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The biology of chemokines and their receptors

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Abstract

This article summarizes the work done by our laboratory and by our collaborators on the biological role of chemokines and their receptors. Using both gain-of-function and loss of function genetic approaches, we have demonstrated that chemokines are important for the homeostatic distribution of leukocytes in tissues and for their mobilization from the bone marrow. We have also shown that chemokines are important players in inflammation and autoimmunity and that they contribute to lymphoid organogenesis, angiogenesis, and immune regulation. Together, our results and those of the literature suggest an important role for chemokines in homeostasis and disease and characterize chemokines as important targets for therapeutic intervention.

Keywords

Chemokines; Inflammation; Cell migration; Lymphoid organogenesis; Cancer; Viruses; Chemokine-binding proteins; Autoimmunity

Introduction

Chemokines are a particular group of cytokines that were originally described as being chemotactic to leukocytes. Currently, there are over 40 such molecules, grouped on 4 distinct families (C, CC, CXC and CX3C). Both chemokines and their receptors can be expressed constitutively and inducibly by virtually all cells in the body. This article summarizes the work done by our laboratory and by our collaborators on the biological activity of chemokines and their receptors. A comprehensive review of the subject is not possible due to space constraints.

Chemokine ligands

CXCL1

We introduced the use of transgenic techniques to study chemokine biology. Our first studies centered on the biology of the chemokine CXCL1 (N51 or KC) [1]. We engineered mice expressing CXCL1 in the thymus and skin and showed that these tissues presented a neutrophilic infiltrate. Surprisingly, the recruitment of neutrophils did not cause tissue

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destruction, suggesting a mobilizing, but not activating role for this chemokine. Subsequent studies showed that CXCL1's ability to promote neutrophil recruitment was not dependent of the tissue in which it was expressed; CXCL1 could also promote recruitment when expressed in the CNS [2], lung [3, 4], heart (unpublished), etc. In contrast, little or no recruitment of neutrophils could be observed in mice expressing CXCL1 in multiple tissues [5]. Such biology was a function of CXCR2 desensitization elicited by the high levels of CXCL1 observed in these animals [5]. These results confirmed the neutrophil chemoattractant properties of CXCL1 and provided a mechanistic explanation for the paradoxical lack of neutrophil infiltration observed in the presence of elevated concentrations of this chemokine.

We used transgenic mice expressing CXCL1 inducibly in the lung to study its role in invasive aspergillosis, a common and devastating pneumonia in immunocompromised hosts. Because neutrophils are critical for defense against this infection, and because CXCL1 promotes recruitment of neutrophils, we hypothesized that transient lung-specific overexpression of CXCL1 in mice with invasive aspergillosis would improve the outcome of disease [4]. Doxycycline administration to the animals induced CXCL1 expression specifically in the lung and promoted an increase in the number of neutrophils in the airways. Doxycycline-induced expression of CXCL1 after the onset of invasive aspergillosis significantly increased the survival and markedly reduced fungal burden. These results were the first to show that local modulation of CXCL1 expression could result in improved host defense and outcome of disease.

To further study the role of CXCL1, we produced *Cxcl1*-deficient mice. The mice were grossly normal, reproduced well, and did not show significant changes in the distribution of neutrophils in circulation [6]. We used these animals to assess the role of CXCL1 in the pathogenesis of atherosclerosis. Previous studies had demonstrated a role for CXCR2 in this process, and it was of interest to determine whether CXCL1 was the ligand mediating this effect. These studies showed that CXCL1/CXCR2 does not play a critical role in the recruitment of macrophages into early atherosclerotic lesions, and that both arterial CXCL1 and leukocyte-specific CXCR2 expression are central to macrophage accumulation in established fatty streak lesions.

Cxcl1-deficient mice were also used to probe the role of CXCL1 in intestinal inflammation. When treated with 2.5 % DSS in their drinking water, *Cxcl1*-deficient mice had significant weight loss, bloody stools, and a complete loss of gut integrity in the proximal and distal colon, accompanied by a predominantly mononuclear infiltrate. Wild-type (WT) littermates showed only minimal histopathology, but significantly more infiltrating neutrophils. This finding suggests that neutrophil infiltration induced by CXCL1 is an important component of the intestinal response to inflammatory stimuli [7].

In the setting of *K. pneumoniae* infection, CXCL1 produced by hematopoietic and lung resident cells is important for the expression of CXCL2 and CXCL5, and activation of NF-κB and MAPKs in the lung. These findings highlight the importance of CXCL1 in regulating pulmonary host defense against a bacterial pathogen via the activation of

transcription factors and MAPKs, as well as the expression of cell adhesion molecules and other neutrophil chemoattractants [8].

