Diverse Inflammatory Cytokines Induce Selectin Ligand Expression on Murine CD4 T Cells via p38 α MAPK

Mark E. Ebel, Olufolakemi Awe, Mark H. Kaplan and Geoffrey S. Kansas

*J Immunol* 2015; 194:5781-5788; Prepublished online 4 May 2015;
doi: 10.4049/jimmunol.1500485
http://www.jimmunol.org/content/194/12/5781

**References**

This article *cites 69 articles*, 38 of which you can access for free at:
http://www.jimmunol.org/content/194/12/5781.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Diverse Inflammatory Cytokines Induce Selectin Ligand Expression on Murine CD4 T Cells via p38α MAPK

Mark E. Ebel,* Olufolakemi Awe,†,‡ Mark H. Kaplan,†,‡ and Geoffrey S. Kansas*

Selectins are glycan-binding adhesion molecules that mediate the initial steps of leukocyte recognition of endothelium. Cytokines control numerous aspects of CD4 Th cell differentiation, but how cytokines control the induction of ligands for E- and P-selectin on Th cell subsets remains poorly understood. Among 20 cytokines that affect Th cell differentiation, we identified six that induce expression of selectin ligands on murine CD4 T cells above the low levels associated with TCR engagement: IL-12, IL-18, IL-27, IL-9, IL-25, and TGF-β1. Collectively, these six cytokines could potentially account for selectin ligand expression on all of the currently defined nonsessile Th cell lineages, including Th1, Th2, Th9, and Th17 cells, as well as regulatory T cells. Induction of selectin ligand expression by each of these six cytokines was almost completely inhibited by pharmacologic inhibition of p38 MAPK, but not other MAPKs, or by conditional genetic deletion of p38α MAPK. Analysis of the expression of key glycosyltransferase genes revealed that p38α signaling was selectively required for induction of Fat1 and Gent1 but not for the induction of St3gal4 or St3gal6. Constitutively active MKK6, an immediate upstream activator of p38 MAPK, induced selectin ligand expression equivalent to that of cytokines, and this induction was completely dependent on the expression of p38α.

Our results identify the repertoire of cytokines responsible for selectin ligand induction on CD4 T cells and provide a mechanistic link between Th cell development and T cell migration. The Journal of Immunology, 2015, 194: 5781–5788.
kindly provided by Dr. Mercedes Rincon (University of Vermont, Burlington, VT) and were crossed with mice expressing the cre recombinase under the control of the CD4 locus, which were originally obtained from The Jackson Laboratory and maintained in our colony. For all experiments involving mice with conditional deletion of p38α, CRE- littermates were used as controls. Mice of both genders were used and were matched within an experiment, and no differences between genders were observed. All experiments were approved by the Northwestern University Institutional Animal Care and Use Committee.

T cell activation and culture

Splenic CD4 T cells isolated from WT C57BL/6 mice by positive selection using CD4 magnetic beads (Miltenyi Biotec) were activated for ~40 h by plate-bound anti-CD3/anti-CD28 and cultured with the indicated cytokines (see Fig. 1 legend), as described (17, 20), with or without pharmacologic inhibitors. Cytokines were added at the initiation of culture, following the addition of pharmacologic inhibitors (or vehicle only; see figure legends), or following retroviral transduction, as indicated in each figure legend. Ecotrophic recombinant retrovirus was produced by transfecting Phoenix-Eco cells. Activated T cells were transduced by spinfection on day 2 of activation with supernatants containing recombinant retrovirus encoding eGFP plus no cDNA, dominant-negative (DN) p38 MAPK, or constitutively active (ca) MKK6, Rac1, or Rac2, as described (21). For all cultures, cells were harvested every 2 d, analyzed by FACScan, and recultured in fresh media with (DN p38, inhibitors) or without (caMKK6, caRac1, caRac2) cytokines. Cytokines (see Fig. 1 legend for concentrations used) were all murine, with the exception of TGF-β1, and were from PeproTech (IL-2, IL-4, IL-6, IL-7, IL-9, IL-12, IL-15, IL-21, IL-23); eBioscience (IL-10, INF-γ, IL-27, TNF-α); R&D Systems (TGF-β1, TSLP, IFN-β); Reprokine (IL-18, IL-25); or Axxora (IL-33).

Flow cytometry

Expression of E- and P-selectin ligands on T cells was determined by staining with E- or P-selectin/IgM chimeras, followed by Alexa Fluor 647–anti-human IgM, as described (17, 19). We and other investigators showed previously that staining with these selectin chimeras closely and quantitatively tracks the ability of cells to roll on these selectins (15, 17, 18, 20, 22–24). Live/dead gating was with SYTOX Blue. Retrovirally transduced cells were identified by eGFP expression. Data were collected on a BD FACScan and analyzed using FlowJo software.

