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Cutting Edge: Differential Requirements for Stat4 in Expression of Glycosyltransferases Responsible for Selectin Ligand Formation in Th1 Cells

Samuel J. White,* Gregory H. Underhill,* Mark H. Kaplan,† and Geoffrey S. Kansas²*

A role for Stat4 in IL-12-induced up-regulation of selectin ligands on Th1 cells was explored. Th1 cells generated from Stat4²/² mice exhibited no IL-12-inducible P-selectin ligands, no up-regulation of core 2 β1,6-galactosaminyltransferase I (C2GlcNAcT-I), and low levels of the Th1 transcription factor T-bet. In contrast, Stat4²/² Th1 cells exhibited only a partial defect in expression of IL-12-inducible E-selectin ligands and expressed equivalently high levels of α1,3-fucosyltransferase VII (FucT-VII) as wild-type Th1 cells. FucT-VII expression was induced by T cell activation, and was enhanced by IL-12 independently of Stat4, whereas C2GlcNAcT-I up-regulation was mediated exclusively by IL-12, acting through Stat4. These data show that FucT-VII and C2GlcNAcT-I are controlled through distinct pathways and imply the existence of at least one other IL-12-inducible glycosyltransferase required for E-selectin and possibly P-selectin ligand formation in Th1 cells. *The Journal of Immunology, 2001, 167: 628–631.

How cytokines control T cell migration remains incompletely understood. Th1 cells express considerably higher levels of functional ligands for both E-selectin and P-selectin than do naive, Th2, or Th0 cells (1–3), implying an important role for IL-12 in the generation of selectin ligands. We have previously shown in activated human CD4 cells that IL-12, the canonical Th1-polarizing cytokine, enhances expression of α1,3-fucosyltransferase VII (FucT-VII), an FucT-VII well established to play an essential role in selectin ligand formation in all leukocytes, but particularly in T lymphocytes (2, 4). In contrast, IL-4, the canonical Th2-polarizing cytokine, inhibited FucT-VII expression, thereby providing a possible molecular basis for the difference in selectin binding capacity of short-term polarized Th1 and Th2 cells (2). An important role for TCR-initiated pathways was also revealed in that study. These results showed that cytokines can influence selectin ligand expression through control of FucT-VII expression, but did not identify signal transduction or transcriptional pathways by which IL-12 or IL-4 controls FucT-VII expression. In addition, these studies did not elucidate how expression of other relevant glycosyltransferases was controlled, or how pathways initiating at the TCR interact with those emanating from cytokine receptors. In this report, we used mice with a targeted disruption of Stat4 to examine the role of Stat4 in induction of selectin ligands and in the induction of FucT-VII and core 2 β1,6-galactosaminyltransferase I (C2GlcNAcT-I), an O-linked branching enzyme also critically involved in selectin ligand biosynthesis (5, 6), in Th1 cells.

Materials and Methods

Mice

Production and characterization of Stat4²/² mice have been described previously (7). Stat4²/² mice used in the present study were backcrossed 10 generations to BALB/c. Wild-type (WT) BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were bred and maintained in the Center for Comparative Medicine at Northwestern University Medical School. All protocols were approved by the local Animal Care and Use Committee.

T cell activation

Splenic CD4 cells were isolated by magnetic bead columns according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). CD4 cells were activated for ~48 h on plates precoated with 1 μg/ml each of anti-CD3 and anti-CD28 in the presence (Th1) or absence (Th0) of 10 ng/ml IL-12 (R&D Systems, Minneapolis, MN) and 10 μg/ml anti-IL-4. Cells were then transferred to fresh medium without Ab and cultured with 20 ng/ml IL-2 in the continued presence (Th1) or absence (Th0) of IL-12 plus anti-IL-4. Fresh cytokines and anti-IL-4 were added every 2 days, and the cell concentration was maintained at ~10⁶ cells/ml for the duration of culture. Analysis of leukocyte rolling was performed on day 8, and RNA for RT-PCR analysis was obtained on day 6.

RT-PCR

Semiquantitative RT-PCR and Southern blotting to determine the relative levels of mRNA for FucT-VII, C2GlcNAcT-I, and dihydrofolate reductase (a housekeeping gene used as an internal control) were conducted as previously described (2, 3).

Flow cytometry

FACS analysis with 1B11 (BD Pharmingen, San Diego, CA) was performed as described elsewhere (6). For experiments using selectin...
chimeras, cells were incubated in empirically determined saturating concentrations of E-RIgM or P-RIgM (4), washed, and Cy5-conjugated goat anti-human IgM was used as a second step. Data were collected and analyzed using a FACSort or FACSCalibur flow cytometer and CellQuest software (BD Biosciences, Mountain View, CA).

**SDS-PAGE and Western blotting**

SDS-PAGE and Western blot analysis of cell lysates was as described previously (8). Cell lysates were made with high-salt radioimmunoprecipitation assay lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM EDTA, and a mixture of protease inhibitors) followed by ultracentrifugation. Rabbit anti-mouse T-bet antiserum (9) was kindly provided by Drs. L. Glimcher and S. Szabo (Harvard School of Public Health, Cambridge, MA).