CCL2

Transgenic mice overexpressing CCL2 in the thymus and central nervous system have a higher number of mononuclear cells in those tissues than do control litter-mates [9]. In the thymus, there is a modest increase in the number of Mac-1- and F4/80-positive cells, but no apparent change in the number of lymphoid cells. A more pronounced mononuclear infiltrate is detected in transgenic mice expressing CCL2 in the brain. The vast majority of the recruited cells in the brain were monocytes and macrophages, as defined by light microscopy, ultrastructural, and immunohistochemical criteria. Such cells were found in a perivascular orientation with minimal parenchymal infiltration, possibly as a consequence of the accumulation of CCL2 in the vessels, as shown by immunohistochemistry. The mononuclear cell infiltrate in the brain could be significantly amplified by LPS treatment, suggesting that the recruitment properties of CCL2 could be potentiated by additional factors [9]. No spontaneous disease could be observed by the expression of CCL2 in the CNS. However, changes in the number of circulating monocytes or DC by conditional expression of Flt3 ligand in animals expressing CCL2 in the CNS promoted parenchymal cell infiltration and ascending paralysis in 100 % of the mice within 9 days of Flt3 ligand induction [10]. The disease could be aborted by depletion of monocytes/macrophages and, different from the classical models of experimental autoimmune encephalomyelitis, did not depend on $CD4^+$ and $CD8^+$ T cells. T cells and demyelinating lesions were observed in the CNS at a later stage as a result of organ-specific inflammation. Together, the results indicate that the pathogenesis induced by CCL2 expression is dependent on changes in the number and function of the circulating myeloid subsets.

Expression of CCL2 by insulin producing cells led to recruitment of monocytes and DCs into the islets and that this effect was dependent on the amount of CCL2 produced [11]. Interestingly, we observed that expression of CCL2 by pancreatic islets was associated with increased numbers of monocytes in circulation and accumulation of macrophages in the islets of transgenic mice. These changes were promoted by combined actions of CCL2 at the level of the bone marrow and of the islets, and were not seen in animals in which the CCL2 receptor (CCR2) was inactivated. Mice expressing higher levels of CCL2 in the islets developed diabetes spontaneously. The development of diabetes was correlated with the accumulation of large numbers of monocytes in the islets and did not depend on T- and B cells. Diabetes could also be induced in normoglycemic mice expressing low levels of CCL2 by increasing the number of circulating myeloid cells, by overexpression of FLT3 ligand systemically [12].

In conclusion, our studies showed that CCL2 promotes monocyte recruitment in vivo to at least three different organs and that it acts both locally (in the tissue) and remotely (at the level of the bone marrow) to promote cellular recruitment.

CCL21

is a potent chemoattractant for lymphocytes and dendritic cells in vitro. In the murine genome, there are multiple copies of CCL21 encoding two CCL21 proteins that differ from each other by one amino acid at position 65 (either a serine or leucine residue). Both serine and leucine forms are expressed in most tissues examined, the serine form being the predominant form in lymphoid organs, while the leucine form is predominantly expressed in nonlymphoid organs [13]. We showed that plt mutant mice, which have a profound deficiency in lymphocytes in the lymph nodes, lack the CCL21 serine form [14]. When expressed in transgenic pancreas, both forms of CCL21 induce of lymph-node-like structures [13]. Such infiltrates were composed primarily by topologically segregated T and B cells and by dendritic cells.

Transgenic mice expressing CCL21 from the thyroglobulin promoter (TGCCL21 mice) have lymphocytic infiltrates in the thyroid, which are topologically segregated into B- and T-cell areas and specialized vasculature, such as high endothelial venules [15]. Although high endothelial venules expressing peripheral lymph node addressin were frequently observed in the thyroid infiltrates, lymphocyte recruitment was independent of L-selectin or lymphotoxin-α but required CCR7 expression. These findings are of interest because CCL21 expression, lymphocytic infiltrates, and lymphoid follicles with germinal centers are often detected in autoimmune thyroid disease (AITD), suggesting a role for CCL21 in the pathogenesis of AITD.