Western blotting

Western blotting of whole-cell lysates representing 1 × 10⁶ cells for either total or phospho-p38 MAPK was as described (17).

Quantitative RT-PCR

Total RNA was isolated from CD4 cells on day 8 using TRizol reagent, and 1 μg was reverse transcribed using the Superscript III system and random hexamers. cDNA was amplified and quantitated with SYBR Green using primers specific for mouse St3gal4, St3gal6, Fut7, and Gene1. Data were normalized to the housekeeping gene Hprt and further normalized by assigning the normalized value for each gene in the absence of cytokines or inhibitors to 1.

Statistical analysis

Comparison between groups was by the multiple t test within Prism 6.

Results

Identification of selectin ligand–inducing cytokines

We selected a panel of cytokines based on their ability to affect Th cell differentiation and tested them individually or in physiologically relevant combinations for their ability to induce selectin ligands above the low levels associated with T cell activation alone. We included IL-2 in all cultures, because inclusion of IL-2 does not alter the level of selectin ligands on viable CD4 T cells but does increase cell yield and viability (data not shown). This screen identified six Th cell–promoting cytokines exhibiting strong selectin ligand–inducing activity: IL-12, IL-18, IL-27, IL-9, IL-25, and TGF-β1 (Fig. 1). No cytokines selectively induced only E- or P-selectin ligands. Selectin ligand expression on CD4 T cells cultured with TGF-β1 was not altered by inclusion of IL-6, and IL-6 alone had no significant selectin ligand–inducing activity, nor did other STAT3-activating cytokines, including IL-10 and IL-21. IL-4 inhibited selectin ligand induction by TGF-β1. Because IL-4 does not induce selectin ligand expression, how Th2 cells in vivo express selectin ligands (25, 26) has been enigmatic. Our finding that IL-25, which is known to promote Th2 development (27), is a strong inducer of selectin ligands suggests that IL-25 could be the major physiologic inducer of selectin ligands on Th2 cells in vivo. Although IL-9 is not known to promote the development of specific Th cell lineages on its own, autocrine IL-9 could amplify selectin ligand expression on Th9 or Th2 cells. Other cytokine-activating cytokines, including IL-2, IL-4, IL-7, and IL-15, had no significant effect. IL-21 and IL-23, both of which promote Th17 cell generation and/or expansion (28), were without effect. Notably, both IFN-β and IFN-γ were without effect, despite being potent inducers of T-bet (29), which is critical for selectin ligand induction in response to IL-12 (20). For each of these cytokines, selectin ligands were expressed on a distinct subpopulation of cells (Fig. 2A) and exhibited identical kinetics of expression, with the percentage of cells expressing selectin ligands increasing throughout the culture until at least day 10 (Fig. 2B). Dose-response curves (Fig. 2C) showed that the doses used were near maximal. Together, these results identify a restricted subset of cytokines that collectively could account for selectin ligand induction on each of the currently defined nonsessile Th cell lineages.

FIGURE 1. Cytokines that induce selectin ligands on murine CD4 T cells. CD4+ T cells were activated with plate-bound anti-CD3/CD28 in the presence of the following cytokines, as described in Materials and Methods: IL-4 (10 ng/ml), IL-6 (20 ng/ml), IL-7 (10 U/ml), IL-9 (20 ng/ml), IL-10 (20 ng/ml), IL-15 (10 ng/ml), IL-18 (50 ng/ml), IL-21 (20 ng/ml), IL-23 (20 ng/ml), IL-25 (25 ng/ml), IL-27 (100 ng/ml), IL-33 (10 ng/ml), TSLP (10 ng/ml), IFN-γ (10 ng/ml), IFN-β (1000 U/ml), TNF-α (20 ng/ml), IL-12 (10 ng/ml), and TGF-β1 (5 ng/ml). FACS analysis for selectin ligands was performed every 2 d; results are from day 8. (A) E-selectin. (B) P-selectin. Values are mean ± SD (n = 3). *p < 0.01, versus IL-2 alone; †p < 0.01, versus TGF-β1 alone.
Cytokines induce selectin ligands on CD4 cells via p38 MAPK

Induction of selectin ligands on human CD4 T cells in response to TGF-β1 is blocked by SB203580, a specific pyrimidazole inhibitor of p38 MAPK (18). IL-12, IL-18, and IL-27 are also known to activate p38 MAPK (30–32). Therefore, we asked whether p38 MAPK was a common pathway for selectin ligand induction by these six cytokines. CD4 T cells were activated by anti-CD3/CD28 overnight in the absence of cytokines to enhance cytokine responsiveness, rested for 6 h, and cultured with the six selectin ligand–inducing cytokines for 15 min. Each of the six cytokines strongly activated p38 MAPK, and for each cytokine, this effect was dependent on prior T cell activation (Fig. 3A).

