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Chemokines and T Lymphocytes: More than an Attraction

Review

Stephen G. Ward,*‡ Kevin Bacon,† and John Westwick*
*Pharmacology Group
Department of Pharmacy and Pharmacology
Bath University
Claverton Down
Bath, BA2 7AY
United Kingdom
†Neurocrine Biosciences
San Diego, California 92121

Not so long ago, most people outside of the field, when asked "What are chemokines?" might have responded, with some apathy, "Aren't they those small proteins involved in the recruitment of leukocytes?" Well, general awareness and understanding of this field has changed considerably in the past 3 years or so. The term "chemokine" was adopted in 1992 to describe a family of closely related *chemo*tactic cyto*kines* with conserved sequences, known to be potent attractors for various leukocyte subsets such as neutrophils, monocytes, or lymphocytes (Lindley et al., 1993). The history of chemokines can be traced back as far as 1977 with the characterization of platelet factor 4, although the prototypical chemokine IL-8 was not described until 1987 (Baggiolini et al., 1997). Identification of other structurally related chemotactic cytokines quickly followed with advances in molecular cloning techniques and availability of bioinformatics-based analyses of nucleotide databases. During the last 10 years, it has become apparent that chemokines are a large superfamily consisting of four subfamilies that display between two and four highly conserved NH_2-terminal cysteine amino acid residues. The CXC (or α) familiy has the first two NH_2-terminal cysteines separated by one nonconserved amino acid residue. In contrast, the CC (or β) family has these cysteines in juxtaposition, and the C (or γ) family has one lone NH_2-terminal cysteine residue, while the CX_3C (or δ) family has these cysteines separated by three intervening amino acids (Baggiolini et al., 1997). There are now well over 40 characterized chemokines (some of which are listed in Table 1 and Table 2) as well as a number of virally encoded chemokine-like proteins, so this family far outnumbers other cytokine families. Most chemokines fall into the CXC and CC groups, since there is only one C and one CX_3C chemokine known in human. The discovery of chemokine receptors also appears to be a modern growth industry. There are now at least 15 known chemokine receptors, many of which exhibit multiple ligand specificity, although the chemokine/ligand promiscuity does not usually cross CC versus CXC chemokine boundaries (Table 2). In addition, there are several orphan and virally encoded chemokine receptors, so the number of chemokine receptors will likely increase substantially over the next few years.

‡To whom correspondence should be addressed (e-mail: prssgw @bath.ac.uk).

Members of all four families are known to attract various subsets of T lymphocytes, and it is by virtue of their diversity that chemokines are ideal molecules for mediating a plethora of events such as selective trafficking of T lymphocyte subsets from intravascular to extravascular compartments, trafficking within lymph node and thymus, and/or recirculation from tissues to lymphatics. However, the large number of chemokines and their receptors, together with the expression of chemokine receptors on cells other than leukocytes (such as epithelial, endothelial, and smooth muscle cells), is probably indicative of the importance of these molecules in a wide range of biological functions. For example, in addition to their role as chemoattractants for leukocytes, chemokines play a role in regulating angiogenesis. However, this review will focus on the current understanding of the biochemical and functional role of chemokines and their receptors in T lymphocyte biology. In this respect, perhaps the most exciting and influential discovery this decade is the finding that human immunodeficiency virus-1 (HIV-1) uses chemokine receptors for entry into cells. Although the sole purpose of chemokines is probably not that of sharing receptors with HIV-1, it is undeniable that this finding is responsible for considerably raising the scientific profile of chemokines and for unleashing an astounding surge of research into this field—so much so that chemokines now attract almost as many researchers as they do leukocytes. Accordingly, there has been rapid progress in understanding how the expression of chemokines and their receptors is regulated, what biochemical events are utilized by the receptors, and the biological role of chemokines and chemokine receptors in T lymphocyte biology, with implications that stretch far beyond the role of chemokine receptors as binding sites for AIDS viruses. So today, when asked "What are chemokines?", most people might venture a somewhat more excited and informed reply than the one suggested earlier.

Chemokine Receptor Expression on Resting and Activated T Lymphocytes

The known chemokine receptors, together with their respective ligands, are shown in Table 2. Chemokine receptors are seven-transmembrane-spanning, pertussis toxin–sensitive, G protein–coupled receptors that are similar to many other seven-transmembrane-spanning receptors and have a number of conserved motifs, including the DRYLAIV motif in the second intracellular loop domain (Baggiolini et al., 1997). The only exception is the Duffy antigen receptor for chemokines (DARC), which has less than 20% amino acid identity with CXC and CC chemokine receptors. DARC is the only known chemokine receptor that can bind both CC *and* CXC chemokines. In addition, DARC lacks the DRYLAIV motif, is not coupled to G proteins, and has not been reported to elicit any detectable signal transduction events. Given the chemotactic effects of chemokines on T lymphocytes, it was a natural assumption that T cells should express chemokine receptors, and there has been intense effort to determine which chemokine receptors

Table 1. Some Common Chemokine Abbreviations

CXC Chemokines	
BCA-1	B cell attracting chemokine-1
GROα	Growth related oncogene-α
IL-8	Interleukin 8
IP-10	Interferon-γ inducible protein
MIG	Monokine induced by interferon-γ
SDF	Stromal cell-derived factor
CC Chemokines	
DC-CK1	Dendritic cell-derived chemokine-1
ELC	EBI1-ligand chemokine
LARC	Liver and activation-regulated chemokine
MCP-1	Monocyte chemotactic protein-1
MDC	Macrophage-derived chemokine
MIP-1α	Macrophage inflammatory protein-1α
SLC	Secondary lymphoid tissue chemokine
RANTES	Regulated on activation, normal T cell expressed and secreted
TARC	Thymus and activation-regulated chemokine
TECK	Thymus-expressed chemokine

are expressed on T cells. Accordingly, it is now known that T cells express most of the known CC, CXC, and CX_3CR chemokine receptors listed in Table 2.