Mechanistically the formation of CCL21-induced lymphoid aggregates in the thyroid did not depend on the presence of lymphoid tissue-inducer cells expressing the *inhibitor of differentiation 2* (Id2) gene, essential for the generation of $CD3^{-}CD4^{+}$ lymphoid tissue–inducer (LTi) cells and development of secondary lymphoid organs [16]. Rather, the formation of the lymphoid aggregates depended on mature $CD3^{+}CD4^{+}$ T cells. The initial stages of this process involved interaction of $CD3^{+}CD4^{+}$ T cells with DCs, the appearance of peripheral-node addressin-positive (PNAd+) vessels, production of chemokines that recruit lymphocytes and DCs, and lymphangiogenesis. Genetic deletion of lymphotoxin-β receptor or lymphotoxin-α abrogated development of lymphatic vessels in the inflamed areas in the thyroid but did not affect the development of neighboring lymphatics [17]. The development of lymphatic vessels within the lymph-node-like aggregates was dependent on the expression of lymphotoxin ligands by host cells, but not by the transferred $CD4^{+}$ T cells. Ablation of host DCs, but not NK cells, reduced the formation of new lymphatic vessels in the thyroid [18]. These results suggest that the formation of lymph-node-like structures induced by CCL21 involves not only recruitment of lymphocytes and DC, but also specific vascular changes that are dependent on the influx of these cells into the tissue. The establishment of specialized vasculature within the tissue may facilitate further recruitment of target cells.

Induction of lymph-node-like structures by CCL21, however, is not observed in all tissues. For instance, no lymphocyte recruitment or accumulation was observed when CCL21 is overexpressed in the skin [13] or in the brain [19], suggesting that formation of lymph-node-like structures is dependent on the tissues in which CCL21 is expressed.

CXCL13

is a chemokine that promotes chemoattraction of B cells in vitro and in vivo [11]. Studies carried out by our group and others have shown that CXCL13, besides being expressed constitutively in lymphoid tissues, is also expressed during inflammatory conditions in other organs, such as the gut [20]. To examine the consequences of CXCL13 expression in the intestine, we created transgenic mice expressing CXCL13 in intestinal epithelial cells. CXCL13 expression promoted a marked increase in the number of B and NK cells in the lamina propria and an increase in the size and number of lymphoid follicles in the small intestine [21]. This process was associated with the presence of cells expressing markers of lymphoid tissue-inducer cells, such as LTα, LTβ and TNF-related activation-induced cytokine (TRANCE). Such LTi-like cells produced IL-22, a cytokine implicated in epithelial repair, and expressed the IL-23 receptor, a key regulator of IL-22 production. These results suggest that overexpression of CXCL13 in the intestine during inflammation promotes mobilization of B cells and of LTi and NK cells that may have immunomodulatory and reparative functions.

CX3CL1 (Fractalkine)

is a chemokine that can exist either as a soluble protein or as a membrane-bound molecule. Both forms of CX3CL1 can mediate adhesion of cells expressing its receptor, CX3CR1. This activity, together with its expression by endothelial cells, suggests that CX3CL1 might mediate adhesion of leukocytes to the endothelium during inflammation.

CX3CL1 is also highly expressed in neurons, by epithelial cells of the terminal ileum, cecum and colon (in particular goblet cells) in the intestine, in bronchoalveolar cells in the lung and in tubular cells in the kidney [22]. In addition, as discussed above, CX3CL1 is also highly expressed in endothelial cells after inflammatory stimulation. To determine the biologic role of CX3CL1, we used targeted gene disruption to generate *Cx3cl1*-deficient mice [23].

Cx3cl1-deficient mice generated by our laboratory did not have overt behavioral abnormalities, and histological analysis of their brains did not reveal any gross changes compared to wild-type mice. In addition, they had normal hematologic profiles except for a decrease in the number of blood leukocytes expressing the cell surface marker F4/80. The cellular composition of their lymph nodes did not differ significantly from that of wild-type mice.

Microglia, the resident inflammatory cells of the CNS express the CX3CL1 receptor (CX3CR1). In collaboration with Richard Ransohoff's group, we have shown that *Cx3cr1* deficiency dysregulates microglial responses, resulting in neurotoxicity [24]. These results suggest that fractalkine may be involved in mediating neuron-glia cross talk.

As described above, epithelial cells in the intestine also express CX3CL1, and there is a population of CX3CR1+ macrophages in the lamina propria that extend dendrites through the intestinal epithelium. Work done by Steffen Jung's group in collaboration with our group has shown that the formation of these dendrites is dependent on the CX3CL1/R1 axis. More recently, in collaboration with Tim Denning's group, we have shown that fractalkine

and its receptor are important in maintaining LP macrophage populations, preventing translocation of commensal bacteria to mesenteric lymph nodes (mLNs) and limiting colitogenic Th17 responses [25].

