Cutting Edge: Differential Expression of Chemokines in Th1 and Th2 Cells Is Dependent on Stat6 But Not Stat4

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The in vivo function of Th cell subsets is largely dependent on the ability of differentiated CD4\(^+\) T cells to be recruited to specific sites and secrete restricted sets of cytokines. In this paper we demonstrate that Th1 and Th2 cells secrete discrete patterns of chemokines, small m.w. cytokines that function as chemoattractants in inflammatory reactions. Th2 cells secrete macrophage-derived chemokine and T cell activation gene 3, and acquisition of this pattern of expression is dependent on Stat6. In contrast, Th1 cells secrete lymphoactin and RANTES, though unlike IFN-\(\gamma\), expression of these chemokines is independent of Stat4. We further show that supernatants from activated Th2 cells preferentially induce the chemotaxis of Th2 over Th1 cells, corresponding with Stat6-dependent expression of CCR4 and CCR8 in Th2 cells. These data provide the basis for restricted and direct T cell-mediated cellular recruitment to sites of inflammation. The Journal of Immunology, 2000, 165: 10–14.

Differentiated CD4\(^+\) Th cells can be divided into Th1 and Th2 subsets based on their cytokine production following Ag stimulation (1, 2). Th1 cells produce IFN-\(\gamma\) and mediate delayed-type hypersensitivity and protection against intracellular pathogens. The Th2 subset produces IL-4, IL-5, IL-10, and IL-13 and is implicated in humoral and allergic responses. The differentiation of these Th subsets is dependent on cytokine-stimulated genetic programs. IL-12-activated Stat4 is required for the development of fully functional Th1 cells (3–5). Similarly, IL-4-activated Stat6 is essential for the differentiation of Th2 cells (6–8).

Although the exact nature of the STAT protein activated genetic program is unknown, it has been demonstrated that genes other than cytokines are differentially expressed between Th1 and Th2 cells. Several of these are chemokine receptors including CXCR3, CCR5, and CCR7, preferentially expressed in Th1 cells (9–11), and CCR3, CCR4, and CCR8, which are preferentially expressed in Th2 cells (9, 10, 12, 13). This provides a mechanism by which Th subsets can be selectively recruited to sites of inflammation. Indeed, Th1 cells selectively undergo chemotaxis to IFN-\(\gamma\)-inducible protein (IP-10), a ligand for CXCR3, and RANTES, macrophage inflammatory protein 1\(\alpha\) (MIP-1\(\alpha\))\(^3\) and MIP-1\(\beta\), ligands for CCR5 (9, 14). Similarly, Th2 cells selectively undergo chemotaxis to macrophage-derived chemokine (MDC), T cell activation gene 3 (TCA3), and thymus- and activation-regulated chemokine (TARC), chemokines that are ligands for CCR4 and CCR8 (9, 13, 15–17).

Because Th subsets are important in regulating inflammatory processes, it seemed likely that they would also secrete discrete patterns of chemokines to recruit restricted types of cells to sites of inflammation. Although chemokine receptor expression in Th subsets has been extensively described, restricted expression of chemokines between Th subsets has not been carefully examined. We have determined the expression patterns of 18 chemokines and shown that there is restricted expression of chemokines between Th1 and Th2 cells that is dependent on the function of Stat6 but not Stat4. We further demonstrate that Th2 supernatants preferentially recruit Th2 rather than Th1 cells.

Materials and Methods

Mice

The generation of Stat4- and Stat6-deficient mice has been previously described (3, 6). Both strains have been backcrossed 10 generations to the BALB/c genetic background and were bred as homozygotes in the Indiana University Laboratory Animal Resource Center. Wild-type BALB/c mice were purchased from Harlan Bioproducts (Indianapolis, IN).