The precise pattern of chemokine receptor expression depends on the activation state of the T cell. For instance, expression of a number of chemokine receptors such as CCR1, CCR2, CCR5, CXCR4, and CX_3CR1 is markedly up-regulated after mitogenic stimulation and/or prolonged treatment of T lymphocytes with IL-2 (Loetscher et al., 1996b; Carroll et al., 1997). Moreover, some receptors such as CXCR3 are restricted to activated T cells, and this correlates well with the observation that its ligands IP-10 and MIG both attract activated T cells (Loetscher et al., 1996a). Indeed, for the most part, chemokine receptor expression does correlate well with the known chemotactic effects of the respective ligands on T lymphocyte subsets. For example, CXCR4 (the receptor for SDF-1), is expressed predominantly on $CD45RA^+$ naive T lymphocytes (Bleul et al., 1997). This finding is consistent with the view that SDF-1, constitutively expressed in a broad range of tissues, is involved in basal trafficking of naive lymphocytes. In contrast, CCR5 is expressed mainly on the $CD45RO^+$ memory subset, which migrates in response to RANTES, the major ligand for CCR5 (Bleul et al., 1997). Furthermore, CCR2 expression on the memory T cell subset correlates with the response of these cells to MCP-1 in chemotaxis assays (Qin et al., 1996). Some controversy surrounds the expression of the two IL-8 receptors CXCR1 and CXCR2 on T cells, since they have been reported to be restricted to the NK-like cells within the T cell lineage that are recruited by IL-8 (Qin et al., 1996). However, other groups have clearly demonstrated high affinity binding of IL-8 to T cell populations that have not been enriched for NK cells. If there were a restriction of CXCR1 and CXCR2 to NK cells, it is unlikely that the chemotactic responses of $CD4^+$ and $CD8^+$ T cells to IL-8 would have been detected (Bacon et al., 1995a).

Chemokine Receptors as Markers of T Lymphocyte Differentiation

The notion that chemokine receptors are expressed on T lymphocytes depending on their state of activation or differentiation is supported by two recent observations: (1) expression of CCR4 and CCR3 (and perhaps CCR7) has been linked to the program of Th type 2 differentiation in vitro (Gerber et al., 1997; Sallusto et al., 1997; Bonecchi et al., 1998); and (2) CXCR3 and CCR5 are prefentially expressed on human Th1 cells and are present on T lymphocytes recovered from the synovial fluid of rheumatoid joints that exhibit a Th1 phenotype, while CXCR4 and CCR2 (and maybe CCR1) are expressed equally on both Th1 and Th2 cells (Bonecchi et al., 1998; Loetscher et al., 1998; Qin et al., 1998). This pattern of receptor expression correlates with the efficient attraction of Th1 but not Th2 cells by the appropriate CC chemokine ligands MIP-1α, MIP-1β, and RANTES. Moreover, SDF-1 and MCP-1, which bind to CXCR4 and CCR2, respectively, exerted chemotactic effects on both Th1 and Th2 cells (Siveke and Hamann, 1998). Chemokine receptor expression and association with Th1 and Th2 phenotypes can be affected by other cytokines known to affect T lymphocyte polarization such as α-interferon (promotes Th1 phenotype) and transforming growth factor β (promotes Th2 phenotype). Hence, transforming growth factor β inhibited CCR3 but enhanced CCR4 expression, while α-interferon inhibited CCR3 but up-regulated CXCR3 expression (Sallusto et al., 1998). These observations imply that T cell differentiation may well require migratory capacity as well as distinct cytokine production patterns. In addition, chemokines may be part of effector and amplification mechanisms of polarized Th1- and Th2-mediated immune responses, and their receptors might serve as Th1 versus Th2 markers (Figure 1) as well as targets for selective modulation of T cell–dependent immunity. There is certainly evidence that differential expression of chemokine receptors may influence functional T cell responses other than chemotaxis, as evidenced by the demonstration that CC chemokines (e.g., RANTES, MIP-1α, and MCP-1) promote lymphocyte activation and/or differentiation (Bacon et al., 1995b; Taub et al., 1996).

The costimulatory T cell molecule CD28 can regulate the expression of a number of chemokine receptors (e.g., CCR1, CCR2, and CCR5), which will be discussed in further detail later. It is tempting to speculate, therefore, that the differential expression of chemokine receptors during T lymphocyte differentiation may be related to the differential immune regulation that is exhibited by the natural ligands for the T cell costimulatory molecule CD28, namely, B7.1 and B7.2, which have been demonstrated to promote Th1 and Th2 differentiation, respectively (Freeman et al., 1995). In this respect, eotaxin (the major ligand for CCR3) deserves a particular mention, since eotaxin expression is up-regulated in tissues known to be sites of allergic reactions, such as the airways, and is important for the attraction of eosinophils leading to lung eosinophilia (Gonzalo et al., 1996). The generation and maintenance of an allergic reaction requires Th2 cells as a source of IL-4 and IL-5, and these cytokines serve as growth and stimulation factors for

Table 2. Human Chemokine Receptor Expression and Ligand Specificity

Receptors	T Lymphocyte Expression[a]	Ligand
CXC Chemokines		
CXCR1	+	IL-8
CXCR2	+	IL-8, GROα, GroB
CXCR3	+	MIG, IP-10
CXCR4 (LESTR/Fusin)	+	SDF-1α/β
CXCR5	−	BCA-1
CC Chemokines		
CCR1	+	MIP-1α,RANTES, MCP-3 MIP-5 (leukotactin-1)
CCR2A/B	+	MCP-1, MCP-2, MCP-3, MCP-4, MCP-5
CCR3	+	Eotaxin-1, Eotaxin-2, MIP-5, RANTES, MCP-2, MCP-3, MCP-4
CCR4	+	TARC
CCR5	+	MIP-1α, MIP-1β,RANTES
CCR6	+	MIP-3α (LARC or Exodus)
CCR7	+	MIP-3β (Exodus-3 or ELC), SLC (Exodus-2)
CCR8	+	I-309, TARC, MIP-1β
D6[c]	?	MIP-1α, MIP-1β, RANTES
(Putatively CCR9 or CCR10)		MCP−1, −2, −3, −4, Eotaxin
C Chemokines		
XCR1 (GPR5)[b]	+	Lymphotactin
CX_3C Chemokine		
CX_3CR1 (V28)	+	Fractalkine
Miscellaneous		
DARC	Unknown	IL-8, GROα, RANTES, MCP-1, TARC