Another cell population affected by *Cx3cl1* deletion is the circulating monocyte population. Survival of these cells is dependent on interaction of the membrane tethered form of CX3CL1 with its receptor. Expression of a soluble form of CX3CL1 does not rescue this defect [22], suggesting that the soluble and membrane functions have unique in vivo activities. The shed form may be important for formation of tissue gradients, whereas the tethered form may be critical in circulation for survival of specific cell populations, such as monocytes [22].

CXCL15 (Lungkine)

is highly expressed in the adult mouse lung. To study CXCL15 biology, we produced *Cxcl15*-deficient mice. *Cxcl15*-deficient mice had normal numbers of leukocytes in the lung, peripheral blood and bone marrow, but were more susceptible to *Klebsiella pneumonia* infection, with a decreased survival and increased lung bacterial burden compared with infected wild-type mice. Neutrophil numbers were normal in the lung parenchyma, but reduced in the airspace. The production of other neutrophil chemoattractants in the *Cxcl15*-deficient mice did not differ from that in wild-type mice, and neutrophil migration into other tissues was normal. These studies thus showed that CXCL15 is an important mediator of neutrophil migration from the lung parenchyma into the airspace [26].

Chemokine receptors

CCR6

is expressed by immature dendritic cells, B cells and effector/memory T cells, and binds with high affinity to a single chemokine ligand, CCL20. Mice lacking *Ccr6* have an impaired humoral immune response to orally administered antigen and to the enteropathic virus rotavirus. In addition, CCR6-deficient mice have a twofold to 15-fold increase in cells of select T lymphocyte populations within the mucosa, including $CD4^+$ and $CD8^+$ $\alpha\beta$TCR T cells. By contrast, systemic immune responses to subcutaneous antigens in CCR6-deficient mice are normal. These findings demonstrate that CCR6 is a mucosa-specific regulator of humoral immunity and lymphocyte homeostasis in the intestinal mucosa [27].

CCR6 also appears to be important for mucosal respiratory responses. In a cockroach antigen (CA) model of allergic pulmonary inflammation, CCL20 is expressed in the lung within hours of allergen challenge. Allergic asthmatic responses in the airway are associated with airway hyperreactivity, eosinophil accumulation in the lung, and cytokine production by allergen-specific, T helper cell type 2 (Th2) lymphocytes. CA-challenged *Ccr6*-deficient mice had reduced airway resistance, fewer eosinophils around the airway, lower levels of interleukin 5 in the lung, and reduced serum levels of immunoglobulin E. [28]. Altogether, the defect in *Ccr6*-deficient mice appears to be primarily due to an alteration in T-cell activation, but also appears to include local pulmonary APC defects [29].

The dependence of allergic responses on CCR6 has also been studied in the gut. Our colleague Cecila Berin and her group have studied the role of CCR6 in T cells expressing Th2 cytokines that are critical for experimental food allergy; *Ccr6*-deficient mice were protected from OVA-induced diarrhea but surprisingly were not impaired in mastocytosis or allergen-specific immunoglobulin E. *Ccr6*-deficient mice were also protected from T-cell-mediated diarrhea induced by anti-CD3 antibody. Allergic diarrhea was associated with an increased expression of Th2 cytokines within the intestinal mucosa that was significantly reduced in *Ccr6*-deficient mice. Finally, T-cell transfer studies demonstrated that CCR6 was required both on the transferred T cells and in the recipient mouse to manifest allergic disease in the gastrointestinal tract [30].

Immature myeloid DCs are the major population of CCR6-expressing cells in the lungs of mice infected with *Aspergillus*. These cells appear to be important in the overall pathogenesis, because *Ccr6*-deficient mice develop a more severe infection when challenged with *A. fumigatus* conidia than WT controls. *Ccr6*-deficient and wild-type DCs do not differ in their phagocytosis of conidia, cytokine response or maturation in vitro, but adoptive transfer experiments, showed that *Ccr6*-deficient DC do not accumulate in the lung. Thus, CCR6-mediated DC influx into the lung is important for host defense in invasive aspergillosis [31]. These results are in contrast to those Vermaelen et al. who showed that CCR2, but not CCR5 or CCR6, directly controls the accumulation of DCs into allergic lungs. Furthermore, the size of inflammatory monocyte populations in peripheral blood was strikingly dependent on CCR2, suggesting that CCR2 primarily mediates the release of monocytic DC precursors into the bloodstream under these conditions [32]. The apparent discrepancy on the ability of CCR6 to mediate trafficking of DC may reflect the different nature of the models (invasive aspergillosis vs. OVA-allergic responses).

