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Øystein Bruserud
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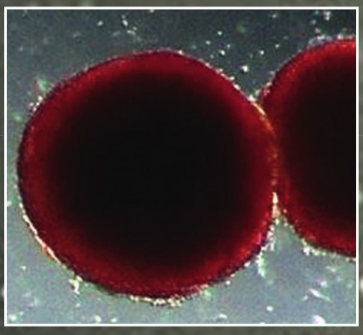
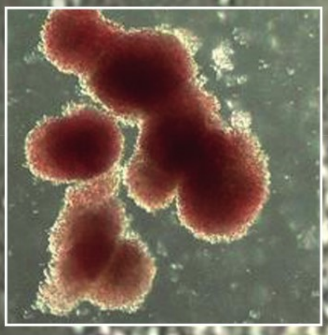
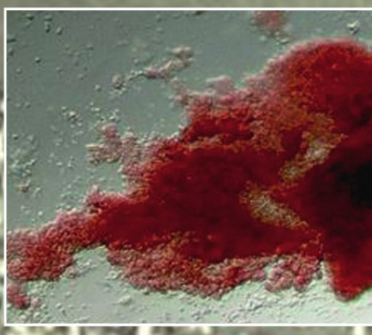
The Chemokine System in Experimental and Clinical Hematology

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The Chemokine System in Experimental and Clinical Hematology

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Preface

The aim of this issue is to describe and explain the importance of the chemokine system in hematology. As described in the "Introduction," the chemokine system is probably important for many aspects of normal and malignant hematopoiesis. A major focus is the development and treatment of hematologic malignancies, including the immunobiology of stem cell transplantation.

The first main section includes three chapters, where the first review by Bonecchi et al. describes the chemokine decoy receptors, a group of receptors that recognize chemokines but are unable to activate transduction events. However, they seem to have important biological functions both in inflammation and possibly in carcinogenesis through removing, transporting, or concentrating their chemokine ligands. The next two chapters describe the function of the chemokine system in chemotaxis of natural killer cells and for regulation of angiogenesis. As described by Magazachi, natural killer (NK) cells are important in allogeneic stem cell transplantation; these cells are important as antileukemic effector cells that influence the risk of posttransplant leukemia relapse, and they are in addition involved in the development of graft-versus-host disease (GVHD). Allogeneic stem cell transplantation is used in the treatment of most hematologic malignancies and also certain nonmalignant hematologic disorders. The three major causes of death after allotransplantation are leukemia relapse, severe GVHD, and serious infections. NK cells seem to be involved in all these complications. Furthermore, increased angiogenesis is common in the pathogenesis of many hematologic malignancies. Leukemic cells often show constitutive release of proangiogenic chemokines, and antiangiogenic therapy is now considered in the treatment of these diseases. The importance of chemokines in this angioregulation is described in detail in Dimberg's review.

The next three chapters focus on the immunobiology of allogeneic stem cell transplantation, an important therapeutic strategy first of all for hematologic malignancies. The treatment is increasingly used and the use of reduced intensity conditioning has made this treatment available for elderly patients. The grafts include both stem cells and immunocompetent cells, and allotransplantation should therefore be regarded as a combination of intensive chemotherapy with stem cell rescue

and antileukemic immunotherapy. As reviewed by Löffler et al., single nucleotide polymorphisms in immunoregulatory genes influence the risk of severe and possibly lethal posttransplant complications. Furthermore, as described by Kittan and Hildebrandt, the transplanted immunocompetent cells are then important for the risk of developing GVHD and for the induction of specific antileukemic reactivity. Studies in animal models and in humans suggest that chemotaxis of immunoregulatory T-cell subsets is important in the development of GVHD. The last review describes the importance of immunosuppressive Treg and proinflammatory Th17 cells in the pathogenesis of GVHD.

The three last contributions describe the importance of the chemokine system in clinical hematology. First, Kittang et al. describe the chemokine system in acute myeloid leukemia (AML). This disease is characterized by accumulation of immature malignant cells that show constitutive release of several chemokines that may contribute to leukemia-associated angiogenesis, chemosensitivity, and regulation of antileukemic immune reactivity. The biological background for the possible use of CXCR4 inhibitors in the treatment of AML is outlined. Second, as described by Calandra et al., pharmacological CXCR4 inhibition can be used for mobilization and harvesting of peripheral blood stem cells that are used for autologous and allogeneic transplantations. These inhibitors are also considered for the treatment of AML. Finally, venous thromboembolic disease is one of the most common hematologic disorders. Cancer patients have an increased risk of this disease and are then treated with heparin. Heparin-induced thrombocytopenia (HIT) is an important complication during this treatment and is caused by antibodies directed against the chemokine CXCL4 (platelet factor 4)–heparin complex on the platelet surface. Previous reviews have focused on the clinical handling of this complication; the present review by Sandset includes a more detailed description of the immunobiology of this complication.