T cell differentiation

Total spleen cells were depleted of CD8\(^+\), B220\(^+\), and FcR\(^+\) cells. CD4\(^+\) cells were positively selected from the resulting populations using magnetic beads from Miltenyi Biotec (Auburn, CA) according to the manufacturer’s instructions. Final CD4\(^+\) populations were >97% CD4\(^+\) as determined by FACS analysis. CD4\(^+\) T cells cultured at 10\(^6\) cells/ml were then differentiated with 2 \(\mu\)g/ml plate-bound anti-CD3 (145-2C11) plus 1

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Cutting Edge: Differential Expression of Chemokines in Th1 and Th2 Cells Is Dependent on Stat6 But Not Stat4

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and PCR probes were labeled using random decamers (Ambion, Austin, TX). CCR4 (a gift of Dr. Byung Youn, Indiana University, Indianapolis, IN), CCR8, and CCR5 were generated by PCR, subcloned, and sequenced to confirm the identity of the probes. The cDNA probes for MDC (a gift of Dr. Uli Schindler, Tularik, South San Francisco, CA), Breast And Kidney, Exodus-1, -2 (gifts of Dr. Rob Hromas, Indiana University, Indianapolis, IN), and CCR4 (a gift of Dr. Byung Youn, Indiana University, Indianapolis, IN), and PCR probes were labeled using random decamers (Ambion, Austin, TX) and hybridized overnight in a formamide hybridization buffer.

Chemokine mRNA analysis

Total RNA samples were isolated from cell populations indicated using Trizol (Life Technologies/BRL, Bethesda, MD). RNase protection assays (RPA) were done according to the manufacturer’s instructions (PharMingen). cDNA probes for monokine induced by IFN-γ (MIG), TARC, and CCR8 were generated by PCR, subcloned, and sequenced to confirm the identity of the probes. The cDNA probes for MDC (a gift of Dr. Uli Schindler, Tularik, South San Francisco, CA), Breast And Kidney, Exodus-1, -2 (gifts of Dr. Rob Hromas, Indiana University, Indianapolis, IN), and CCR4 (a gift of Dr. Byung Youn, Indiana University, Indianapolis, IN), and PCR probes were labeled using random decamers (Ambion, Austin, TX) and hybridized overnight in a formamide hybridization buffer.

ELISAs

ELISAs for IL-4 and IFN-γ were performed as described previously (5, 6). Rabbit anti-murine MDC Abs were prepared by multiple-site immunization of New Zealand White rabbits with recombinant murine MDC (R&D Systems, Rochester, MN) in CFA. Polyclonal Abs were titrated by direct ELISA and specifically verified by the failure to cross-react to other chemokines. The IgG portion of the serum was purified over a protein A column and used in a sandwich ELISA. Goat anti-murine RANTES was made as previously described (18). The levels of chemokines in cell-free supernatants were measured by specific ELISA using a modification of a double-ligand method as previously described (19).

Chemotaxis assay

Chemotaxis assays were performed in a Costar (Cambridge, MA) Transwell with a 3-μm pore filter. Assays were performed in chemotaxis buffer (RPMI 1640, 1% BSA, and 20 mM HEPES). Supernatants from the indicated Th population were diluted 1:2 with chemotaxis buffer in the lower chamber and 5 × 10^6 Th2 cells in the upper chamber. After 4 h, cells in the lower chamber were counted using trypan blue exclusion. Rabbit anti-MDC serum was purified using protein G and used in the assay at 10 μg/ml. Anti-TC3 was purchased from PharMingen. Control Ig was purified by protein G from normal rabbit serum. Supernatants were incubated with anti-MDC for 1 h on ice before adding to Transwell culture.

