Targeting of Th1-Associated Chemokine Receptors CXCR3 and CCR5 as Therapeutic Strategy for Inflammatory Diseases

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Abstract: CXCR3 and CCR5 are chemokine receptor that are predominantly expressed on the surface of Th1 polarized T cells. In a variety of human and experimental autoimmune diseases the enhanced expression of CXCR3 and CCR5 binding chemokine ligands is followed by the recruitment of CXCR3- and CCR5-positive T cells, indicating an important role for these chemokine receptors in T cell-mediated tissue damage. In this review, we summarize a number of *in vivo* studies available on the neutralization of CXCR3 and CCR5 in inflammatory disease, and specifically focus on the potential therapeutic effects of CXCR3 and CCR5 blockade in human autoimmune disease and organ transplantation.

Key Words: Chemokine receptor, T cell, autoimmune disease, transplantation, inflammation.

INTRODUCTION

Chemokines are a family of small (8 to 12 kDa) secreted proteins that control leukocyte trafficking under homeostatic and inflammatory conditions. The effects of chemokines are mediated through interaction with G protein-coupled receptors that are predominantly expressed on leukocytes. CXCR3 and CCR5 are chemokine receptors that are highly expressed on the surface of Th1-polarized T cells. In a variety of human inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, glomerulonephritis, inflammatory bowel disease and solid organ allograft rejection, the enhanced expression of CXCR3 and CCR5 chemokine ligands is followed by the recruitment of CXCR3- and CCR5-positive T cells, indicating an important role for these chemokine receptors in T cell-mediated tissue damage. Pharmacological interventions with CXCR3 and CCR5 signaling using neutralizing antibodies or modified small chemokine analogues with antagonistic properties reduce inflammatory infiltrates and ameliorate disease severity in experimental models of inflammatory disease. Today, several new neutralizing chemokine fusion proteins are being developed for blocking of CXCR3 and CCR5 action, and small molecule antagonists that interfere with CCR5 are being tested in the clinical setting. Chemokine receptors are promising targets in the development of novel therapeutic strategies for human inflammatory diseases.

CHEMOKINES AND THEIR RECEPTORS

Chemokines are a family of structurally related cytokines that serve as key regulators of directional leukocyte trafficking under homeostatic and inflammatory conditions. Their main function in inflammation is to mediate leukocyte migration to the site of tissue injury, while in physiological, non inflammatory conditions they are crucial for maintenance of immune surveillance and spatial organization of secondary lymphoid organs. Apart from leukocytes, the expression of chemokine receptors has been detected on many different cell types, and chemokine signaling has been implicated in processes such as angiogenesis, fibrosis, proliferation, and tumor metastasis (reviewed in [1]).

The chemokine family is divided into two major subclasses based on the position of the two conserved cysteine residues in the N-terminal region of the molecule: CC chemokines (with the two residues adjacent to each other) and CXC chemokines (with a single amino acid between the two residues). The two other chemokine subfamilies are C chemokines (lacking the first of the two cysteines) with, to date, only two representatives and CX3C chemokine fractalkine (with three interposed amino acids) [2]. According to the ligands they bind, chemokine receptors are named and classified into CC, CXC, C, or CX3C receptors [2]. Most chemokine receptors bind more than one ligand. CXC receptors, however, exclusively bind CXC chemokines, while CC receptors are specific for CC chemokines.

The diverse biological effects of chemokine-induced signaling are communicated through G protein-coupled receptors (GPCR) with seven transmembrane domains, which link to the $G\alpha_i$ isotype of pertussis toxin-sensitive heterotrimeric G proteins. Upon ligand binding, chemokine receptors exert their effects through multiple intracellular signaling pathways (reviewed by [3, 4]). These include (I) activation of phospholipase C with subsequent formation of diacylglycerol, which acts on protein kinase C, and of inositol-1,4,5-trisphosphate, which induces calcium release from intracellular stores, (II) activation of mitogen-activated protein kinases (MAPK), (III) signaling through phosphatidylinositol 3 kinase and protein kinase B, (IV) activation of small GTPases of the Rho family, (V) focal adhesion kinases, and (VI) G protein-independent activation of the Janus kinase / signal transducer and activator of transcription (Jak/Stat) pathway.