We then activated CD4 T cells with anti-CD3/CD28 in the absence of cytokines and cultured them with each of the six cytokines in the presence or absence of specific inhibitors of different MAPK pathways. Selectin ligand induction in response to each of these six cytokines was nearly completely blocked by 5 μM SB203580 (Fig. 3B), but was not affected by pharmacologic inhibition of either ERK MAPK activation by 10 μM PD98059 (Fig. 3C) or JNK MAPK activation by 5 μM SP100265 (Fig. 3D). No defects in T cell proliferation or viability were observed in cultures containing SB203580 (data not shown). Because this dose of SB203580 also was reported to affect the PI3K pathway (33), we directly tested whether pharmacologic inhibition of PI3K by GSK1059615 would have any effect on selectin ligand induction in response to these cytokines and found that it did not (Fig. 3E). In addition, retroviral overexpression of a DN p38 blocked selectin ligand induction in response to all six cytokines (data not shown). These results indicate that the p38 MAPK pathway is
selectively involved in selectin ligand induction in response to diverse inflammatory cytokines.

Four p38 isoforms, p38α, p38β, p38γ, and p38δ, have been identified, three of which (p38α, p38β, and p38δ) are expressed in CD4 T cells (34). Of the expressed isoforms, p38β is expressed at very low levels (34), and p38δ is insensitive to inhibition by SB203580 (35). Taken together, these considerations clearly implicate p38α as the primary isoform responsible for cytokine-induced selectin ligand expression. To test this, we analyzed CD4 T cells from mice with a conditional deletion of p38α driven by CD4-cre. Consistent with previous reports (36–38), these mice exhibit no detectable alterations in T cell development or in peripheral CD4 or CD8 T cell numbers (data not shown). CD4 T cells were isolated from cre+ and cre− littermates and analyzed as above for selectin ligand induction by these six cytokines. The results (Fig. 4) show that conditional genetic inactivation of p38α in T cells abrogates the expression of selectin ligands in response to these six cytokines, phenocopying the results obtained with pharmacologic inhibition. Taken together, our results offer strong evidence that p38α MAPK is required for selectin ligand induction on CD4 T cells in response to this panel of cytokines.

p38α MAPK is required for induction of Fut7 and Gcnt1

To identify mechanisms of p38 MAPK–dependent selectin ligand induction, we next examined the expression of GTases known to be involved in selectin ligand biosynthesis in murine CD4 T cells (39–41). CD4 T cells activated and cultured with each of the six cytokines or IL-2 only, both with and without SB203580, were analyzed by quantitative RT-PCR for expression of genes encoding Fut7, Gcnt1, St3gal4, and St3gal6. The results (Fig. 5) show that expression of each of these GTase genes was coordinately increased by these six cytokines, with the most potent induction in response to IL-12 and TGF-β1, roughly corresponding to the percentage of selectin ligand–positive cells generated in the presence of each cytokine (Fig. 1). Inhibition of p38 MAPK by SB203580 strongly inhibited the expression of Fut7 and Gcnt1 but had no significant effect on St3gal4 or St3gal6 (Fig. 5). Essentially identical results were obtained using p38α-deficient CD4 T cells,
with which we also observed strong inhibition of Fut7 and Gcnt1 gene expression but no significant effect on St3gal4 or St3gal6 (Fig. 6). These results show that p38α MAPK signaling is selectively required for cytokine-driven upregulation of two key GTases in response to Th cell–promoting inflammatory cytokines.

**Constitutive activation of p38α is sufficient for induction of selectin ligands**

Finally, to determine whether activation of p38 MAPK was sufficient for induction of selectin ligands, CD4 T cells were activated and transduced with RV expressing caMKK6, the immediate upstream activator of p38 MAPK, and cultured in the absence of selectin ligand–inducing cytokines. We found that expression of caMKK6 induced levels of selectin ligands similar to those of cytokines (Fig. 7). Similar results were found using caRac1 or caRac2, which activate MEKK4, the upstream activator of MKK6 (Fig. 7). To ensure that this response was entirely dependent on p38α, these experiments were also carried out with p38α-deficient CD4 cells. Induction of selectin ligands by each of these upstream p38 activators was absent in cells deficient in p38α (Fig. 7). Thus, activation of p38α is essential for induction of selectin ligands on CD4 T cells in response to these six inflammatory cytokines and is sufficient for induction of selectin ligands in the absence of inducing cytokines.