Results and Discussion

Differential expression of chemokines in Th1 and Th2 cells

Because chemokines are known to recruit cells to sites of inflammation, and because Th subsets mediate distinct types of inflammation, we examined whether Th1 and Th2 cells secreted distinct sets of chemokines. CD4^+ T cells (>97% pure), purified from wild-type spleen cells, were differentiated to Th1 or Th2 phenotypes. After 1 wk in culture, cells were restimulated with anti-CD3. Restimulation of Th1 and Th2 cultures resulted in secretion of IFN-γ and IL-4, respectively (Fig. 1A). RPA were used to assess the expression of a panel of chemokines in cultures that were unstimulated or activated for the time periods indicated with anti-CD3. Expression of RANTES and lymphotactin was induced by anti-CD3 specifically in Th1 populations (Fig. 1B). By contrast, Th2 cells selectively expressed TCA3 following stimulation with anti-CD3. To determine whether expression of these chemokines in Th subsets was dependent on the genetic programs activated by Stat4 and Stat6, we performed the differentiation described above with CD4^+ T cells from mice deficient in either Stat4 or Stat6. These cultures secreted cytokines as predicted from previous results (Fig. 1A). TCA3 expression was dependent on Stat6, as Stat6-deficient CD4^+ Th2 cultures did not express TCA3 (Fig. 1B). Surprisingly, and in contrast to IFN-γ, RANTES and lymphotactin were expressed in Stat4-deficient Th1 cultures (Fig. 1B). This suggests that Stat4 is not required for RANTES and lymphotactin expression in Th1 populations and also correlates with the ability of Stat4-deficient lymphocytes to mediate reduced but significant tissue inflammation (5).

We also tested the differential expression of several other chemokines by Northern blot analysis. Th cells were differentiated as above and RNA was isolated at the time points indicated following stimulation with anti-CD3. Of the chemokines tested, MDC (also known as STCP-1 and ABCD-1) was inducibly expressed specifically in Th2 cells and not in Th1 cells (Fig. 2A). Because IL-4 induces, and IFN-γ represses, MDC expression from macrophages, dendritic cells and B cells (16, 20, 21) we tested whether anti-CD3 induced cytokines were responsible for Th2-restricted MDC expression. Wild-type Th2 cells restimulated with anti-CD3 in the presence of anti-IL-4 showed that the absence of IL-4 reduced but did not abrogate MDC expression (Fig. 2B). Thus it is likely that both the anti-CD3 and IL-4 signals contribute to MDC expression in Th2 cells. Th1 cells stimulated with anti-CD3 expressed barely detectable levels of MDC and even in the presence of neutralizing anti-IFN-γ were not inducible by IL-4 (Fig. 2B). The restricted expression of MDC was also dependent on Stat6,
because Stat6-deficient Th2 cultures did not express MDC even when supplemented with IL-4 (Fig. 2B).

To demonstrate that the differentially expressed chemokines were indeed being secreted, we also performed ELISAs on supernatants from Th1, Th2, Stat4-deficient Th1, and Stat6-deficient Th2 cultures stimulated for 24 h with anti-CD3. Secretion of RANTES and MDC was detectable in supernatants (Fig. 3) with a similar restricted pattern of expression as seen in RPA and Northern blot analysis (Figs. 1 and 2).

**Common expression of chemokines in Th1 and Th2 cells**

MIP-1α and MIP-1β are often associated with Th1-mediated inflammatory lesions (22–24). However, our RPA analysis demonstrated that Th1 cells express only about 2-fold more RNA for these chemokines than Th2 cells. Importantly, expression of both MIP-1α and MIP-1β was readily detectable in Th2 cells (Fig. 1B). This difference was also reflected in ELISAs where MIP-1α was readily detectable in supernatants of Th2 cells, but accumulated in Th1 supernatants at 2- to 5-fold higher levels than in Th2 supernatants (data not shown). Furthermore, the absence of either Stat4 or Stat6 had only minor effects on the expression of MIP-1α and MIP-1β (Fig. 1B) or the secretion of MIP-1α (data not shown).

Other chemokines had undetectable expression in either Th1 or Th2 cells. These included chemokines that were analyzed by RPA in Fig. 1B (eotaxin, monocyte chemotactant protein-1 (MCP-1), MIP-2, and IFN-γ-inducible protein (IP-10)), Northern blot analysis (MIG, TARC, Exodus-1 (LARC/MIP-3α), Exodus-2 (TCA4/SLC6Ckine), Exodus-3 (ELC/MIP-3b/CKβ11) and BRAK; data not shown), and ELISA (C10 and MCP-3; data not shown).