[a] Expression may vary between T cell subsets and/or T cell lines or activation state of the T cells.
[b] Designation of GPR5 as XCR1 awaits confirmation.
[c] Sequence of human D6/CCR9 is identical to sequence submitted for human CCR10. Hence, designation of human D6 as CCR9 awaits confirmation.
The most commonly used names are used for the well-characterized chemokines listed, although some alternative names are indicated in parentheses for recently identified chemokines. See Table 1 for abbreviations.

basophils and eosinophils. Hence, the presence of CCR3 on both eosinophils and Th2 cells, as well as the observation that T cells colocalize with eosinophils in diseased tissues, may suggest a possible pathogenetic mechanism for T cell recruitment in the airways and may also provide a potential target for therapeutic intervention. Given that CD28-dependent costimulation has been demonstrated to be required for induction of a lung mucosal Th2 immune response and airway eosinophilia, a close relationship between CD28 and regulation of CCR3 expression is an interesting possibility (Harris et al., 1997).

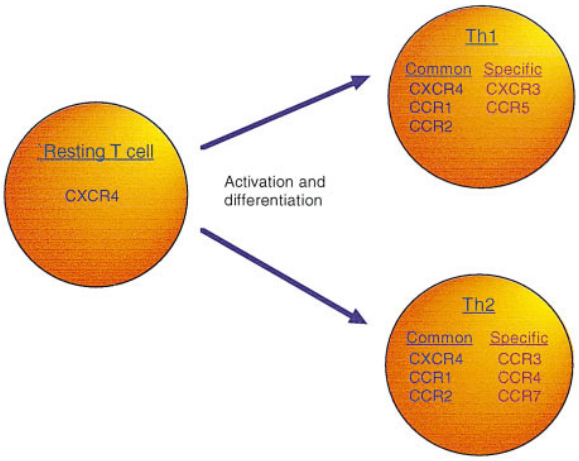

Figure 1. Schematic Model for Chemokine Receptors as Markers for T Lymphocyte Differentiation

Chemokines and Lymphocyte Development

The importance of chemokines in T lymphocyte recruitment during allergic inflammation is well documented (Baggiolini et al., 1997). With the discovery and characterization of new chemokines, it is becoming clear that control of T lymphocyte recruitment by chemokines is not limited to inflammatory situations. For example, during their development and differentiation, lymphocytes move through different tissue compartments, although the mechanisms by which this complex cellular trafficking is regulated are not fully understood. However, a group of recently identified chemokines including TARC, ELC, SLC, LARC, and DC-CK1 are constitutively expressed at high levels in the thymus, lymph nodes, and other lymphoid tissues and/or attract T lymphocytes, which bear selective receptors, namely, CCR4 (predominantly binds TARC), CCR6 (binds LARC), and CCR7

(binds ELC) (Table 2). DC-CK1 is produced by dendritic cells of germinal centers and T lymphocyte areas of secondary lymphoid organs and is chemotactic for naive T lymphocytes, while TECK is produced by thymic dendritic cells and is chemotactic for macrophages, dendritic cells, and thymocytes (Adema et al., 1997; Vicari et al., 1997). The restricted and constitutive production of these chemokines in lymphoid tisues and/or their selective binding to chemokine receptors that are expressed by T lymphocytes suggests that they may be heavily involved in the regulation of lymphocyte trafficking during development.

Chemokine Receptors Act as Coreceptors for HIV-1

An exciting and important advance in recent chemokine research was the finding that certain CC chemokines (e.g., RANTES, MIP-1α, and MIP-1β) can suppress infection of T cells with the M-tropic HIV-1 strains (Cocchi et al., 1995). Several groups subsequently showed that CCR5 (the receptor for RANTES, MIP-1α, and MIP-1β), is the coreceptor for M-tropic HIV-1. In contrast, CXCR4 is the coreceptor responsible for the efficient entry of T-tropic strains of HIV-1 into target cells. Accordingly, the CXCR4 ligand SDF-1 blocks infection with T tropic, but not M- or dual-tropic, HIV-1 (reviewed by Fauci, 1996). At around the same time, homozygosity for a 32 bp deletion in the human *CCR5* gene (*CCR5*Δ32 allele), which produces a mutant truncated protein that is not expressed on the cell surface, was found to confer resistance to infection by HIV-1 with no obvious deleterious phenotype (Liu, et al., 1996; Samson et al., 1996). Surprisingly, the Δ32 allele is quite common in individuals of northern European descent. Another much rarer mutation, *CCR5-m303* (which contains a premature stop codon at position 303), has also been reported to confer resistance to HIV-1 infection (Quillent et al., 1998). Although CCR5 mutations clearly confer a very high level of resistance to HIV infection, they are not infallible and it seems likely that there are other important resistance factors. Indeed, later during the course of infection, T-tropic HIV-1 variants emerge that can also use CCR3, CCR2, and CCR5 in addition to CXCR4 (Connnor et al., 1997). The emergence of these viruses has been suggested to coincide with a less favourable clinical prognosis. Furthermore, a mutation (valine to isoleucine switch at position 64) in the transmembrane region of CCR2 also has a significant protective effect on HIV disease progression. The precise mechanism by which this *CCR2–64I* mutation influences protection against HIV-1 remains unclear, although it has been speculated that it may be related to CCR5 heterozygosity, since there is strong linkage disequilibrium between *CCR2–64I* and a mutation (*CCR5–59653T*) in the regulatory region of the closely linked CCR5 gene (Kostrikis et al., 1998). It is also interesting to note that other CC chemokines such as MDC (binds CCR4) can inhibit HIV-1 infection of T lymphocytes, while I-309 (binds CCR8) inhibits CCR8-dependent infection of monocytes by diverse HIV-1 strains, further suggesting that a number of chemokine receptors are involved in HIV-1 entry (Pal et al., 1997; Horuk et al., 1998).