**Discussion**

Although cytokines control numerous aspects of the immune response and host defense, how cytokines control leukocyte traffic, particularly T cell traffic, remains poorly understood. Similarly, it has been known for some time that specific cytokines or combinations of cytokines drive the differentiation of specific Th cell subsets, but how this is integrated into the regulation of T cell migration has been largely unclear. Selectins are critically involved in control of T cell traffic, and expression of selectin ligands is critical for recruitment of multiple classes of inflammatory Th cells to migrate to at least the skin and gut. Th1 cell migration to the skin during delayed type hypersensitivity (42, 43) and to the gut (44), Th2 cell recruitment to the skin in atopic dermatitis (45, 46), and Th17 cell migration to the gut (47, 48) all require selectin ligand expression on inflammatory T cells, and Th9 cells are also skin-tropic and proinflammatory (49). Tregs also require the ability to access peripheral tissues to downregulate immune responses (50). Thus, how selectin ligand expression is regulated on Th cells of all classes is a key question in understanding the pathogenesis of a range of chronic inflammatory disorders.

In this report, we identify a small group of inflammatory cytokines, IL-12, IL-18, IL-27, IL-9, IL-25, and TGF-β1, most of which have defined roles in the differentiation of specific Th cell subsets that induce selectin ligands on activated CD4 T cells. We further show that induction of selectin ligands by this group of cytokines requires p38 MAPK activity, mediated specifically by the p38α isoform, despite otherwise distinct signaling mechanisms. Our findings identify a common signaling mechanism underlying expression of selectin ligands on distinct classes of CD4 T cells and provide a foundation for further dissecting molecular mechanisms that coordinate the regulation of Th cell differentiation and Th cell migration.

Although MAPK p38β is expressed at much lower levels than p38α in CD4 T cells (34), more recent research showed that p38β represents as much as one third of the active (i.e., phosphorylated) species following activation specifically through the TCR (51). Whether this degree of relative phosphorylation of p38β is also induced by cytokines is unknown. As mentioned above, p38β activity is also inhibited by SB203580. However, our results using p38α conditional-knockout mice show clearly that p38α accounts for essentially all of the activity in response to cytokines, indicating that p38β plays little or no role in these responses. Data showing that caMKK6 or caRac1/2 can induce selectin ligands also suggest that cytokines trigger primarily the classical pathway of p38 MAPK activation.

At least four GTases contribute to selectin ligand formation: FucT-VII, C2GlcNAcT-I, ST3Gal-IV, and ST3Gal-VI (39–41, 52, 53). Although expression of the genes encoding each of these enzymes was upregulated by these six cytokines, we found that only Fut7 and Gcnt1 induction is dependent on p38 MAPK signaling. This finding implies that these cytokines trigger additional, p38α MAPK–independent signaling pathways that are responsible for upregulation of St3gal4 and St3gal6, which will be important to identify. Our results implicate Fut7 and Gcnt1 as targets of a p38α MAPK–dependent genetic program that controls T cell migration in diverse settings of inflammation.

**FIGURE 5.** Selective inhibition of Fut7 and Gcnt1 by p38 MAPK inhibition. CD4 T cells were activated and cultured with cytokines or IL-2 only in the presence (open bars) or absence (filled bars) of 5 μM SB203580, harvested at day 8, and resuspended in TRizol, and 1 μg of total RNA was reverse transcribed. Expression of Fut7, Gcnt1, St3gal4, and St3gal6 was quantitated using SYBR Green. Levels of mRNA for each enzyme in the absence of cytokines and inhibitors was assigned a value of 1. Data are mean ± SD (n = 3), *p < 0.01 versus IL-2, †p < 0.01 for both groups versus all other groups.
It is possible that our results can be explained, in part, by a requirement for p38 MAPK in the expression of cell surface receptors for the selectin ligand–inducing cytokines that we identified. Little is known about signaling pathways that control the expression of most of these cytokine receptors. However, inhibition of p38 activity does not affect the ability of IL-12 to trigger STAT4 phosphorylation (30), and receptors for TGF-β1 are constitutively expressed on T cells. In addition, caMKK6 induced high levels of selectin ligand expression, which are absolutely dependent on p38 MAPK. These findings make it unlikely that inhibition of receptor expression is a common mechanism underlying our results.