**Th2 supernatants preferentially recruit Th2 cells**

The chemokines that are preferentially expressed in Th2 cells are also ligands for receptors that are differentially expressed between Th subsets. The Th2 chemokines MDC and TCA3 signal through CCR4 and CCR8, respectively, which are also expressed at greater levels in Th2 cells than in Th1 cells and are expressed in a Stat6-dependent manner (Fig. 4A). This led us to the prediction that Th2-generated chemokines would be more effective at stimulating the chemotaxis of Th2 cells. To test this, we used Th2 supernatants generated as above, in a standard, two-chamber migration assay. As shown in Fig. 4B, Th2 supernatants were significantly more effective in stimulating chemotaxis in Th2 cells than were Th1 supernatants or media alone (p < 0.01). Th2-induced chemotaxis could be inhibited by coinoculation of the supernatant with anti-MDC, demonstrating that Th2-derived MDC is an important mediator of Th2 chemotraction. Incubation with anti-TCA3 did not significantly affect Th2 migration in response to Th2 supernatants. Thus, Th2-derived chemokines can differentially recruit Th2 subsets.

This report describes the differential expression of specific CC chemokines in Th1 and Th2 subsets. The issue of differential expression of chemokines in Th subsets has been addressed in several reports with differing results. MDC expression has been observed in neither or both Th1 or Th2 populations/clones (16, 25, 26). TCA3 was expressed in transgenic Th2 cell populations but was also seen expressed in both Th1 and Th2 clones (27, 28). MIP-1α, MIP-1β, and RANTES have also been shown to be both differentially and similarly expressed in Th1 and Th2 populations (25, 28). Some of these discrepancies may be simply explained by the use of methods that were not quantitative enough to distinguish preferential expression in Th subsets or by differences in cultured primary T cells vs long-term T cell clones. Our report describes the systematic and quantitative examination of the expression of 18 chemokines in primary, polyclonally activated, and purified CD4+ cells that have been polarized to the Th1 or Th2 phenotype.

The differential dependence on STAT proteins for the acquisition of chemokine secreting phenotypes is distinct from that required for cytokines. Although Stat6 appears to be necessary for the development of Th2 cells and secretion of IL-4, MDC, and TCA3, there is not the same requirement for Stat4. In the absence of Stat4, Th1 cells can still acquire a chemokine-secreting phenotype that includes RANTES and lymphotactin. We have previously suggested that Th1-like populations can be generated in the absence of Stat4 (5). However, this observation was complicated by the direct effects of Stat4 on IFN-γ expression, which served as the sole marker for Th1 cells (3, 5). Using RANTES and lymphotactin as representative chemokines of Th1 and not Th2 cells, we provide further evidence here that cells with some characteristics of Th1
cells can develop in the absence of Stat4. Thus, the differential expression of chemokines in Th1 and Th2 cells offers additional markers for analysis of Th subsets that may be important for tracking and detecting Th cells in vivo and in vitro.

Our data also provide a novel view of the biology of Th1 and Th2 cells. In many instances Th cells have been characterized as recruits rather than recruiters to sites of inflammation. This study suggests that Th cells can contribute to inflammation not simply by secretion of cytokines such as IFN-γ and IL-4, which regulate inflammatory processes, but also by secreting specific subsets of chemokines to recruit additional cells to an inflamed site. For example, both MDC and Th2 cells are required for allergic airway hypersensitivity (17, 26, 29, 30), raising the possibility that Th2 cells may be a relevant source of this chemokine during allergic inflammation. Similarly, anti-RANTES decreases migration into inflamed sites. These observations provide the basis for understanding specific T cell-mediated recruitment of leukocytes to sites of inflammation. They also suggest additional targets for modulating chemokine-mediated inflammation in vivo.

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