Signal Transduction Pathways Activated by Chemokines in T Lymphocytes

The biochemical events underlying the chemotactic effect as well as other biological roles of chemokines have been a major focus of interest in many cell types for several years, initially in the hope of discovering a worthwhile therapeutic anti-inflammatory target. In this respect, however, the stakes have been raised considerably given the diversity of biological outcomes increasingly attributed to chemokines. One of the most extensively investigated chemokines with respect to signal transduction mechanisms in cells other than T lymphocytes is the CC chemokine MCP-1. Several biochemical events are stimulated by MCP-1, including inhibition of adenylate cyclase, activation of phospholipase C, calcium flux, and inositol trisphosphate generation by G protein–dependent mechanisms (Baggiolini et al., 1997). In marked contrast, the signaling events initiated by chemokines in T lymphocytes are only now beginning to be understood. In this respect, the best-characterized chemokines in terms of both their functional influence on T lymphocytes and regulation of signal transduction mechanisms are the CC chemokine RANTES and the CXC chemokine IL-8. As a result, these chemokines serve as prototypes and reference points for studying signaling events elicited by other chemokines in T lymphocytes.

Biochemical Signals activated by RANTES

RANTES is an extremely versatile chemokine in terms of the functional T cell consequences that it has been reported to influence. These range from control of uropod formation (a cytoplasmic projection indicative of cellular polarization) and expression of adhesion molecules during chemotaxis, to the regulation of cytokine release and T cell proliferation as well as protection against HIV-1 (Figure 2). It is therefore critical to understand whether these diverse functional outcomes are controlled by discrete biochemical signaling cascades, since these may lead to the identification of a selective therapeutic target.

RANTES stimulates biphasic calcium mobilization in T lymphocytes with the initial transient peak associated with chemotaxis. This initial transient calcium elevation is initiated by nanomolar concentrations and mediated by a heterotrimeric G protein–coupled pathway as evidenced by sensitivity to pertussis toxin. The second, sustained peak of calcium influx is elicited by micromolar concentrations of RANTES and is dependent on protein tyrosine kinases (PTKs), as evidenced by its sensitivity to PTK inhibitors. This second peak of calcium influx is associated with Ca^{2+} channel opening, IL-2 receptor expression, cytokine release, and T cell proliferation (Bacon et al., 1995b). Interestingly, RANTES-induced calcium mobilization depends on the expression of CD3, but the full significance of this observation has yet to be demonstrated, although it may imply that RANTES may engage the TCR complex as a way of effecting cellular activation (Dairaghi et al., 1998). Given the partial sensitivity of RANTES-stimulated calcium mobilization to PTK inhibitors, it is perhaps surprising that RANTES stimulation has not been reported to induce tyrosine phosphorylation of phospholipase C, even though it induces the tyrosine phosphorylation and activation of a

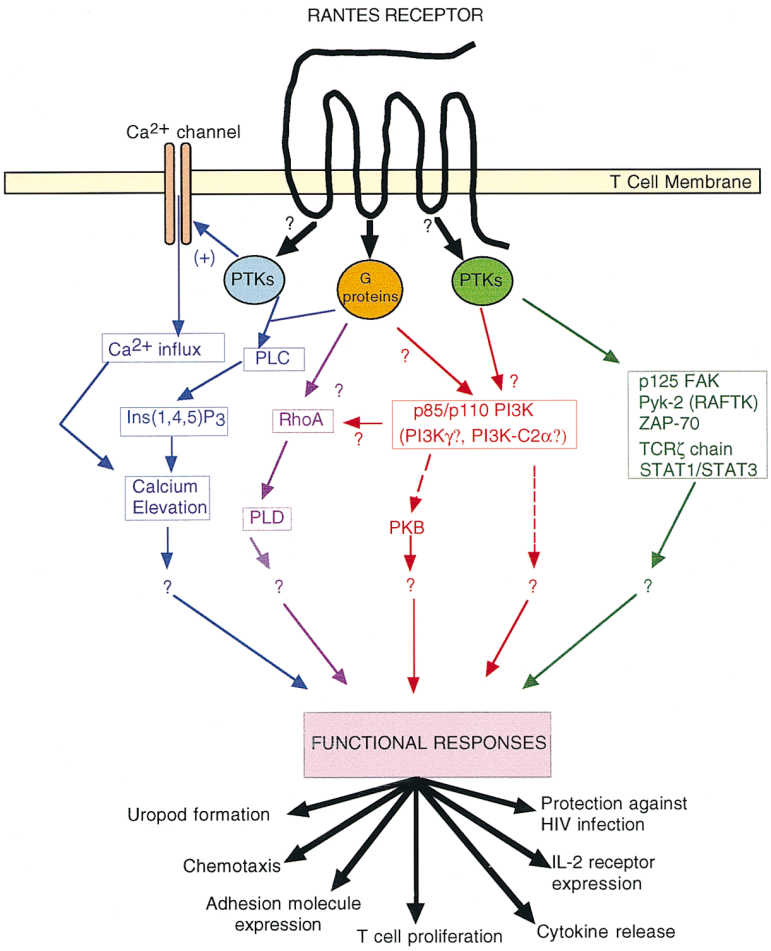

Figure 2. Schematic Representation of the Signaling Pathways Activated by RANTES in T Lymphocytes

