 cells are restimulated with Ag in the presence of IL-12, they gain expression of functional P-selectin ligands. These findings are consistent with a critical role of IL-12 in inducing functional P-selectin ligand expression. Interestingly, IL-12 has been previously implicated in the induction of the E-selectin ligand called cutaneous lymphocyte-associated Ag during bacterial superantigen stimulation of human T cell (41). In contrast, IFN-γ is not sufficient to induce expression of functional P-selectin ligands in this model. In addition, the current results indicate that the ability of effector T cells to make significant amounts of IFN-γ, a characteristic of Th1 cells, does not strictly correlate with enhanced expression of functional P-selectin ligands. This is most evident when DO.11 cells are differentiated in the presence of anti-IL-4 but no added cytokines (Fig. 3 and Table II).

Our data indicate that the regulation of selectin ligand expression is not tightly linked to regulation of cytokine gene expression. For example, although highly polarized Th1 populations interact with P-selectins under flow to a much greater extent than do polarized Th2 cells, we observed significant P-selectin interactions by Th0-like populations that produced both IFN-γ and IL-4, as long as those populations were differentiated from naive T cells in the presence of IL-12. These findings are consistent with a recent report describing the selectin binding and cytokine expression properties of lymph node T cells draining a skin DTH site (42). In that study, both IFN-γ-producing and -nonproducing cells bound soluble P-selectin. Far fewer IL-4-producing cells were found in the lymph nodes, but again both IL-4-producing and -nonproducing cells bound P-selectin. It is likely that in the skin DTH model, few highly polarized Th2 cells are generated, but IL-12-driven Th1 differentiation does occur. Those in vivo findings and the findings reported here support the notion that there is heterogeneity in both selectin binding and the T cell cytokine secretory responses among Ag-activated T cells. Furthermore, there is no obligate coexpression of both IFN-γ and functional selectin ligand. Nonetheless, both phenotypic characteristics are promoted by the presence of IL-12 during T cell differentiation from naive T cells, and therefore IL-12 will lead to the generation of IFN-γ-producing cells that are capable of binding to selectins on activated endothelium at inflammatory sites.

Other recent studies have found that regulation of the expression of functional P-selection ligands differs from regulation of functional E-selectin ligands (43, 44). The results reported here support the hypothesis that E- and P-selectin ligand expression is distinctly regulated during effector $CD4^+$ T cell differentiation. While ligands for E- and P-selectin are detected in both flow cytometry and in vitro flow adhesion assays on polarized Th1 cells (but not on Th2 cells), only E-selectin ligand expression (but not P-selectin ligand) is detected following Ag stimulation of naive T cells in the absence of exogenous IL-12 (Fig. 4). Furthermore, the loss or gain in adhesion to P-selectin under flow did not match that of E-selectin adhesion when differentiated Th1 and Th2 cells were redifferentiated by a second Ag challenge and cytokine stimulation (Figs. 5 and 6). Interestingly, although the expression of functional E-selectin ligand could be induced by Ag stimulation of naive T cells, it was rapidly down-regulated by IL-4 (Figs. 4 and 6*C*). Indeed, in all experimental conditions where IL-4 was present, whether exogenously added or endogenously produced, the resultant effector T cells minimally interacted with E-selectin. This is in contrast to the expression of functional P-selectin ligands, which were maintained in at least two experimental conditions where IL-4 was present: 1) when naive DO.11 T cells were differentiated in the presence of IL-4 and IL-12 (Fig. 3); and 2) when polarized Th2 cells were redifferentiated in the presence of IL-12 (Fig. 5*B*). This demonstrates that the expression of functional E-selectin ligand is sensitive to the presence of IL-4. This finding is consistent with results reported by Teraki et al. (45) that showed the expression of the skin-homing E-selectin ligand on T cells, cutaneous lymphocyte Ag, is rapidly down-regulated by exogenous IL-4. Taken together, we speculate that the expression of functional E-selectin ligands on effector T cells is regulated by a negative feedback mechanism triggered by the presence of IL-4 during T cell differentiation.

Several recent reports support the notion that the expression of functional selectin ligands correlates closely with elevated levels of fucosyltransferase enzymes, in particular FucT-VII (26, 27). A recent study by Knibbs et al. (46) has shown that T cells derived from FucT-VII knockout mice do not synthesize ligands for E- or P-selectin. Consistent with these reports, we have found that the level of mRNA for FucT-VII was 10-fold higher in Th1 cells than in Th2 and naive T cells (Fig. 7). A report published while this paper was in revision also indicates that Th1 cells express more FucT-VII mRNA than Th2 cells, and this correlates with more binding of soluble P-selectin to Th1 cells (42). Interestingly, Knibbs et al. also have reported that synthesis of P-selectin ligands required lower levels of FucT-VII activity than the level required for E-selectin ligand synthesis, and the authors did not observe T cell E-selectin ligand expression in the absence of P-selectin ligand. The latter finding contrasts with the data reported here indicating that E-selectin ligand can be induced without P-selectin ligand when DO.11 T cells are simulated with Ag but no added cytokines. The culture conditions reported by Knibbs et al. were very different from those used here, and the activated T cell populations analyzed in that study were largely $CD8^+$. Nonetheless, it is reasonable to infer that TCR activation without exogenous cytokines can induce the elevation in FucT-VII that is necessary to synthesize functional E-selectin ligands. Indeed, studies using T cell lines have shown that TCR activation of T cells alone can induce an increase in FucT-VII mRNA expression (26). It is also possible that FucT-IV, which in certain cells can generate E-selectin ligands (26), is induced by TCR stimulation in DO.11 T cells, and this may be sufficient to generate functional levels of E-selectin ligands but not P-selectin ligands. In contrast, for the expression of functional P-selectin ligands, other signals in addition to TCR activation, appear to be required. In fact, our data show that the presence of IL-12 during TCR activation of naive T cells or restimulation established Th2 cells is critical to generate P-selectin ligands. We infer from these new data that an elevated level of FucT-VII per se is not the sole requirement for generation of both E- and P-selectin ligands, but another level of control is likely involved. Other potential point(s) of control by cytokines include regulation of FucT-IV and/or core 2 expression and enzyme(s) that mediate posttranslational sulfation reactions that are critical for PSGL-1 function, the major P-selectin ligand (40).

In summary, this study establishes that the expression of physiologically relevant P- and E-selectin ligands differ in Th1 and Th2 populations and that this is a consequence of the regulatory influences by exogenous cytokines during effector T cell differentiation. These findings are consistent with the hypothesis that IL-12 is a key regulator of functional P-selectin ligand expression during Th1 differentiation, and IL-4 may be an important down-regulator of E-selectin ligand. Further investigations will be necessary to precisely define the signals involved in cytokine regulation of selectin

ligand expression and the possible direct effects of cytokines on the expression of glycosyltransferases involved in ligand production.

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