The issue of mobilization of immature DC into the skin was further examined by Le Borgne et al. [33], who showed that recruitment of DC precursors to the buccal mucosa or skin in response to adjuvant is highly dependent on CCL20/CCR6 and that these newly recruited DC are responsible for efficient cross-priming of CD8+ CTL after mucosal or skin immunization.

Detailed analysis of the peritoneal cell populations showed that *Ccr6*-deficient mice have significantly lower number of both F4/80(+) macrophages and dendritic cells, but higher number of B cells in the peritoneal cavity than controls. In an experimental model of peritonitis induced by cecal ligation and puncture (CLP), *Ccr6*-deficient mice were protected when compared with WT controls, a fact that was associated with significantly lower levels of inflammatory cytokines/chemokines in both the peritoneal cavity and blood. Interestingly, DC recruitment into the peritoneal cavity was impaired in *Ccr6*-deficient mice during the evolution of CLP-induced peritonitis. This study showed that *CCR6* deficiency attenuates both local and systemic inflammatory response during CLP-induced peritonitis [34].

Lukacs et al. investigated the role of CCL20 and CCR6 in a pulmonary viral infection caused by respiratory syncytial virus (RSV). Neutralization of CCL20 or CCR6-deficiency during RSV infection significantly reduced lung pathology and favored a Th1 effector

response. No differences were observed in migration of T cells to the lungs of *Ccr6*-deficient mice, but a significant reduction was observed in numbers of conventional (cDC), but not plasmacytoid DC. A pathogenic phenotype could be reconstituted in CCR6-deficient mice by supplying cDC into the airway, indicating that the number of cDC dictates the adverse response in this model [35].

While CCR6 does not seem important for the migration of pDC in the context of RSV infection of the lung, there is evidence to suggest that it may be important to migration of pDC into mucosa and skin. Sisirak et al. [36] report that pDC are recruited to imiquimod-treated skin tumors in WT but not *Ccr6*-deficient mice. Treatment of human blood pDC with IL-3 induces expression of CCR6 and CCR10 expression making them responsive to CCL20 and CCL27/28, respectively. IL-3-differentiated CCR6(+) CCR10(+) pDC secrete high levels of IFN-α in response to virus. The authors propose that following CCR7-mediated extravasation into lymphoid tissues draining inflamed epithelia, blood pDC are instructed to upregulate CCR6 and/or CCR10 allowing their homing into inflamed epithelia (in mucosae or skin). At this site, pDC can then produce IFN-α contributing to pathogen clearance and/or local inflammation.

CCR6 is highly expressed by interleukin 17–producing T helper cells (Th-17 cells), which are important in experimental autoimmune encephalomyelitis. Reboldi et al have found that mice lacking *Ccr6* develop normal Th-17 responses but are highly resistant to the induction of experimental autoimmune encephalomyelitis [37]. Disease susceptibility can be reconstituted by transfer of wild-type T cells that enter into the CNS before disease onset and trigger massive CCR6-independent recruitment of effector T cells across activated parenchymal vessels. The observation that the CCR6 ligand CCL20 is constitutively expressed in epithelial cells of choroid plexus in mice and humans led the authors to propose that the CCR6-CCL20 axis in the choroid plexus controls immune surveillance of the CNS. Elhofy et al. [38], however, using a similar protocol to induce disease, have obtained strikingly different results. In their hands *Ccr6*-deficient mice developed a severe chronic EAE as compared to wild-type immunized animals. Furthermore, CCR6 expression was not required by T cells to induce EAE. The reason for discrepancy between these studies is unclear.

CCR8

To address the biological role of CCR8, we generated *Ccr8*-deficient mice. Using three different models, we showed that CCR8-deficient mice had defective Th2 immune responses in vivo. Mechanistic analyses indicated that Th2 cells developed normally in vitro [39] but that there were pronounced deficits in the number of eosinophils in the tissues in all models tested, suggesting a potential defect in the function or migration of Th2 cells. Recent studies by Andy Luster et al. tested this initial hypothesis. Using *Ccr8*-deficient mice derived by our group, they have shown that CCR8 is a key regulator of Th2 cell recruitment into allergen-inflamed skin. CCL8, a ligand for CCR8, is highly expressed in the allergic skin, and CCR8 expression is detected in a population of Th2 cells enriched for interleukin (IL)-5. *Ccr8*- and *Ccl8*-deficient mice had markedly less eosinophilic inflammation than

wild type in a model of chronic atopic dermatitis. These studies define CCR8 as a crucial regulator of Th2 cell homing that drives IL-5-mediated chronic allergic inflammation [40].