The present reviews illustrate that chemokines can be involved in leukemogenesis. The chemokine system is also important both for the crosstalk between malignant cells and their neighboring nonmalignant stromal cells (including endothelial cells) and for the immunoregulation in patients treated with allogeneic stem cell transplantation. Thus, chemokines are important both for the pathogenesis and treatment of hematological diseases.

Bergen, Summer 2010 Øystein Bruserud

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Part I
Introduction

The Chemokine System in Experimental and Clinical Hematology

Øystein Bruserud and Astrid Olsnes Kittang

Contents

Abstract The chemokine family consists of approximately 50 small (8–14 kDa), basic proteins that are expressed and released by a wide range of normal and malignant cells. Based on their molecular structure, these cytokines are divided into the two major subgroups CCL and CXCL chemokines that bind to CCR or CXCR receptors, respectively. These mediators are important for regulation of cell viability, proliferation, differentiation, and migration. Chemokines are important for cell migration during embryogenesis; they are involved in the regulation of complex processes like local recruitment of inflammatory cells, angiogenesis, and

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regulation of normal as well as leukemic hematopoiesis. Chemokines can be constitutively released by malignant hematopoietic cells as well as by bone marrow stromal cells. This bidirectional crosstalk between malignant hematopoietic cells and neighboring stromal cells may therefore be important for the development and clinical presentation of malignant diseases, and the chemokines or their receptors may also represent a target for specific anticancer therapy at the molecular level.

Abbreviations

AML	Acute myeloid leukemia
DARC	Duffy antigen receptor for chemokines
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft vs. host disease
IFN	Interferon
IL	Interleukin
MMP	Matrix metalloprotease
SNP	Single nucleotide polymorphism

1 The Chemokine System

1.1 Nomenclature

The chemokine family consists of approximately 50 small (8–14 kDa), basic, secreted proteins that are expressed by a wide range of normal and malignant cells (Laurence 2006; Olsnes et al. 2009b; Dimberg 2010). Chemokines are defined by their molecular structure with four conserved NH_2-terminal cysteine residues; the first cysteine forms a disulphide bond with the third and the second and fourth cysteine forms an additional bond. The chemokines are subclassified into two major and two minor subfamilies depending on this structure (Laurence 2006; Olsnes et al. 2009b; Dimberg 2010): (1) CXC chemokines (CXCL1–16) have one amino acid separating the first two cysteines, (2) CC chemokines (CCL1–27) have two conserved cysteine residues in the juxtaposition, (3) C chemokines (XCL1–2) lack two of the four conserved cysteine residues, and (4) the CX3C chemokine (CX3CL1) has three amino acids intervening between the two first cysteine residues. The CXC subfamily is further divided into ELR-CXC and non-ELR-CXC chemokines, depending on the presence of a tripeptide (Glu-Leu-Arg) ELR-motif preceding the first cysteine (Dimberg 2010). Finally, chemokines were previously classified as homeostatic-constitutive and inflammatory-inducible. Many of the homeostatic chemokines bind only to one receptor, while inflammatory chemokines often bind

to several receptors, and each of these receptors often bind several chemokines (Olsnes et al. 2009b; Dimberg 2010; Maghazachi 2010; Löffler et al. 2010). A systematic nomenclature was introduced in 2000 (Murphy et al. 2000), but many chemokines are still referred to by their previous functional names (Olsnes et al. 2009b).

1.2 Chemokines

Most genes encoding CXC chemokines are located in a common cluster on human chromosome 4q12-21, and most CC chemokine genes are located on 17q11-32 (Christopherson and Hromas 2001). The molecular size is 67–127 amino acids, and chemokines belonging to the same class have a similar monomeric tertiary structure (Fernandez and Lolis 2002). The NH_2-terminal region contains the chemokine receptor binding site (Fernandez and Lolis 2002). CXCL1, CXCL16, and CX3CL1 exist as additional membrane-bound forms at the cell surface, and CX3CL1 then acts as an adhesion molecule (Moser et al. 2004; Allen et al. 2007); these molecular forms have an additional transmembrane domain with a cytoplasmic tail (Johnson et al. 2004) (Table 1).