The mechanisms by which the RANTES receptor(s) couples to the PTKs are unclear (represented by "?") and the diagram is not meant to imply interaction of PTK(s) or G proteins with any specific intracellular loop. Receptor engagement by RANTES stimulates PTK activity, which results in the tyrosine phosphorylation of the substrates indicated (shown in green). Elevation of intracellular calcium (shown in blue) is due in part to activation of a G protein–coupled PLC and opening of a PTK-dependent calcium channnel. RANTES also stimulates the p85/p110 heterodimeric form of PI3K (shown in red), although it is unknown how the RANTES receptor(s) couples to and activates PI3K in T lymphocytes (denoted by "?"). Given the known coupling of the chemokine receptors to G proteins, it is also possible that RANTES may activate the G protein–coupled isoform of PI3K, namely, PI3Kγ. The RANTES receptor couples to and activates Rho and PLD (shown in purple) by unknown routes, although G protein coupling and PI3K-dependent routes are suggested. PTK-dependent mechanisms may also operate by analogy with other systems but are not indicated. The effector targets downstream of PI3K, calcium mobilization, PLD, $p125^{FAK}$, and ZAP-70 following RANTES stimulation are unknown, although several functional outcomes have been attributed to RANTES as shown and may be differentially regulated by one or more of the depicted signaling cascades. Dotted lines indicate putative pathways. See text for further details and references.

number of other substrates including the PTKs ZAP-70, $p125^{FAK}$, and the related Pyk-2 (Bacon et al., 1996; Davis et al., 1997). Activation of these PTKs by RANTES gives it the potential to interface with a number of downstream biochemical events and so to potentially influence T cell activation, gene transcription, cell cycle progression, and even cell survival. Certainly, RANTES induces a functional molecular complex consisting of $p125^{FAK}$, ZAP-70, and the focal adhesion protein Paxillin, indicating that these molecules may be required for T cell focal adhesions formed in response to RANTES stimulation (Bacon et al., 1996; Davis et al., 1997). RANTES also stimulates the tyrosine phosphorylation and activation of STAT1 and STAT3 that correlates with the induction of gene expression of the STAT-inducible proto-oncogene c-*fos* in T cell lines (Wong and Fish, 1998). Both MIP-1α and MIP-1β exhibit receptor specificity similar to that observed to RANTES, and it is interesting to note that tyrosine phosphorylation of Pyk-2, STAT1, and STAT3 are observed in response to these chemokines (Davis et al., 1997; Wong and Fish, 1998). Although the precise mechanisms involved remain obscure, RANTES clearly stimulates PTK activation, and in this respect RANTES is similar to other G protein–coupled receptors such as Bombesin and Vasopressin that can also couple to PTKs (Zachary et al., 1991).

RANTES has also been demonstrated to stimulate activation of a distinct phosphatidylinositol 3-kinase (PI3K), namely, the p85/p110 heterodimeric PI3K. Coupling of receptors to the p85/p110 heterodimeric PI3K is known to require interaction of SH2 domains within the p85 regulatory subunit with specific phosphotyrosine-containing binding motifs (Turner et al., 1995; Vanhaesebroeck et al., 1997). Since no such recognized motifs are present in known RANTES receptors, it is unclear how the receptor couples to this PI3K, although other G protein–coupled receptors such as fMLP receptors are able to activate the p85/p110 heterodimeric PI3K (Stephens et al., 1993; Kurosu et al., 1997). In light of the demonstration that both PTK-dependent and G protein–dependent signaling events are triggered by RANTES in T cells, it is interesting to note that the p85/p110 PI3K is synergistically activated by tyrosine-phosphorylated peptides and βγ subunits of G proteins (Kurosu et al., 1997). Given the well-established role for RANTES as a chemotactic agent and evidence that RANTES promotes T lymphocyte activation, it seems appropriate that RANTES can activate a PI3K-dependent signaling cascade, since this cascade has been implicated in both the regulation of chemotaxis and as a pivotal signal involved in T cell activation (Wennstrom et al., 1994; Ward et al., 1996). Indeed, RANTES-induced chemotaxis

and polarization of normal human T lymphocytes is inhibited by pretreatment with the PI3K inhibitor wortmannin (Turner et al., 1995).

There are several outstanding questions relating to the activation of PI3K by RANTES. First, the effect, if any, of RANTES on the G protein–coupled form of PI3K (referred to as PI3Kγ) has yet to be elucidated. Certainly, there is evidence that both the p85/p110 heterodimer and PI3Kγ can be activated by a single receptor stimulus, since stimulation of THP-1 cells with concanavolin-A can activate both the p85/p110 heterodimer as well as a distinct pertussis toxin–sensitive PI3K (Matsuo et al., 1996). It will therefore be important to assess the effect, if any, of chemokines such as RANTES on either PI3Kγ or other distinct PI3Ks such as the recently identified PI3K-C2α or the PtdIns-specific 3-kinase (Vanhaesebroeck et al., 1997). Second, the possible activation by RANTES of protein kinase B, a known downstream effector of both p85/p110- and PI3Kγ-dependent signaling cascades, has yet to be reported. Finally, recent studies have revealed that RANTES also activates phospholipase D (PLD) in Jurkat cells, and this activation is dependent on the GTP-binding proteins ARF and RhoA (Bacon et al., 1998). It is tempting to speculate that PI3K may be involved in regulating RhoA and hence PLD activation, since PI3K activity is known to be essential for receptor-mediated activation of the related GTPase Rac in mammalian cells, and the PI3K homolog TOR2 controls Rho1 activation in *Saccharomyces cerevisiae* (Hawkins et al., 1995; Schmidt et al., 1997). Rho has been implicated in regulating actin filament organization and cell motility, so it is possible that Rho plays an important role in mediating the chemotactic effects of RANTES (Hall, 1998). PLD and the ARF family of proteins have been implicated in the vesicle formation and retrograde transport of proteins between Golgi and endoplasmic reticulum, and they may therefore operate a mechanism for receptor down-regulation (Ktistakis et al., 1996). It is interesting to note, therefore, that PI3K has been shown to be involved in growth factor receptor down-regulation and that agonist-stimulated receptor internalization is a feature of several chemokine receptors including CCR5 (Joly et al., 1994; Aramori et al., 1997). However, little is currently known about the involvement of PI3K(s) in agonist-stimulated chemokine receptor internalization.