**Leukocyte rolling assays**

The interaction of T cells with E-selectin and P-selectin was assayed in a parallel plate flow chamber (GlycoTech, Rockville, MD) at 1.5 dynes/cm² on monolayers of Chinese hamster ovary cells stably transfected with either human E-selectin or human P-selectin as described (6, 10). Data analysis was performed using Celltrak software developed by Compex (Mars, PA), as previously described (6, 10). Briefly, a rolling event is defined as a rolling cell that can be tracked by morphology and trajectory between paired sequential images separated by a defined time delay, here 2 s. The total number of rolling events was collected for 50–100 sequential images. Data are represented as the mean ± SD of duplicate runs and represents one of at least four separate experiments.

**Results and Discussion**

WT and Stat4−/− CD4 cells were activated in the presence (Th1) or absence (Th0) of IL-12 plus anti-IL-4 and analyzed for rolling on P- and E-selectin, expression of glycosyltransferases required for selectin ligand formation, and expression of T-bet, a recently described Th1 transcription factor (9). WT Th1 cells rolled well on E- and P-selectin, at levels 6–10-fold higher than WT Th0 cells (Fig. 1A). This loss of the IL-12-induced component of rolling activity on P-selectin was not due to delayed induction of P-selectin ligands on Stat4−/− Th1 cells, because FACS analysis of P-selectin ligands by P-selectin chimera (P-RIgM) staining showed a steady increase in the fraction of P-RIgM+ cells in the WT Th1 group, but low and relatively constant levels of P-RIgM+ cells in Stat4−/− Th1 cells and in both WT and Stat4−/− Th0 cells (Fig. 1B). In contrast, rolling of Stat4−/− Th1 cells on E-selectin was only partially inhibited. This level of rolling was clearly intermediate between that of Th0 cells and the high levels observed on WT Th1 cells (Fig. 1C). Kinetic analysis of E-selectin ligands confirmed this partial effect and showed that the partial loss of E-selectin ligands was not due to altered kinetics (Fig. 1D). Induction of P-selectin ligands in CD4 cells in response to IL-12 therefore absolutely requires Stat4, whereas Stat4 is only partially required for induction of E-selectin ligands.

We therefore examined the levels of C2GlcNAcT-I and FucT-VII mRNA in these same sets of activated CD4 cells. C2GlcNAcT-I mRNA was detectable in unstimulated CD4 cells, and levels were substantially enhanced in WT Th1 cells (Fig. 2A). These enhanced levels of C2GlcNAcT-I mRNA were strikingly absent from Stat4−/− Th1 cells. Both WT and Stat4−/− Th0 cells showed approximately the same low level of C2GlcNAcT-I mRNA as seen in unstimulated cells (Fig. 2A). Lack of up-regulation of C2GlcNAcT-I mRNA was strikingly absent from Stat4−/− Th1 cells, because FACS analysis of these same sets of activated CD4 cells in response to IL-12 therefore requires Stat4. In contrast, activation of both WT and Stat4−/− CD4 cells led to substantial increases in FucT-VII mRNA levels in both Th0 and Th1 cells, with higher levels observed in Th1 cells than in Th0 cells (Fig. 2A). Importantly, for both Th1 and Th0 cells, no difference was seen between WT and Stat4−/− cells. Stat4 is therefore not essential for induction of

**FIGURE 1.** Expression of selectin ligands on WT and Stat4−/− Th1 and Th0 cells. WT and Stat4−/− Th1 and Th0 cells were analyzed for expression of ligands for P-selectin (A and B) or E-selectin (C and D) under defined shear flow (A or C) or over time by flow cytometry (B and D). Each panel represents one of at least four experiments.
Presence or absence of reverse transcriptase in the reverse transcription reaction is indicated. Naive refers to unactivated CD4 cells isolated at time 0, which was analyzed by semiquantitative RT-PCR (B), and induction of C2GlcNAcT-I was analyzed by flow cytometry over time by staining with 1B11 (B). In contrast, low levels of T-bet, equivalent to Th0 cells, and high levels were found in WT Th1 cells. Induc- tion of T-bet in CD4 cells in response to IL-12 therefore requires Stats4, at least under these culture conditions. The concordant loss of both IL-12-inducible C2GlcNAcT-I and T-bet, coupled with the ability of T-bet to enhance binding to P-selectin, suggests that T-bet may play an important role in controlling expression of C2GlcNAcT-I.

C2GlcNAcT-I is absolutely required for formation of P-selectin ligands (5, 6, 12). The loss of IL-12-inducible C2GlcNAcT-I is therefore sufficient to explain the loss of IL-12-inducible P-selectin ligands in Stats4−/− CD4 cells. However, loss of IL-12-inducible C2GlcNAcT-I in Stats4−/− CD4 cells is not responsible for the partial loss of functional E-selectin ligands. Although C2GlcNAcT-I plays a detectable role in E-selectin ligand formation, at least in neutrophils, recent work has shown that, under identical assay conditions as used in the present work, Th1 cells from C2GlcNAcT-I−/− mice roll on E-selectin as well as WT Th1 cells, with no change in the number or velocity of rolling cells (6).