A role for CCR8 in the innate response has also been appreciated from studies with *Ccr8*-deficient mice. CCR8 is also expressed by monocytes [41], macrophages [42] and DC [41]. CCR8 is expressed in resident peritoneal macrophages and elicited leukocytes during septic peritonitis induced by CLP. *Ccr8*-deficient mice were resistant to CLP-induced lethality compared to controls. In vitro, peritoneal macrophages from *Ccr8*-deficient mice, but not neutrophils, had augmented bactericidal activities relative to those from controls and produced several cytokines and chemokines known to augment bactericidal activities of leukocytes, including TNF-α, IL-12, CCL22, CXCL1 and CXCL2 [42].

Interestingly, CCR8 is upregulated in peritoneal macrophages undergoing aggregation, and this process is increased by the presence of CCL1. This mechanism may be important for the development of peritoneal adhesions because *Ccr8*-deficient mice or mice treated with anti-CCL1-neutralizing Ab exhibited significantly reduced postoperational peritoneal adhesion [43]. These results suggest that CCR8 mice have an amplification of the innate immune response. Indeed, experiments involving treatment of *Ccr8*-deficient mice with *A. fumigatus* have shown that all characteristic airway physiology, inflammatory, and remodeling parameters of fungal asthma were significantly decreased or abolished in the *Ccr8*-deficient mice compared to controls [44].

Tacke et al. have studied the role of CCR8 in liver fibrosis. They showed that CCR8 is strongly upregulated in experimental liver injury models. *Ccr8*-deficient mice have attenuated liver damage and are protected from liver fibrosis compared to control mice. *Ccr8*-deficient mice had reduced infiltrates of liver macrophages, neutrophils and NK cells, while hepatic $CD4^+$ T cells were increased. The main CCR8-expressing cells in the liver were hepatic macrophages, and CCR8 was functionally necessary for CCL1-directed migration of inflammatory monocytes. Liver damage and fibrosis could be elicited in *Ccr8*-deficient mice by transferring $CCR8^+$ macrophages at the onset of experimental injury. These studies suggest that CCR8 blockade may be an important approach to protect the liver against injury and fibrosis [45].

Viral chemokine receptors

The genome of herpes viruses encodes chemokine ligands and chemokine receptors. While the biology of the chemokine ligands is relatively unclear, our laboratory has identified important oncogenic roles for two such receptors.

ORF74

The genome of human herpesvirus 8 (HHV8, also known as Kaposi's sarcoma [KS]-associated herpesvirus), encodes a highly constitutively active G protein–coupled receptor that is regulated both positively and negatively by endogenous CXC chemokines. This receptor referred to as vGPCR or ORF74 been implicated as an important oncogene causing Kaposi's sarcoma, an angiogenic tumor composed of endothelial, inflammatory and spindle cells. Transgenic mice generated by our group expressing ORF74 developed

angioproliferative lesions in multiple organs that morphologically resembled KS lesions [46]. Analysis of transgenic mice carrying mutated receptors deficient in either constitutive activity or chemokine regulation showed that induction of the KS-like disease in transgenic mice by ORF74 required not only high constitutive signaling activity but also modulation of this activity by endogenous chemokines [47].

We also showed that conditional transgenic expression of ORF74 by cells of endothelial origin triggered expression of the angiogenic factors and inflammatory factors that were necessary for the development of the disease [48]. Continued ORF74 expression was required for the progression of the KS-like phenotype, because its downregulation resulted in reduced expression of angiogenic factors and regression of the lesions [48]. Using this system, we were also able to monitor expression of ORF74 via the use of the surrogate marker LacZ. Upon treatment with doxycycline (DOX), cells expressing ORF74 and LacZ (ORF74/LacZ+ cells) progressively accumulated in areas where angioproliferation was observed [49]. Sorted ORF74/LacZ+ cells from angiogenic lesions expressed markers characteristic of endothelial progenitor cells, which produced angiogenic factors, and proliferated in vitro. This was an unexpected finding given that the promoter used in our studies was the CD2 promoter that targets expression of transgenes to hematopoietic cells. Expression of ORF74 in lymphocytes was not required for the development of disease, because KS-like disease was never observed in the thymus, spleens or lymph nodes, and more importantly, generation of ORF74 transgenic mice in a lymphocyte-deficient background did not prevent disease development. To prove that the endothelial cells expressing ORF74 caused disease, we sorted them from the ears from DOX-treated mice and transferred them into *Rag1*-deficient mice. With time, we observed angioproliferation and development of tumors in mice treated with DOX, but not in the group that did not receive DOX. These studies showed that (1) ORF74 promoted autocrine and paracrine effects, (2) development of disease depended directly on chemokines (ORF74 mutants without the chemokine-binding domain did not develop disease, even if they preserved high constitutive activity), and (3) disease development depended on ORF74 expression on a particular cell type (an endothelial progenitor). Together, our studies helped to establish ORF74 as the first chemokine receptor oncogene.