The signaling events elicited by RANTES that we have just described are schematically depicted in Figure 2, along with several known functional effects of RANTES. To date there is insufficient evidence to attribute control of individual functional consequences to any one signaling cascade. Nevertheless, RANTES-induced homotypic aggregation has been shown to be sensitive to the tyrosine kinase inhibitor herbimycin A but resistant to pertussis toxin, while lymphocyte uropod formation induced by chemokines can be completely inhibited by pertussis toxin treatment (Del Pozo et al., 1996; Szabo et al., 1997). Hence, it is possible that the activation of both G protein–dependent and PTK-dependent signaling pathways by RANTES reflects differential regulation of different functional events by these signaling cascades.

Biochemical Signals Activated by IL-8

IL-8 is the best studied CXC chemokine with respect to its activation of signal transuction events in T lymphocytes. Several studies have indicated roles for phosphoinositide hydrolysis, protein kinase C activation, transmembrane calcium flux, and PLD as signaling events underlying T lymphocyte and NK cell responses to IL-8 (Bacon et al., 1993, 1995a). Interestingly, IL-8 does not stimulate PLD activation in neutrophils, suggesting that IL-8 may discriminate between its cellular targets by differentially engaging distinct signaling pathways in neutrophils and T lymphocytes (Bacon et al., 1995a). In contrast to RANTES, the effects of IL-8 on PI3K activity in T lymphocytes remains unknown, although IL-8-stimulated migration of neutrophils is inhibited by the PI3K inhibitor wortmannin (Knall et al., 1997). Finally, IL-8 stimulation of mouse lymphocyte cell lines expressing CXCR1 results in activation of RhoA (Laudana et al., 1997). It is an exciting prospect, therefore, that like the CC chemokine RANTES, IL-8 may activate RhoA in normal T lymphocytes, although this has yet to be confirmed. Little is known about the signaling events elicited by other CXC chemokines, although MIG, IP-10, and SDF can generally stimulate increases in intracellular calcium. In addition, the CXC chemokine SDF-1 induces tyrosine phosphorylation of Pyk-2 (Davis et al., 1997).

At present, therefore, there is insufficient evidence available to indicate one way or the other as to whether distinct signaling cascades are preferentially activated by CC or CXC chemokines. Nevertheless, the knowledge of chemokine signaling garnered from studies on RANTES and IL-8 should provide useful points of reference for studying the signals activated by other chemokines.

What Is the Relevence of Chemokine Receptor Signaling to HIV-1 Viral Entry?

Viral entry is a complex phenomenon in which glycoprotein attachment to CD4 creates a high affinity binding site for the chemokine coreceptor leading to membrane fusion. The HIV envelope protein CD4 and the chemokine coreceptor can form a heterotrimeric complex on the surface of cells, and the molecular mechanisms by which CXCR4, CCR3, and CCR5 facilitate internalization of HIV-1 are now beginning to be understood (Lapham et al., 1996; Trkola et al., 1996; Wu et al., 1996). Using a panel of CCR5-CCR2B receptor chimeras, it has been determined that regions of CCR5 involved in chemokine ligand specificity and in the cofactor usage for various HIV-1 strains are not identical. For instance, chemokine selectivity is mediated by the second extracellular loop, while cofactor usage is dependent on the NH_2 terminus and first extracellular loop (Rucker et al., 1996; Samson et al., 1997). Consistent with this observation, antibodies binding to the NH_2-terminal region of CCR5 block HIV-1 infection but have no effect on chemokine activity. However, one antibody that recognizes the second extracellular loop of CCR5 blocks both the binding and the biological activity of the ligands RANTES, MIP-1α, and MIP-1β as well as infection by M-tropic and dual-tropic HIV-1 (Wu et al., 1997). Hence, whereas the specificity of CC chemokine binding to CCR5 is determined by a single domain, the gp120-binding site is more complex

and probably involves at least two domains, although changes in the NH_2 terminus can be sufficient to significantly disrupt gp120 binding and HIV entry.

The significance of G protein–linked signaling to viral entry remains unclear, but there are at least two lines of evidence to indicate that chemokine signaling and HIV-1 entry are separable functions of CCR5. First, pertussis toxin, genistein, and herbimycin A are all able to inhibit RANTES signaling, but do not block the ability of RANTES to inhibit HIV-1 infection (Oravecz et al., 1996; Alkhatib et al., 1997). Second, truncation mutants of CCR5 (which are unable to elicit intracellular calcium fluxes and chemotaxis in response to RANTES, MIP-1α, and MIP-1β) or certain CCR5-CCR2 chimeras (which are lacking domains necessary to signal in response to their natural chemokine ligands) are still able to function as HIV-1 coreceptors (Atchison et al., 1996; Rucker et al., 1996; Farzan et al., 1997; Samson et al., 1997). However, the involvement of pertussis-toxin-insensitive, G protein–linked signaling pathways in viral entry cannot be entirely ruled out. One possibility in this respect is that chemokines could inhibit viral entry by down-regulating the expression of their receptors. Indeed, both CCR5 and CXCR4 are endocytosed upon ligand binding. Although the mechanisms of agonist-stimulated chemokine receptor internalization are not fully understood, this event is also pertussis toxin insensitive (Oravecz et al., 1996; Alkhatib et al., 1997; Amara et al., 1997). However, it seems that internalization is not required for the coreceptor function of either CCR5 or CXCR4 for HIV-1 entry, since mutations of these receptors that prevent agonist-stimulated internalization do not impair HIV-1 entry. In the case of both CXCR4 and CCR5, although HIV-1 entry was unaffected, the impairment of receptor internalization correlates with a decreased potency of their respective ligands as inhibitors of HIV-1 infection (Alkhatib et al., 1997; Amara et al., 1997). Hence, it appears that the ability of a coreceptor to internalize is not required for HIV-1 entry, although it may contribute to the HIV-1 suppressive effect of CXC and CC chemokines, possibly by reducing the density of coreceptors. In this respect, it is tempting to speculate that one or more PI3K isoforms may have some role to play, given the recognized role of PI3K in mediating growth factor receptor internalization and the ability of some chemokines to activate PI3K (Joly et al., 1994; Turner et al., 1995). From our knowledge of the mechanisms for activation of the p85/p110 heterodimeric PI3K or PI3Kγ, the lack of effect of PTK inhibitors and pertussis toxin on HIV-1 entry would appear to argue against this possibility. Nevertheless, the growing diversity within both the PI3K family of enzymes and the functional consequences known to require PI3K activation, together with severe gaps in our knowledge regarding receptor regulation of certain classes of PI3K, suggests that this possibility merits further investigation.