IL-12 promotes Th1 development, and both IL-18 and IL-27 can also induce or augment Th1 development (31, 32, 54, 55). We and others have previously shown (15–17) that IL-12 potently induces selectin ligands, and we show in this study that IL-18 and IL-27 also do so. Th1 cell development and selectin ligand induction by IL-12 require both Stat4 and T-bet (17, 20, 56, 57). Like IL-12, IL-18 and IL-27 also induce T-bet (58, 59), which may be required for selectin ligand induction by these cytokines, and induction of T-bet by these cytokines appears to require p38 MAPK signaling (32, 60). Also like IL-12, both IL-18 and IL-27 are products of APCs, suggesting multiple ways through which APCs can regulate selectin ligand expression in CD4 T cells. Recent evidence indicates that IL-27 also may be important for the modulation of inflammation, in part via its ability to induce IL-10 (61), suggesting a mechanism for the maintenance of T cell recruitment during the resolution phase of an inflammatory response.

The downstream targets that link p38 MAPK signaling to induction of Fut7 and Gent1 in T cells are presently unclear and may not be identical for each of these cytokines. A number of widely expressed transcription factors have been identified as direct targets of p38 MAPK, including ATF-2, CREB, CHOP, MEF2C, Runx, and STATs (62, 63). IL-12 induces phosphorylation of STAT4 serine 721 by p38 MAPK, and this enhances transcriptional potential for a subset of STAT4 gene targets (64), which may include Gent1, because STAT4 is essential for IL-12–induced expression of Gent1 (17). IL-9 and IL-27 activate STAT1, STAT3, and/or STAT5 in various cell types, including T cells, suggesting that one or more of these STAT proteins could function downstream of IL-9 and IL-27 in selectin ligand induction, analogous to STAT4 in IL-12–induced responses. Consistent with this, p38 MAPK phosphorylates serine 727 in both STAT1 and STAT3, which is homologous to serine 721 in STAT4. These serine residues are located in a highly conserved region of the transactivation domains of STAT1, STAT3, and STAT4, and p38-mediated phosphorylation of serine 727 in STAT1 and STAT3 also enhances their transcriptional activity (65, 66).

Distinct mechanisms involving other p38 targets seem likely, at least for TGF-β1 and IL-25. TGF-β1 signaling uses multiple MAPK pathways in addition to Smad transcription factors, and both p38 and ERK MAPK were shown to phosphorylate Smad proteins (67), modulating their activity. Determining whether phosphorylation of Smad proteins by p38 MAPK is involved in selectin ligand induction or whether the requirement for p38 MAPK signaling is independent of Smad transcriptional activity is a key question going forward. The E3 ubiquitin ligase Act1/CIKS is an adaptor protein essential for at least some signaling through IL-25R and related family members (68). Act1 couples IL-25R to multiple downstream effectors, including Traf6 (69, 70).

**FIGURE 6.** Inhibition of Fut7 and Gent1 expression by genetic inactivation of Mapk14. Identical set-up as in Fig. 5, except that instead of treating cells with SB203580, RNA was isolated on day 8 from the p38α MAPK–deficient CD4 and cre− control cells in Fig. 4. Data are mean ± SD (n = 3). *p < 0.01 versus IL-2 only.

**FIGURE 7.** Induction of selectin ligands in response to caMKK6 requires p38α. CD4+ T cells from p38α MAPK–deficient CD4 and cre− control cells were activated, transduced with retrovirus (RV) expressing caMKK6, caRac1, caRac2, or no cDNA, and cultured with IL-2 only. Cells were analyzed by FACS for E-selectin ligands and P-selectin ligands as above; data are from day 8. Data are mean ± SD (n = 3). *p < 0.01, versus control RV.
important to determine whether Act1 or Traf6 is required for p38 MAPK activation by IL-25. It also will be important to determine how IL-9R couples to p38 activation.

In summary, we have shown that a diverse group of inflammatory cytokines can potentially collectively account for the expression of selectin ligands on all defined murine Th cell subsets and that selectin ligand induction in response to these functionally distinct cytokines requires p38 MAPK. The undoubtedly diverse mechanisms underlying the requirement for p38 MAPK in GfTα gene induction in response to these inflammatory cytokines will require further investigation. Our results identify p38 MAPK as a common and essential pathway through which a select group of cytokines responsible for promoting the development of diverse Th cell lineages can couple the differentiation of specific Th cell lineages to the expression of key homing molecules required for effector function in peripheral tissues.

Acknowledgments
We thank numerous colleagues for provision of cytokines used in this study.

Disclosures
The authors have no financial conflicts of interest.

References