Since the complete absence of C2GlcNAcT-I has no detectable effect on expression of functional E-selectin ligands on Th1 cells, the lack of inducible C2GlcNAcT-I cannot be responsible for the partial loss of E-selectin ligands seen in Stats4−/− Th1 cells. Taken together with the lack of effect of Stats4 deficiency on FucT-VII levels, these results therefore imply the presence of other IL-12-inducible, Stats4-dependent glycosyltransferases involved in biosynthesis of E-selectin ligands.

FucT-VII is absolutely required for formation of all selectin ligands (4), but equivalent levels of FucT-VII were observed in WT and Stats4−/− T cells. These results show that lack of FucT-VII cannot be responsible for the selectin ligand defects in Stats4−/− Th1 cells. Our results also show that enhancement of FucT-VII in response to IL-12 does not require Stats4. Although Stats4 is well established as crucial for IL-12 signaling and Th1 development, IL-12 can also activate p38 mitogen-activated protein kinase (MAPK) pathways (13–15), which may also be important for Th1 development. TGF-β1 is also a potent inducer of FucT-VII and selectin ligands in human CD4 cells, and the induction of FucT-VII in activated CD4 cells in response to TGF-β1 requires p38 MAPK (16), further implicating this pathway in control of FucT-VII expression. Whether p38 MAPK is responsible for up-regulation of FucT-VII expression and/or selectin ligand formation in response to T cell activation and/or IL-12 is an important question for further experimentation.

Our results suggest a new level of regulation of lymphocyte traffic through independent control of expression of glycosyltransferases which are differentially required for formation of E-selectin ligands vs P-selectin ligands (Fig. 4). Expression of FucT-VII, which is absolutely required for both E- and P-selectin ligand formation in T cells, is induced by T cell activation and is enhanced and/or maintained by IL-12 independent of Stats4. In contrast, induction of C2GlcNAcT-I, which is absolutely required only for formation of P-selectin ligands, requires Stats4. As mentioned above, C2GlcNAcT-I−/− Th1 cells roll as well as WT Th1 cells on E-selectin (6). In addition, IgG plasma cells express high levels of FucT-VII and low levels of C2GlcNAcT-I, and roll well on E-selectin but poorly on P-selectin (4). IgG plasma cells, C2GlcNAcT-I−/− Th1 cells (6), and Stats4−/− Th1 cells therefore share a similar functional modification of P-selectin glycoprotein ligand 1, the P-selectin ligand. We therefore examined the expression of T-bet from WT and Stats4−/− Th0 and Th1 cells by Western blotting of whole-cell lysates. As expected, low levels of T-bet were detected in WT Th1 cells (Fig. 3). In contrast, low levels of T-bet, equivalent to those found in Th0 cells, were seen in Stats4−/− Th1 cells. Induction of T-bet in CD4 cells in response to IL-12 therefore requires Stats4, at least under these culture conditions. The concordant loss of both IL-12-inducible C2GlcNAcT-I and T-bet, coupled with the ability of T-bet to enhance binding to P-selectin, suggests that T-bet may play an important role in controlling expression of C2GlcNAcT-I.

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Since the complete absence of C2GlcNAcT-I has no detectable effect on expression of functional E-selectin ligands on Th1 cells, the lack of inducible C2GlcNAcT-I cannot be responsible for the partial loss of E-selectin ligands seen in Stats4−/− Th1 cells. Taken together with the lack of effect of Stats4 deficiency on FucT-VII levels, these results therefore imply the presence of other IL-12-inducible, Stats4-dependent glycosyltransferases involved in biosynthesis of E-selectin ligands.

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FIGURE 4. Independent pathways control expression of glycosyltransferases involved in selectin ligand biosynthesis in CD4+ T cells. IL-12 activates Stat4, which directly and/or indirectly controls expression of C2GlcNAcT-I, possibly through T-bet. We propose that IL-12 also controls expression of other, still unidentified glycosyltransferases (GT; boxed) through Stat4, either directly or through other transcription factors (TF). Although not depicted, T-bet could also control other glycosyltransferases in addition to C2GlcNAcT-I. T cell activation through the TCR/CD28 and IL-12 each independently control FucT-VII expression, and do so through still unidentified pathways independently of Stat4. Thus, multiple independent pathways control expression of distinct glycosyltransferases involved in selectin ligand biosynthesis. P-selectin glycoprotein ligand 1 (PSGL-1), the leukocyte ligand for P-selectin. E-selectin ligands have not been definitively identified at the molecular level.

E-selectin-binding, P-selectin-nonbinding phenotype. Other enzymes such as members of the ST3Gal family of α2–3 sialyltransferases (17) may also be independently regulated in activated CD4+ cells and/or differentially required for ligands for E-selectin vs P-selectin, allowing further fine tuning of the level of functional selectin ligands on activated T lymphocytes. Independent control of expression of these various enzymes, along with distinct requirements for individual enzymes in biosynthesis of E-selectin ligands vs P-selectin ligands, represents a novel level of regulation of lymphocyte traffic.

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References