US28

is a constitutively active chemokine receptor encoded by CMV (also referred to as human herpesvirus (5), a highly prevalent human virus that infects many cells, including intestinal epithelial cells. When expressed in intestinal epithelial cells of transgenic mice, US28 promoted development of intestinal dysplasia and adenomas in transgenic mice [50]. Mechanistically these changes were related to specific changes in the intestinal epithelial cells. They included inhibition of glycogen synthase 3β (GSK-3β) function, accumulation of β-catenin protein and increased expression of Wnt target genes involved in the control of the cell proliferation. The transgenic mice were more susceptible to cancer development when exposed to an inflammation-driven tumor model (azoxymethane/dextran sodium sulfate). Similar to what was observed in the case of the ORF74 transgenic mice, tumor development appeared to be modulated by chemokines. Transgenic coexpression of the US28 ligand CCL2 (an inflammatory chemokine) increased epithelial cell proliferation as well as tumor

burden, suggesting that the oncogenic activity of US28 could be modulated by inflammatory factors. Together, these studies indicate that US28 has tumorigenic properties in vivo and suggest that CMV infection may facilitate the development of intestinal neoplasia in humans.

Viral chemokine–binding proteins

The finding of chemokine ligands and receptors in the genome of several herpes viruses immediately suggests that they have been hijacked from mammalian genomes and optimized through selection to promote viral replication and immune evasion. Many of such receptors and ligands do resemble endogenous molecules. However, one group of decoy receptors has also been identified in the genomes of viruses (pox and herpes viruses) and even parasites and ticks that have no sequence homology to any known molecules. These are the chemokine-binding proteins. We will summarize below our studies with these molecules.

M3

The *M3* gene present in the murine gammaherpesvirus 68 (MHV-68) genome encodes a secreted 44-kDa protein that binds with high affinity to many murine and human chemokines and has been shown to block chemokine signaling in vitro. We first examined its interaction with CCL19 and CCL21 chemokines expressed in lymphoid tissues, where gammaherpesviruses characteristically establish latency. M3 blocked in vitro chemotaxis induced by these molecules [51]. We also tested whether M3 could directly interfere with diverse chemokines in vivo. We performed experiments in which we promoted cell-specific or systemic expression of M3. We generated mice expressing M3 in insulin expressing cells in the pancreas and showed that it did not promote any biological change. We then intercrossed these animals with animals expressing CCL21 [51], CXCL13 [52] or CCL2 [11]. As shown above, these animals have significant lymphocytic and myeloid infiltrates, respectively. Co-expression of M3 markedly reduced these infiltrates in vivo indicating that M3 could neutralize each and every one of these chemokines when co-expressed in vivo.

These results prompted us to study whether M3 expression could alter development of disease. We first studied the susceptibility of RIP-M3 mice to multiple low-dose streptozotocin treatment. Such treatment promotes expression of several chemokines, including CXCL9, CCL1, CXCL10 and CCL21, lymphocytic insulitis and diabetes in mice. RIP-M3 mice were remarkably resistant to diabetes induced by MLDS. Islets from MLDS-treated RIP-M3 mice had fewer inflammatory cells and expressed lower levels of chemokines than those from MLDS-treated controls. The role of M3 in chemokine blockade during insulitis was further supported by in vitro experiments, demonstrating that multiple chemokines upregulated during islet inflammation are high-affinity M3 ligands that can be simultaneously inhibited by M3. These results confirmed M3 as a chemokine multiblocker and implicated chemokines as key mediators of insulitis [53]. To further test the role of chemokines in insulitis and diabetes and the ability of M3 to suppress disease, we tested the development of diabetes in NOD mice expressing M3 in pancreatic islets. Pancreatic islets of NOD mice express multiple chemokines before development of diabetes [54]. Islet-specific expression of M3 dramatically reduced leukocyte infiltration and islet destruction and completely blocked development of diabetes in NOD-M3 mice. M3 blocked diabetes by

inhibiting the priming of diabetogenic cells in the pancreatic lymph nodes and their recruitment into the islets. This effect was specific to the pancreatic islets because M3 expression did not affect other ongoing autoimmune processes [54].