Chemokines undergo post-translational proteolysis by CD26 and by matrix metalloproteases (MMPs) (Murdoch and Finn 2000; Comerford and Nibbs 2005). Proteolytic cleavage may then lead to activation, no functional alteration, formation

Table 1 Human CCR and CXCR chemokine receptors and their ligands

Chemokine receptor	Ligands
CCR1	CCL3,CCL5, CCL6, CCL7, CCL9–10, CCL13–16, CCL23
CCR2	CCL2, CCL6, CCL7, CCL8, CCL12, CCL13, CCL27
CCR3	CCL5, CCL6, CCL7, CCL8, CCL11, CCL13, CCL15, CCL24, CCL26–28
CCR4	CCL17, CCL22
CCR5	CCL3–5, CCL8, CCL11
CCR6	CCL20
CCR7	CCL19, CCL21
CCR8	CCL1
CCR9	CCL25
CCR10	CCL27, CCL28
CXCR1	CXCL1, CXCL6–8
CXCR2	CXCL1–3, CXCL5–8
CXCR3A	CXCL9–11
CXCR3B	CXCL4, CXCL9–11
CXCR4	CXCL12
CXCR5	CXCL13
CXCR6	CXCL16
CXCR7	CXCL12

References: Fernandez and Lolis (2002), Moser et al. (2004), Allen et al. (2007), Johnson et al. (2004), Tanaka et al. (2005)

of antagonists, or loss of receptor binding. Additional effects of cleavage can be the release of membrane-bound chemokines or a change in the ability to form oligomers.

1.3 Chemokine Receptors

Chemokines from one subfamily bind only to receptors from the corresponding family; CCL chemokines bind to CCR receptors and CXCL chemokines to the CXCR receptors (Laurence 2006; Olsnes et al. 2009b; Dimberg 2010; Maghazachi 2010). Similarly, one receptor may bind several chemokines, although restricted to the same family. Nearly 20 signaling chemokine-binding receptors have been described (Allen et al. 2007). In addition, the Duffy antigen receptor for chemokines (DARC), D6, and CCX-CKR are nonsignaling or Decoy receptors that may be involved in chemokine transport or neutralization (Bonecchi et al. 2010). DARC differs from other receptors by its ability to bind both CXC and CC chemokines, while D6 binds at least 12 CC chemokines that are transported intracellularly and become degraded (Bonecchi et al. 2010).

2 Chemotaxis of Immunocompetent Cells

Chemokines have a general role in local recruitment and retention of immunocompetent cells, and this is also true in hematological malignancies. Three aspects of this recruitment should be emphasized. First, immunocompetent cells often express several chemokine receptors; the NK cells are an example of this as described in detail by Maghazachi in this issue (Maghazachi 2010). Several chemokines can then contribute to the local recruitment of the same immunocompetent cells, and the predominating chemokine seems to differ depending on the biological context. Second, even though anticancer immune reactivity is important especially after allogeneic stem cell transplantation but probably also after autotransplantation and conventional intensive chemotherapy, local recruitment and retention of immunocompetent cells in the cancer cell microenvironment does not necessarily represent an anticancer effect. For example, cancer-specific T cells release hematopoietic growth factors that may stimulate the growth of myeloid malignant cells (Ersvaer et al. 2007; Liseth et al. 2010), and immunocompetent cells may also release proangiogenic mediators and thereby contribute to leukemogenesis/carcinogenesis (Dimberg 2010). Finally, the results from single nucleotide polymorphisms (SNPs) analysis of chemokines as well as other cytokines and immunoregulatory molecules in patients with hematological malignancies clearly illustrate that the chemokines should be regarded as a part of an extensive immunoregulatory network. This genetic influence on immune reactions has been most extensively investigated in patients treated with allogeneic stem cell transplantation, and SNPs then influence the risk of fungal infections, graft vs. host disease (GVHD), and possibly also

leukemia relapse (Löffler et al. 2010). SNPs may also be important for cancer cell migration and thereby for the clinical presentation of hematological malignancies (Kittang and Hildebrandt 2010).

3 Chemokines in Normal and Leukemic Hematopoiesis

3.1 Effects of Chemokines on Normal Hematopoiesis

Bone marrow infiltration of the malignant cells is common in hematological malignancies, and it is mandatory for some of these diseases (Jaffe et al. 2001). Suppression of normal hematopoiesis is therefore common in these diseases and may lead to life-threatening hemorrhages due to thrombocytopenia or severe infections due to neutropenia.