TH1-ASSOCIATED CHEMOKINE RECEPTORS IN HUMAN DISEASE

The main function of chemokines is the tuning of selective migration of leukocyte subsets to their sites of action. Depending on inflammatory stimulus, costimulatory events,

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and disease stage, different populations of leukocytes initiate and maintain the inflammatory process [5].

T lymphocytes play a major pathophysiological role in human inflammatory diseases. In particular, CD4-positive T cells of the Th1 subtype are critical to tissue damage in a variety of autoimmune disorders. One reason for the specific composition of the inflammatory infiltrate is the differential expression of chemokine receptors on the surface of T lymphocyte subsets. T helper cell polarization of the Th1 type promotes surface expression of the chemokine receptors CXCR3 and CCR5, while Th2 lymphocytes commonly express CCR4 and CCR3 [6-10]. These expression patterns of chemokine receptors, however, are not strict markers of Th1 or Th2 type differentiation, since minor subsets of polarized T cells also co-express CXCR3 and CCR4 *in vivo* [11]. The chemokine receptor profile expressed by the lately discovered Th-IL17 helper cell population so far remains completely unknown. It is noteworthy that the expression of CCR5 is also detectable on human monocytes and natural killer (NK) cells, while the CXCR3 can be found on a subset of plasmacells.

Inflammatory infiltrates in rheumatoid arthritis, multiple sclerosis, proliferative glomerulonephritis, inflammatory bowel disease and in solid organ transplant rejection contain numerous CXCR3- and CCR5-positive T lymphocytes. The recruitment of these Th1 cells is preceded by selectively upregulated expression of CXCR3 ligands and CCR5 ligands in the inflamed tissue.

Recent studies have furthermore shown some new implications for the CCR5 in inflammatory disease. Production of the chemokines CCL3 and CCL4 is important for accumulation of naive CCR5-positive CD8+ T lymphocytes at sites of interaction between CD4+ T cells and antigen presenting cells (APCs). CCR5 signaling thereby increases antigen-dependent activation of CD8+ T cells in early immune response [12]. Moreover, functional CCR5 on the surface of CD4+ T lymphocytes translocates to the immunological synapse forming between T cells and APCs during costimulation. The secretion of CCR5 ligands by APCs is required to establish a sustained and "productive" interaction. This chemokine receptor-dependent conjugation reduces responsiveness of the engaged T cell to other chemoattractant sources and enhances T cell activation and proliferation [13]. Noteworthy, a possible role for CCR5 also during the resolution of inflammation has recently been described [14]. The enhanced CCR5 expression of the surface of neutrophils and T cells undergoing apoptosis promotes CCR5 ligand scavenging in experimental peritonitis and is further enhanced by "pro-resolution" lipid mediators. CCR5 may therefore also be important in the termination of chemokine signaling after cessation of the inflammatory stimulus [14].

The inhibition of Th1-associated chemokine receptor signaling as a therapeutic strategy to reduce the inflammatory infiltrate and to limit T cell-mediated tissue damage has been shown in multiple animal models of inflammation with variable success.

Rheumatoid Arthritis

Various studies provide evidence for the important role of CXCR3 and CCR5 receptor ligand interaction in rheumatoid arthritis [reviewed in [15]. Synovial fluid levels of the CXCR3 ligands CXCL9 and CXCL10 [16] and of the CCR5 ligands CCL3 [17], CCL4 [16], and CCL5 [18] in patients with rheumatoid autoimmune synovitis exceed those observed in patients with other forms of arthritis. Using immunohistochemistry and *in situ* hybridization, the production of CCL4, CCL5, and CXCL9 has been localized to synovial lining cell layers and inflammatory infiltrates. The cell types responsible for chemokine production seem to be $CD3^+$ T cells and macrophages for CCL4 and CCL5 [18-20]. CCL3 and CXCL10 are produced by infiltrating macrophages, and can also be detected in resident synovial fibroblasts [17, 21]. FACS analysis of synovial fluid revealed an accumulation of CXCR3/CCR5-double-positive T cells compared to peripheral blood in patients with rheumatoid arthritis [8] and juvenile idiopathic arthritis [22]. CCR5 is also highly expressed on the surface of monocytes and NK cells in the synovial fluid of patients with rheumatoid arthritis [23]. CXCR3 immunoreactivity in the inflamed synovial tissue is restricted to the perivascular T cell infiltrate [16, 24], while CCR5 immunoreactivity also localizes to macrophages [19]. Taken together, it is appropriate to conclude that during rheumatoid arthritis CXCR3 and CCR5 ligand production in the synovial tissue recruits CXCR3/CCR5-positive T cells of the Th1 subtype into the affected joint, where they promote tissue damage.