There is growing evidence, however, to indicate that HIV-1 envelope glycoproteins may be more versatile with respect to their interaction with signaling pathways than anticipated up to now. For instance, the HIV-1 envelope protein gp160, like secretory proteins and most membrane proteins, is synthesized with a signal sequence that is usually cleaved from the nacent polypeptide during transport into the lumen of the endoplasmic reticulum. A signal peptide fragment of gp160 binds calmodulin, but the functional consequences of this interaction has not been elucidated (Martoglio et al., 1997). Furthermore, a recent study has indicated for the first time that HIV-1 envelope proteins such as gp120 can induce the rapid, pertussis toxin–sensitive tyrosine phosphorylation of the PTK Pyk-2 (Davis et al., 1997). Since Pyk-2 can feed into both the MAP kinase and Jun kinase signaling pathways and can modulate ion channel function, there may be a link between HIV-1 binding to chemokine receptors and several signaling cascades known to regulate cell growth, cell survival, and cell differentiation. In addition, a recent report has also demonstrated that recombinant gp160 envelope proteins from M-tropic (but not T-tropic) HIV-1 can actually induce a calcium signal through CCR5 on $CD4^+$ T cells and that envelope-mediated signal transduction through CCR5 induces chemotaxis of T cells (Weissman et al., 1997). Hence, although signal transduction through CCR5 may not necessarily be required for efficient fusion and entry of HIV-1, it is possible that this envelope protein–stimulated chemotactic response may contribute to the pathogenesis of HIV in vivo by chemoattracting activated $CD4^+$ cells to sites of viral replication and/or that CCR5 signaling may enhance viral replication by increasing the activation state of the target cells.

What Is the Relationship between T Cell Activation, Chemokine Production, and Susceptibility to HIV-1 Infection?

CXC (e.g., IL-8), CC (e.g., RANTES, MIP-1α, and MIP-1β), and C (e.g., lymphotactin) chemokines have all been reported to be up-regulated after T cell activation. Comparison of the stimuli required for chemokine production by T cells has shown that mitogenic stimulation elicits only small, temporal amounts of MIP-1α/β and RANTES, whereas higher sustained amounts are elicited by CD3 ligation (Wechsler et al., 1994; Riley et al., 1997). Under physiological conditions, commitment to optimal T cell activation requires TCR/CD3 complex engagement in combination with a costimulatory signal such as that provided by CD28 (Ward, 1996). Indeed, costimulation of anti-CD3 MAb-stimulated T cells with anti-CD28 MAb, results in a major augmentation of chemokines over levels elicited by CD3 ligation alone, although other costimulatory receptors such as CD2, CD4, or CD5 can also costimulate the production of these chemokines, albeit less effectively than CD28 (Riley et al., 1997). Furthermore, in genetically modified murine systems, full production of MIP-1α is dependent on CD28 costimulation interactions and is controlled by pathways different from those regulating CD28-dependent Th1 and Th2 differentiation (Herold et al., 1997). However, in contrast to findings using human T cells, murine T cell production of RANTES production appears to be constitutive and independent of anti-CD3 and anti-CD28 costimulation (Herold et al., 1997; Riley et al., 1997). Hence, the regulation of RANTES production may differ between human and murine cells. Interestingly, IL-2 cannot induce chemokine production, even though it can induce T cell expression of several chemokine receptors such as CCR1 and CCR2. However, stimulation of these T cells

with anti-CD3 and anti-CD28 Abs reduced CCR1 and CCR2 expression (Loetscher et al., 1996b; Herold et al., 1997). Thus, T cell production of chemokines and their responses to secreted chemokines appear to be regulated by the costimulatory signals that are available. If CD28 is ligated, T cells produce chemokines as well as cytokines such as IL-2, but as a consequence they lose responsiveness to chemotactic molecules. It might be predicted, therefore, that cells specific for the presented antigen will remain at the site of antigen and will recruit other cells that have not yet encountered antigen to that location, thus serving to ultimately amplify the immune response.

In view of the fact that HIV-1 infection of T lymphocytes is critically influenced by CC chemokines, and because several chemokines are strongly up-regulated upon CD28 ligation, the effect of CD28 ligation on HIV-1 infection of T cells was investigated. Indeed, activation of CD28 with immobilized anti-CD28 Abs or with its natural ligand B7.1 in combination with anti-CD3 Abs induces a potent anti-HIV-1 effect. These stimuli can still promote the long-term polyclonal proliferation of T cells from HIV-1-infected donors in the absence of exogenous cytokines or feeder cells. The CD28-dependent anti-viral effect acted early in the viral life cycle. This effect is, however, due only in part to the enhanced production of the CC chemokines RANTES, MIP-1α, and MIP-1β, which block infection by M-tropic isolates via CCR5 (Levine et al., 1996; Riley et al., 1997). The mechanisms by which CD28 protects against HIV-1 infection also involve regulation of CCR5 expression, as discussed further in the next section. The resistance of anti-CD3 and anti-CD28 costimulated T cells to some strains of HIV-1 in vitro is somewhat paradoxical, however, since it is well established that antigen presentation in vivo leads to increased viral replication, indicating that viral presentation in vivo is much more complex and dependent on many other cellular events.