Chemokines can have both stimulatory and suppressive effects on normal hematopoiesis (Jaffe et al. 2001). CXCL4 and XCL1 have stimulatory effects, but suppression seems to be most the common and has been described for several CCL and CXCL chemokines, for example, CCL2, CCL3, CXCL8, CXCL5, and CXCL12 (Kittang et al. 2010). These chemokines can be constitutively released by malignant hematological cells (Bruserud et al. 2007), and the local release may thereby contribute to the general bone marrow failure that is common in patients with bone marrow infiltration of malignant myeloid or lymphoid cells. This seems to be likely in acute myeloid leukemia (AML) (Bruserud et al. 2007) and may also be possible in other malignancies.

3.2 Angiogenesis in the Development of Hematological Malignancies

Angiogenesis is important in the development of solid tumors and also in leukemogenesis with diffuse infiltrations of malignant hematological cells throughout the bone marrow compartment (sometimes referred to as “liquid tumors”) (Hatfield et al. 2005). Increased microvascular density is observed in leukemic bone marrow, and proangiogenic signaling mediated by a wide range of soluble mediators probably contribute to this process (Dimberg 2010; Hatfield et al. 2005). These mediators include several chemokines that can be released by the malignant cells themselves as well as the nonleukemic stromal cells and normal myeloid cells (Olsnes et al. 2009b; Dimberg 2010; Bruserud et al. 2007; Hatfield et al. 2005). The same angioregulatory cytokines seem to be involved in solid tumor and bone marrow angiogenesis (Dimberg 2010), and targeting of angiogenesis may therefore represent a possible therapeutic strategy not only for the solid tumors but also for the hematological malignancies.

There are several chemokine-mediated interactions between carcinogenesis, angiogenesis, and infiltration of immunocompetent cells in the cancer microenvironment. First, infiltrating T cells may release Interferon (IFN)-γ that modulates the release of angioregulatory chemokines; in AML, IFN-γ causes decreased release of proangiogenic CXCL8, whereas the angiostatic CXCL9–11 show increased release (Ersvaer et al. 2007; Kittang et al. 2010). Second, even though cancer-reactive immunocompetent cells usually are regarded to mediate anticancer effects, the cytokine responses of these cells may enhance carcinogenesis/leukemogenesis either directly through the release of hematopoietic growth factors (e.g., GM-CSF and IL-3 release by T cells) or indirectly through the release of proangiogenic cytokines by, for example, activated T cells and macrophages (Dimberg 2010; Ersvaer et al. 2007; Kittang et al. 2010). Third, tumor-infiltrating macrophages may have anticancer effects (Kittang et al. 2010). Cancer-directed immune responses thus do not necessarily mean anticancer immune reactivity.

4 Humoral Immune Responses Against Cytokines and Cytokine Receptors: The Relevance for the Chemokine System

Cancer patients may show humoral immune responses against tumor-associated antigens, that is, antigens with a relatively high expression in the malignant cells (Reuschenbach et al. 2009). Even though chemokines and chemokine receptors can be expressed by malignant cells, autoantibodies against these cancer-associated molecules are relatively uncommon. Anti-CXCL8 has been described in healthy individuals, and increased levels of these autoantibodies have been detected in patients with ovarian cancers (Lokshin et al. 2006). However, no reports on anti-chemokine antibodies in hematological malignancies have been published (Reuschenbach et al. 2009).

Autoantibodies have been detected mainly in solid tumor patients and they seem relatively uncommon in hematological malignancies. Only a few studies have investigated autoantibodies in these patients (Reuschenbach et al. 2009). The studies have included patients suffering from acute leukemia, lymphoma, myeloma, and chronic myeloid leukemia. Usually autoantibodies have been detected only for a minority of patients, and anticytokine or cytokine receptor antibodies have not been described.

Even though induction of autoantibodies against chemokine or chemokine receptors seems to be uncommon in patients with malignant diseases, a few studies indicate that anti-CXCL8 and anti-CCL2 antibodies can be detected in healthy individuals (Lokshin et al. 2006; Leonard 1996). On the other hand, a recent study described granulocyte-macrophage colony-stimulating factor (GM-CSF)-specific antibodies in patients with myeloid malignancies, and the results suggested that these antibodies could be used as markers of disease activity (Sergeeva et al. 2008). In this context, it is not surprising that therapeutic interventions can also induce antibody responses, the best example being the heparin-induced antibody response

directed against the heparin–CXCL4 complex (Sandset 2010). However, this is a rare event that requires specific clinical intervention only for a small minority of heparin-treated patients.