Further evidence for the importance of CCR5 signaling in the pathogenesis of rheumatoid arthritis comes from individuals with impaired CCR5 expression due to a 32 basepair deletion in the encoding gene (CCR5 Δ32 allele), which in the homozygous form leads to the complete absence of CCR5. The incidence of the CCR5Δ32 allele is not significantly different from that reported in patients with rheumatoid arthritis and healthy controls. The *homozygous* CCR5 deletion, in contrast, was completely absent in a population of 673 rheumatoid arthritis patients, compared to a frequency of 0,9 % in controls [25]. This statistically significant difference suggests a protective effect of the homozygous CCR5Δ32 genotype, and thus the absence of CCR5, against rheumatoid arthritis.

Multiple Sclerosis

Chemokine-driven recruitment of T lymphocytes to demyelinating lesions is a central mechanism in the pathogenesis of multiple sclerosis [reviewed in [26]. Within the inflammatory brain lesions of multiple sclerosis patients, resident astrocytes show enhanced immunoreactivity for CXCL10, especially in their perivascular end-feet processes lining the blood brain barrier [27, 28]. CCL3, in contrast, is predominantly expressed by infiltrating macrophages [28]. CXCL9, CXCL10, and CCL5 levels in the cerebrospinal fluid of multiple sclerosis patients are significantly higher compared to healthy controls. Accordingly, the cerebrospinal fluid of multiple sclerosis patients is enriched in CXCR3-positive and CCR5-positive T lymphocytes [27]. Perivascular lymphocytic infiltrates, too, are CXCR3 and CCR5 positive. CCR5 is the only chemokine receptor that is additionally expressed on macrophages and microglia [27, 28]. It is tempting to speculate that the production of interferon-inducible chemokines in astrocyte processes forming the blood brain barrier is a critical step in goal-directed migra-

tion of T lymphocytes into the parenchyma during chronic neuroinflammation.

The homozygous CCR5 deletion, however, fails to protect against disease susceptibility in multiple sclerosis [29], suggesting a redundancy in the chemokine system that, even in the absence of CCR5, may lead to the development of self-directed immune responses [26].

Renal Inflammation

The importance of chemokine expression for recruitment of leukocytes in glomerulonephritis and in renal involvement of systemic vasculitides is unquestionable [reviewed in[30]. It has been reported that the CXCR3 ligands CXCL9 and CXCL10 are expressed in the glomerular capillary tuft during human proliferative glomerulonephritis, colocalizing with proliferating mesangial cells and glomerular mononuclear infiltrates by *in situ* hybridization experiments [31]. Other authors, however, could not detect glomerular expression of CXCL9, CXCL10, or CXCL11 using the microdissection technique combined with RT-PCR [32], but a strong expression of CXCR3 ligands in the tubulointerstitial mononuclear infiltrates was confirmed by the use of different techniques in several forms of proliferative glomerulonephritis [31, 32].

Glomerular and periglomerular production of the CCR5 ligands CCL3, CCL4, and CCL5, as well as their focal expression in perivascular infiltrates, was found in several forms of crescentic glomerulonephritis [33]. T lymphocytes infiltrating the periglomerular area and the tubulointerstitium show CXCR3 and CCR5 positivity [32, 34, 35]. Renal expression of these chemokine receptors is apparently restricted to infiltrating lymphocytes and macrophages. The CXCR3 signal on mesangial cells described by Romagnani *et al.* [35] could not be reproduced by others [32, 36], and virtually no CCR5 expression was detected on resident renal cells [34, 37]. Interestingly, there was a correlation between clinical parameters such as serum creatinine and proteinuria and the number of infiltrated CXCR3/CCR5-double-positive cells in renal biopsy specimens [32].