Up-regulation of chemokine production following T lymphocyte activation is poorly understood, but is likely to involve effects on transcriptional mechanisms. RANTES is the best-studied CC chemokine in terms of transcriptional regulation of chemokines in T cells and is up-regulated late (3–5 days) after PHA activation in normal T lymphocytes. In contrast, RANTES mRNA is up-regulated quickly (24–48 hr) by TNF-α in fibroblasts, renal epithelial and mesangial cells, and smooth muscle cells (Ortiz et al., 1997). The RANTES promoter contains several important regions (termed A–E) that can bind novel transcription factors and that identify both early and late-acting transcriptional regulatory pathways contributing to RANTES gene expression. The RANTES promoter also contains four NF-κB binding sites, one of which serves as a CD28-responsive element (CD28RE) similar to that decribed in the IL-2 promoter (Ortiz et al., 1997). Interestingly, a CD28RE has also been identified in the IL-8 promoter. Indeed, stimulation with anti-CD3 MAb alone is able to induce RANTES and IL-8 promoter activity, while costimulation with anti-CD3 and anti-CD28 Ab further increased the activity. Moreover, mutation of the CD28RE prevented RANTES promoter activity induced by CD28 ligation (Moriuchi et al., 1997; Wechsler et al., 1994). The biochemical signaling cascades required for receptor regulation of the RANTES promoter have not so far been investigated, but this is an intriguing area that will hopefully be addressed in the very near future.

Regulation of CCR5 Expression by CD28 Contributes to HIV-1 Resistance

Although other accessory molecules such as CD2, CD4, and CD5 can produce levels of CC chemokines similar to those produced by CD28 ligation, the induction of HIV-1 resistance is specific to CD28, suggesting that CD28 can exert an additional effect(s) (Riley et al., 1997). Indeed, CD3/CD28 stimulation induces an HIV-1-resistant T cell phenotype characterized by a lack of expression of the CCR5 HIV-1 receptor. In contrast, levels of CXCR4 are abundant in CD28 costimulated T cells, while PHA-activated cells express both CCR5 and CXCR4 (Carroll et al., 1997). These effects of CD28 ligation on CCR5 and CXCR4 expression are consistent with the observation that only the M-tropic viruses are susceptible to the CD28 antiviral effect, whereas CD28-costimulated cells are sensitive to the T-tropic virus. Furthermore, CD4 expression and the binding of HIV-1 gp120 to CD28-stimulated cells are normal, and the antiviral effect appears to be blocked at the level of membrane fusion. Overall, it appears that CD28 costimulation in vitro induces a form of cellular resistance to HIV-1 infection independent of the effects of CD28 on T cell proliferation, and this resistance is similar to that recently described in individuals with a genetic disruption of the CCR5 gene (Liu et al., 1996; Samson et al., 1996). The identification of the CD28-dependent regulation of CC chemokines and CCR5 may therefore have important therapeutic implications both for preventing infection and for limiting viral spread in patients with HIV-1 infection. The CD28 homolog CTLA-4 plays a critical inhibitory role during T cell activation, but there are no reports concerning the effects of CTLA-4 on CC chemokine production or chemokine receptor regulation; one might predict that CTLA-4 may promote HIV-1 infection by up-regulating CCR5 expression (Chambers et al., 1996). The outcome of CTLA-4 on CCR5 expression is therefore eagerly awaited.

Chemokines as Therapy for HIV

Several modified chemokine derivatives that act as receptor antagonists and do not induce chemotaxis have been shown to inhibit M-tropic strains of HIV-1 including the NH_2-terminal truncated RANTES (9–68), Met-RANTES (which has a single extra methionine residue at the NH_2 terminus), and aminooxypentane (AOP)-RANTES (which has chemically added hydrocarbons at the NH_2 terminus) (Arenzana-Seisdedos et al., 1996; Simmons et al., 1997). There are, however, several drawbacks to using chemokine antagonists, since their use could be generally immunosuppressive. Most recently, three unrelated small molecular weight compounds, previously known for their inhibitory effects on HIV-1 replication, have been shown to block entry of T- but not M-tropic strains by interacting with CXCR4, probably by competition for coreceptor binding by the virus (Baggiolini and Moser,

1997). In addition, a viral chemokine-like protein, vMIP-II, may serve as a tantalizing lead compound for the development of broad spectrum anti-HIV agents. This protein blocks calcium mobilization and chemotaxis induced by several other chemokines as well as entry of HIV-1 mediated through CCR3, CCR5, or CXCR4 (Kledal et al., 1997). Finally, genetically modified CC (e.g., RANTES and MIP-1α) and CXC (e.g., SDF-1) chemokines have been targeted to the endoplasmic reticulum lumen to intracellularly bind the newly synthesized CCR5 or CXCR4, respectively, and which consequently prevents their transport to the cell surface. The T lymphocytes expressing these "intrakines" were subsequently found to resist M- and T-tropic HIV-1 infection (Chen et al., 1997; Yang et al., 1997). This gene-based intrakine therapy using genetically modified lymphocytes may have a potent and longer-lasting anti-HIV-1 action than that achieved by the adminstration of chemokines that have a short half-life in vivo.

In just a few years, there has been an explosion of interest and understanding of what chemokines are and what they do in a number of functional settings, and it is now clear that their role in T lymphocyte biology is not restricted just to cell attraction. In contrast, progress in understanding the biochemical events underlying chemokine receptor ligation in T cells has been much slower, although an interesting and rather complex picture of chemokine receptor signaling machinary is now beginning to emerge. The generation and use of transgenic and knockout mice models of chemokines and/or chemokine receptor(s) should ultimately prove essential tools in plugging the gaps in our knowledge regarding precise functional importance of individual chemokines. However, we are some way from being able to attribute functional consequences to discrete pathways, which would be beneficial in identifying future specific therapeutic drug targets. One can only hope that as the discovery of more chemokines and their receptors continues unabated, understanding of their functional relevence and chemokine receptor signal transduction mechanisms continues to progress at a comparable rate.

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