We have also used M3 to study the relevance of the chemokine system to leukocyte populations in the gut during homeostatic and inflammatory conditions. In the gut, chemokines are differentially expressed, some chemokines are expressed throughout the intestine (CCL28, CCL6, CXCL16 and CX3CL1), whereas others are expressed preferentially in the small (CCL25 and CCL5) or large intestine (CCL19, CCL21, and CXCL5). Expression of M3 in intestinal epithelial cells resulted in reduced numbers of B and T cells in Peyer's patches, reduced numbers of intraepithelial CD8$\alpha\beta$/TCR$\alpha\beta$ and CD8$\alpha\alpha\beta$/TCR$\alpha\beta$ T cells, and reduced numbers of lamina propria $CD8^+$ T cells. M3 expression markedly reduced the number of eosinophils and macrophages in the small and large intestines. Dextran sulfate sodium treatment of control mice led to marked changes in the expression of chemokines and in the number of myeloid cells in the colon. These cellular changes were significantly attenuated in the presence of M3. This study suggests that the presence of specific populations in the gut is controlled by chemokines and that they are critical to promote recruitment during inflammatory conditions [20].

The generation of mice in which M3 expression is conditional to doxycycline treatment allowed us to study the contribution of chemokines to the development of other disease processes such as intimal hyperplasia in response to femoral arterial injury. Intimal hyperplasia appears to be a critical factor in the development of restenosis after coronary angioplasty and in the progression of atherosclerosis. Induction of M3 expression resulted in a 67 % reduction in intimal area and a 68 % reduction in intimal/medial ratio after femoral artery injury, suggesting that chemokine blockade may be effective in attenuating this process [55].

CrmD

is a protein encoded by the ectromelia virus that binds TNF-α and a limited number of chemokines. To test the biological function of this chemokine-binding protein, we generated transgenic mice expressing it in intestinal epithelial cells (vCrmD mice). Expression of CrmD did not alter the normal composition of leukocytes in the intestine, except for reduction in B cells in the lamina propria [56]. To test whether CrmD could block TNF-α function in vivo, we crossed the vCrmD mice to TNF(ΔARE) mice, which overexpress TNF-α in stromal and hematopoietic cells and develop arthritis and ileitis. TNF(ΔARE) mice have a marked dysregulation of chemokine expression in the ileum, which is consequence of TNF-α expression [56]. Expression of CrmD in the intestine significantly decreased expression of chemokines and attenuated the inflammatory infiltrates in the ileum of TNF(ΔARE) mice. Because CrmD binds to and inhibits TNF-α, it is likely that direct inhibition of TNF-α function is key to the reduction in the expression of both TNF-α and chemokines that are upregulated in the TNF(ΔARE) mice. However, inhibition of TNF-α is not the only mechanism underlying this response; we observed that CrmD has a direct inhibitory action on B-cell-mobilizing chemokines such as CXCL13, which could partially explain the decreased number of B cells observed in the mice. An additional, important

finding of these studies is that the inhibitory effect of CrmD is local. Ileitis was markedly reduced, but the development or arthritis induced by TNF-*a* was not affected [56].

Conclusions

Our studies have helped identify biological properties to many chemokine ligands and their receptors. Using both gain-of-function and loss of function approaches, we have demonstrated that chemokines are important for the homeostatic distribution of leukocytes in tissues and for their mobilization from the bone marrow. We have also shown that chemokines are important players in inflammation and autoimmunity and that they contribute to lymphoid organogenesis, to angiogenesis, and to immune regulation. We have also characterized important biological functions for chemokine-like elements encoded by viruses. In particular, we have documented a role for chemokine receptors encoded by herpes viruses in cancer. Finally, our studies on the biology of chemokine-binding proteins encoded by herpes and poxviruses have demonstrated an important role for multiple chemokine ligands in disease development. Together, our results and those of the literature suggest an important role for chemokines in homeostasis and disease and characterize chemokines as important targets for therapeutic intervention.

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Biographies

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