Patients heterozygous for the CCR5Δ32 allele have an impaired chemotactic response of monocytes to CCR5 ligands *in vitro*. In agreement with this finding, the CCR5Δ32 deletion was found to be an independent prognostic factor for renal survival in patients with IgA nephropathy [38]. However, a recent study could not confirm a protective effect of the heterozygous CCR5Δ32 mutation in IgA nephropathy [39].

Inflammatory Bowel Disease

The expression of chemokines plays a key role in the chronic inflammatory condition characteristic for Crohn`s disease and ulcerative colitis [reviewed in [40]. CCR5 and CXCR3 chemokine ligands can be detected by *in situ* hybridization and immunohistochemistry in inflamed mucosal tissue from patients with inflammatory bowel disease [41]. The expression of these chemokines is mainly localized to infiltrating and endothelial cells in actively inflamed mucosa and shows a correlation with histopathologic severity of inflammatory lesions [41]. The associated infiltration of CXCR3+ CD4+ T lymphocytes into the lamina propria and submucosa was confirmed by immunohistochemistry [42]. The intramucosal granulomas developing in Crohn`s disease are also positiv for CCL5 mRNA and the surrounding lymphocytes are mostly CCR5/CXCR3-double-positiv [43]. The flowcytometric analysis of CD4+ T lymphocytes isolated from the lamina propria or the draining mesenterial lymphnodes from surgical specimens of inflammatory bowel disease patients shows enrichment of CXCR3-postive cells [44]. Moreover, it was demonstrated by intracellular cytokine staining that the isolated population of CXCR3+ CD4+ lymphocytes from mesenterial lymphnodes exhibits a Th1 type cytokine production profile [44].

The CCR5Δ32 mutation, however, fails to protect against the development of inflammatory bowel disease [45], possibly indicating the predominant role of CXCR3-mediated lymphocyte recruitment in this context.

Solid Organ Transplantation

Leukocyte infiltration of solid organ allografts induced by upregulation of chemokine expression is characteristic of cellular transplant rejection [reviewed in [46]. The enhanced expression of CXCR3 and CCR5 ligands has been demonstrated in renal [36, 47, 48], cardiac [49, 50] and hepatic [51, 52] allografts with acute cellular rejection.

Immunohistochemical examination of rejecting renal allograft biopsies reveals massive tubulointerstitial T lymphocyte infiltrates that are double-positive for CXCR3 and CCR5 [36]. Expression patterns of the CXCR3 ligands CXCL10 and CXCL11 differ remarkably during acute rejection. CXCL10 is expressed by tubular cells and infiltrating leukocytes, while CXCL11 is mainly expressed in interstitial cells, with apparent exception of mononuclear infiltrates. Interestingly, for both chemokines, some expression was found by *in situ* hybridization in parietal cells of the Bowman's capsule [36]. The production of CCR5 ligands was predominantly localized to tubular epithelial cells [47].

The expression of CXCL9, CXCL10, CXCL11, CCL5 and of their corresponding receptors CXCR3 and CCR5 in endomyocardial biopsies of heart transplant patients is increased during acute rejection [49, 50]. Furthermore, expression levels of these ligand receptor pairs correlate with the severity of rejection episodes and return to baseline after effective treatment of rejection with steroid pulse therapy [50]. The cellular origin of CXCR3 ligands seems to be the small vessels of cardiac allografts. CXCL10 is predominantly expressed in vascular smooth muscle cells and infiltrating mononuclear cells. CXCL11 originates from endothelial cells and, in contrast to infiltrates of renal allografts, is also expressed by intragraft leukocytes. CXCL9 expression in cardiac allografts is, however, exclusively localized to infiltrating mononuclear cells [53].

As demonstrated by immunohistochemistry and chemotaxis assays, lymphocytes infiltrating into rejecting liver allografts express CXCR3 and CCR5 on their surface [51]. In immunohistochemical staining, CXCL9, CXCL10, and CXCL11 are mainly detectable on endothelial cells in normal hepatic tissue, with markedly enhanced signals during episodes of acute rejection. Immunopositivity for CXCL10 and CXCL11 on biliary epithelium is only detectable during

allograft rejection. In agreement with this finding, CXCR3-positive lymphocytes have been shown to surround the bile ducts in this condition [51]. Other cell types that produce CXCR3 ligands in the rejecting liver transplant are the monocytic Kupffer cells expressing CXCL9 and CXCL10 and, similar to rejecting cardiac allografts, mononuclear infiltrates, which specifically express CXCL10 and CXCL11 [51]. In the immunohistochemical examination of normal liver tissue, vascular endothelium and biliary epithelium are positive for CCL3 and CCL4, showing enhanced immunopositivity during liver allograft rejection [52]. Interestingly, only the endothelium and infiltrating cells demonstrate mRNA production for these chemokines, while bile ducts are negative in *in situ* hybridization. This indicates that the presence of chemokines on the surface of bile duct epithelial cells is spatially independent of the actual chemokine transcription [52]. CCR5 ligand expression in liver transplant rejection is effectively reduced by corticosteroid pulse treatment [52].

The homozygous CCR5Δ32 mutation is correlated with prolonged graft survival in renal transplant recipients [54]. In heart and liver transplantation, however, CCR5 deficiency has not been confirmed to be associated with a favorable clinical outcome [55, 56].

CXCR3 AND CCR5 TARGETING IN ANIMAL MODELS OF AUTOIMMUNE DISEASE

Manipulation of CXCR3 and CCR5 signaling has been studied extensively in various animal models resembling human autoimmune disease and organ transplantation. Blocking of chemokine receptor / ligand interaction has been attempted by using chemokine or chemokine receptor-specific antibodies, modified chemokine analogues with antagonistic properties, and non-peptide chemokine receptor antagonists. Other possible sites of interaction with chemokine signaling include inhibition of second messenger pathways and reduction in chemokine binding to extracellular glycosaminoglycans [57], which is essential for correct recognition by immune cells (summarized in Fig. (**1**)). Furthermore, gene inactivation of either chemokines or their corresponding receptors in knock-out mice has provided important insights into the mechanisms of leukocyte trafficking.

As the N-terminal integrity of CCL5 is crucial for receptor binding and activation, aminoterminal fusion of a single methionine (Met) or an aminooxypentane (AOP) residue results in a protein with antagonistic properties on CCR5, CCR1, and CCR3 [58]. Treatment with aminoterminally modified Met-CCL5 or AOP-CCL5 (Met-RANTES or AOP-RANTES) leads to an improved course of disease and reduced monocyte infiltration in rat models of rheumatoid arthritis [59], mesangioproliferative glomerulonephritis [60], and renal transplant rejection [61]. In mouse models of glomerulonephritis, the ameliorative effect of Met-CCL5 treatment appears to be less pronounced [62] or even converse, resulting in aggravation of tissue damage [63]. In experimental autoimmune encephalitis of mice, an animal model of multiple sclerosis, treatment with Met-CCL5 fails to improve the acute phase of the disease and has only a

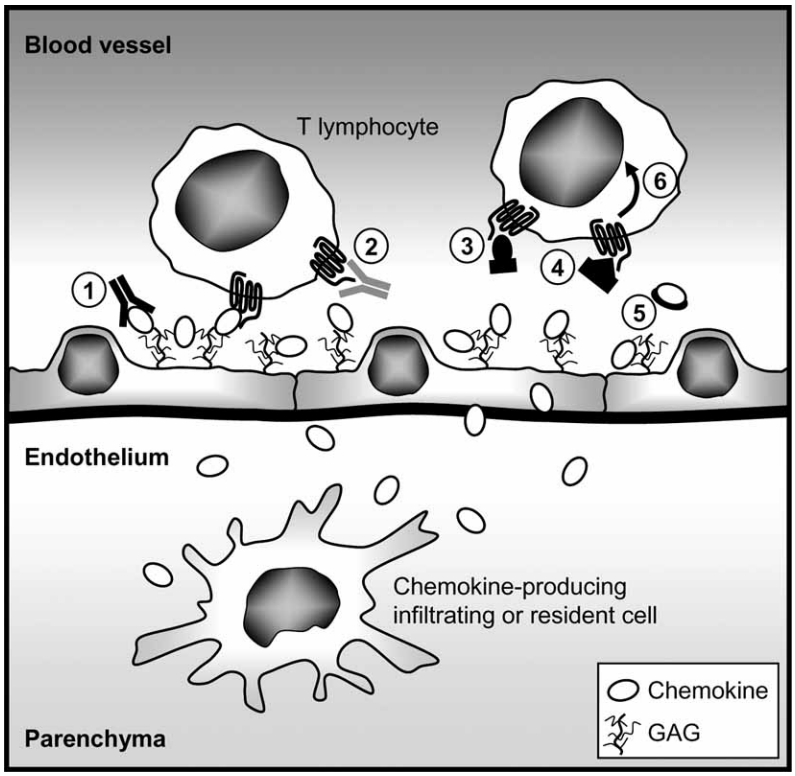

Fig. (1). Potential strategies in therapeutic targeting of chemokine signaling. (1) Blockade of chemokine signaling by neutralizing antibodies, directed against either chemokine ligands or (2) their receptors. (3) Inhibition of ligand receptor interaction by modified chemokine analogues with antagonistic properties or (4) small molecule antagonists. (5) Interference with chemokine binding to glycosaminoclycans (GAG), which is indispensable for correct signal recognition by immune cells. (6) Interaction with chemokine-induced intracellular singaling.

modest ameliorative effect on the chronic-relapsing phase [64].

Positive results, however, have been obtained with neutralizing antibodies against CCL5 in adjuvant-induced arthritis in rats [65]. Another elegant method of interfering with chemokine signaling is the vaccination with DNA vectors encoding chemokine sequences. Subsequent heterotopic chemokine expression leads to the production of self-directed antibodies against the specific chemokine gene product, and is utilized to treat adjuvant-induced arthritis in rats. DNA vaccination with either CXCL10, CCL3, or CCL5 sequences generates a "protective immunity" against these chemokines, resulting in the suppression of manifest arthritis [66, 67].

Neutralization of the CXCR3 chemokine CXCL10 with a specific antibody leads to a marked reduction in T lymphocyte infiltration and improved renal function in a rat model of renal endothelial injury [68]. Furthermore, mice deficient in CXCR3 show a favorable clinical outcome and reduced renal leukocyte infiltration in a mouse model of glomerulonephritis (Panzer *et al*., in press).

Heart transplant survival in mice is prolonged by application of a monoclonal CXCL10 antibody. Unexpectedly, mice deficient in CXCL10 develop acute rejection of wild-type heart allografts in a similar way to that seen in wild-type recipients [69]. Wild-type mice receiving an allograft from a CXCL10-deficient donor, in turn, show long-term graft survival, indicating an important role for local intragraft production of the CXCR3 ligand in the initiation of acute rejection [69]. Further evidence for the importance of CXCR3-mediated signaling in acute rejection comes from studies on CXCR3-deficient mice, which also show a profound resistance to allograft rejection and prolongation of graft survival [70].

In the dextran sodium sulfate-induced colitis, a murine model of ulcerative colitis, neutralization of CXCL10 with a monoclonal antibody amelioriates disease activity and mucosal lesions [71]. Interestingly, the inflammatory infiltrate of macrophages and CD4+ T lymphocytes remains unaltered, proposing a mechanism of CXCL10 blockade distinct from impaired leukocyte recruitment. The dramatically decreased number of apoptotic cells in the colonic crypt mucosa and the higher epithelial turnover under CXCL10 neutralization suggests that CXCL10 may directly inhibit crypt cell proliferation and thereby promote epithelial injury [71].

In experimental autoimmune encephalitis, mice deficient in CXCL10 remain susceptible to the induction of the disease [72]. In rats, a monoclonal antibody against CXCL10 even exacerbates encephalitis, increasing CNS infiltration of CD4+ T lymphocytes [73]. Both, treatment of murine autoimmune encephalitis with a monoclonal antibody against CXCR3 and CXCR3 gene deficiency aggravate the clinical symptoms, while leaving the amount of infiltrating leukocytes unaltered [74]. One explanation for these unexpected results might lie in the impaired antigen-driven INF-γ production of activated T lymphocytes under conditions of defective CXCL10 / CXCR3 signalling [72-74]. INF-γ ameliorates murine experimental autoimmune encephalitis by inhibiting the proliferation of antigen-specific T cells. This mechanism possibly depends on the induction of nitric oxide synthase in periphery and target tissue [74, 75]. In contrast, inactivation of the CXCR3 ligand CXCL10 by DNA vaccination and subsequent self-directed antibody production suppresses disease activity and even ongoing autoimmune encephalomyelitis in rats and mice [76].

Several possible reasons may account for the variable effectivity of different blocking regimes. First, compensatory changes of the immune system in (non-conditional) gene-deleted mice cannot be underestimated. Second, the redundancy of chemokine system (i.e. multiple ligands bind to the same chemokine receptor) must be considered, especially in targeting of one specific chemokine ligand. Third, the systemic application of antibodies specific for either chemokine ligands or their receptors may only incompletely block the local receptor / ligand interaction.

The T cell-mediated hepatitis induced by application of concanavalin A shows a deleterious outcome in CCR5-deficient mice [77-79]. The proposed mechanism for aggravated liver injury includes augmented production of INFγ and IL-4 by liver NKT cells with subsequent activation of NK cells which promote the hepatocyte cell death [77, 78]. Furthermore, increased IL-4 levels induce excess production of CCL5 recruiting CD4+ T cells and NK cells *via* its alternate receptor CCR1 [79].

In the dextran sodium sulfate-mediated colitis of mice CCR5 deficiency shows a protective effect with reduced clinical severity and decreased number of epithelial ulcerations [80]. This beneficial effect is contradictorily accompanied by an increased number of infiltrating CD4+ T lymphocytes and NK cells. The elevated expression of IL-4 and IL-10 together with decreased expression of INF-γ in the inflamed mucosa of CCR5-deficient mice compared to control might indicate a shift towards a Th2 type pattern of intestinal T lymphocyte activation in these mice [80].

In a mouse model of viral CNS infection, CXCR3 deficiency partially protects from the otherwise fatal CD8+ T lymphocyte-mediated tissue damage [81]. The course of this disease in CCR5-deficient mice is generally unaltered. Contrary to expectations, CXCR3/CCR5-double-deficient mice are more susceptible to intracerebral infection, showing a poor clinical outcome [81]. This opposing effect of the two chemokine receptors, putatively synergistic in directing T lymphocyte migration, is speculated to be caused by augmented generation of CD8+ effector T cells in genetically modified mice lacking the CCR5 [81]. The exact role of the CCR5 and its ligands in viral CNS inflammation remains therefore to some extend unclear.

Interestingly, disruption of the CCR5 gene in mice has neither positive nor negative effects on the characteristics of collagen-induced arthritis [82] or autoimmune encephalitis [83].

Given the beneficial effects of interference with the CCR5 and CXCR3 pathways in a number of animal models and the existing data obtained from human homozygous CCR5Δ32 mutation carriers, the strategy of targeting CXCR3 and CCR5 simultaneously for effective prevention of directed Th1 lymphocyte migration in autoimmunity seems a suitable approach. A couple of recent studies address the

issue of therapeutic "double blockade" of Th1-associated chemokine receptors in different animal models. A nonpeptide synthetic CCR5 antagonist named TAK-779 has been particularly useful in this context, because it also inhibits ligand binding on and signaling through CXCR3 [84]. Subcutaneous application of this antagonist in mice mitigates collagen-induced arthritis [85], prevents allograft rejection in cardiac and islet transplantation [86], and improves disease outcome in models of intestinal ischemia reperfusion injury [87] and inflammatory bowel disease [88]. Lymphocyte infiltration in murine cardiac allografts and inflamed intestinal mucosa is effectively reduced by TAK-779 [86, 88] and, likewise, by antagonization of CXCR3 with a polyclonal antibody in CCR5-deficient mice [89].

Taken together, targeting of the Th1-associated chemokine receptors CXCR3 and CCR5 as well as their ligands is a promising therapeutic strategy for human autoimmune disease and organ transplantation. However, care has to be taken as the data available from experimental animal models show inefficacy of CCR5 blockade [63, 64] and even aggravation by CXCR3 inhibition [73, 74] in certain autoimmune diseases, as for example, neuroinflammation. The reasons for the discrepancies among different animal species and different models of CD4+ T cell-mediated autoimmune injury remain elusive. Likewise, the effects on the systemic immune response by Th1 chemokine inhibition are still incompletely understood.

FUTURE DIRECTION - NEW SUBSTANCES

Since macrophage-tropic HIV-1 strains use CCR5 as a coreceptor for viral cell entry, recent interest has focused on blocking the interaction of CCR5 with the viral gp120 protein. Maraviroc (UK-427,857) and vicriviroc (SCH 417690), two novel compounds from HIV research, bind selectively to CCR5 and potently antagonize ligand-induced increase in intracellular calcium, leukocyte chemotaxis, and receptor internalization [90, 91]. Results of short-term monotherapy of M-tropic HIV-infected individuals show that both compounds have sufficient oral bioavailability when given twice per day, are generally well tolerated, and effectively reduce the viral load [92, 93]. The occupancy of CCR5 on peripheral leukocytes by maraviroc was >80% after 10 days of therapy and >60% even after 5d after drug discontinuation [93].

TAK-779, another nonpeptide compound, effectively inhibits CCL5 binding to CCR5 and ligand-mediated intracellular calcium release from intracellular stores [94]. Later on, it was found that TAK-779 does not only inhibit chemokine binding to CCR5 but also to CXCR3. The compound shows profound inhibition of receptor internalization and chemotaxis mediated by CCR5 and CXCR3 ligands *in vitro* [84], and has been successfully used in animal models of inflammation [85-87]. A CXCR3-specific small molecule antagonist has recently been developed by means of high throughput screening. It shows a distinct inhibitory potency in chemotaxis assays, but poor oral bioavailability is still a limiting factor. Prior to *in vivo* experiments, further structural optimization will be needed [95].

In vivo depletion of CCR5-expressing cells is considered another potential therapeutic strategy for HIV infection and severe autoimmune disease [96]. An anti-CCR5-anti-CD3-bispecific single-chain antibody was created to redirect cytotoxic T cells to CCR5-positive target cells. *In vitro* application of this fusion protein to freshly isolated human peripheral blood mononuclear cells or synovial fluid of rheumatoid arthritis patients results in depletion of monocyte and lymphocyte populations [96]. Furthermore, a fusion protein of CCL5 linked to a truncated version of the Pseudomonas exotoxin A (PE38) was generated. Incubation of CCR5-expressing Chinese hamster ovary cells with the CCL5-PE38 construct led to complete cytolysis of these cells, while CXCR4-transfected cells, serving as a control, remained unaffected [96]. Selective down-modulation of CCR5 on CD4+ cells (but not on CD8+ cells) *in vitro* is achieved by application of a fusion protein of CCL5 and the variable domain of a CD4-specific antibody [97]. In the future, this particular experimental approach could be useful in immune cell-specific targeting of certain chemokine receptors, and it might help to minimize the anticipated side effects caused by unwanted inhibition of chemokine receptors on "protective" immune cells and resident cells.

Engineered proteins equipped with a glycosylphosphatidylinositol (GPI) domain can be transferred into cell surface membranes retaining their native protein function [98]. This method, previously referred to as "cell painting", was used to create a local CCR5 antagonist that consists of a Met-CCL5 dimer linked to a GPI anchor at the C-terminus [99]. The resulting fusion protein Met-CCL5(dimer)-GPI was demonstrated to be incorporated into the cell membrane of microvascular endothelial cells *in vitro.* Furthermore, it locally inhibited CCL5-induced transendothelial migration of a monocyte line [99]. Local application of such an antagonist to a vascularized graft before engraftment could provide protection from leukocyte-mediated vascular damage in the initial phase of transplantation, which is critical to long-term prognosis of transplant survival.

CONCLUSIONS

In summary, accumulating data from experimental and clinical studies clearly indicate that the chemokine receptors CXCR3 and CCR5 are among the main regulators of Th1 cell recruitment in autoimmune disease. Since chemokine-induced chemotaxis is a multi-step process, involving a large number of molecules and signaling cascades, several potential targets exist for innovative antiinflammatory therapies in human inflammatory disease. At present, much effort is being made by the pharmaceutical industry to identify and characterize small molecule chemokine receptor antagonists without intrinsic activity, such as the CCR5 receptor blocker used in the treatment of HIV patients [92, 93]. This class of agents seems to be most promising for the therapeutic blockade of CXCR3 and CCR5 action in the treatment of T cell-mediated inflammatory disease and transplant rejection. It will be very interesting to see whether CCR5 and CXCR3 targeting will be established as a new therapeutic tool for the treatment of human T cell-mediated inflammatory disease in the next years.

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