5 The Chemokine System in Clinical Hematology

5.1 Inhibition of Chemokine Responses

Inhibition of chemokine receptors is the most commonly used therapeutic strategy. Inhibition of cell adhesion through CXCR4 inhibition is now used for stem cell mobilization (Calandra et al. 2010), and other possible approaches is to use chemokine-inhibitory agents as antiangiogenic or immunosuppressive drugs. Several SNPs exist in genes encoding chemokines or chemokine receptors (Löffler et al. 2010), and the possibility of individualizing the use of chemokine-targeting therapy therefore has to be considered.

5.1.1 Anticancer Treatment

Chemokines can be important for survival, proliferation, and migration of malignant cells, including malignant myeloid and lymphoid cells. Especially, CXCL12 is important for both normal and malignant immature hematopoietic cells (Kittang et al. 2010; Calandra et al. 2010). CXCR4/CXCL12 seems important for homing of normal and leukemic stem cells to the endosteal and vascular niches in the bone marrow, and CXCR4 inhibition is used for mobilization and harvesting of normal stem cells from peripheral blood (Calandra et al. 2010). The first clinical studies on the use of these agents as anticancer therapy have now been reported (Calandra et al. 2010), and based on the initial phase I/II studies, this therapeutic strategy seems to be safe when used in combination with intensive chemotherapy. Thus, CXCR4 inhibition may become a therapeutic strategy to target leukemic stem cells in the bone marrow.

5.1.2 Immunosuppressive Therapy

The chemokine system is important for migration of immunocompetent cells, including T cells, macrophages, and NK cells (Olsnes et al. 2009b; Maghazachi 2010; Kittang et al. 2010; Bruserud et al. 2007; Calandra et al. 2010). The possible importance of the chemokine system in autoimmune hematological diseases has not been characterized in detail, but both experimental and clinical studies have demonstrated that chemokines are important in the development of GVHD following allogeneic stem cell transplantation (Engelhardt and Crowe 2010). Recent

studies have demonstrated that the risk of acute and chronic GVHD is dependent not only on the degree of antigenic mismatches between donor and recipient, but also on the immunological status of the patient and the balance between pro- and anti-inflammatory immunocompetent cells in the stem cell recipient (Paczesny et al. 2010; Kittan and Hildebrandt 2010). Acute GVHD develops especially in the skin, liver, and gastrointestinal tract (Paczesny et al. 2010). The future treatment of this potentially life-threatening complication should possibly include specific targeting of defined subsets of immunocompetent cells and their migration into the affected organs, and it may then become possible to orchestrate the immune response through targeting of the chemokine system.

5.2 Enhancement of Chemokine Responses

Based on animal studies, systemic administration of several chemokines suppresses normal hematopoiesis and thereby protects myeloid cells against the toxicity of intensive chemotherapy (Kittang et al. 2010). Enhancement of chemokine effects can be achieved by systemic chemokine administration similar to the animal models. Other strategies could be to inhibit chemokine binding to degrading Decoy receptors and thereby increase local chemokine levels, or the use of pharmacological agents that increase local chemokine release (Olsnes et al. 2009a).

5.3 Biomarkers of Cancer

The experience from heparin-induced thrombocytopenia suggests that chemokines can serve as autoantigens (Sandset 2010). Furthermore, several chemokines show increased expression in malignant cells and may therefore function as cancer-associated antigens. Two approaches are thus emerging as possible strategies for the diagnostic use of chemokine expression in cancer cells. First, detection of chemokine mRNA expression as a part of a larger gene expression profile may be used to detect minimal residual disease (Kittang et al. 2010). Alternatively, even though cancer-associated humoral responses against chemokines seem to be uncommon, such responses may be used as a marker of certain malignancies, for example, ovarian cancer (Reuschenbach et al. 2009; Lokshin et al. 2006; Leonard 1996).

6 Conclusion

The chemokine system is important for regulation of viability, proliferation, and migration of normal as well as malignant cells. Studies in Drosophila and zebrafish have in addition demonstrated that chemokines are important for cell migration

during embryogenesis (Mahabaleshwar et al. 2008; Kunwar et al. 2003; Molyneaux et al. 2003), and these animal studies in addition demonstrate that experimental models are available for further detailed studies of the complex roles of the chemokine system in intercellular communication. However, even our present knowledge of the chemokine system allows the conclusion that this system represents a basis for further development of diagnostic tools as well as targeted therapy directed against normal immunocompetent or malignant hematological cells.

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References

Allen SJ, Crown SE, Handel TM (2007) Chemokine: receptor structure, interactions, and antagonism. Annu Rev Immunol 25:787–820

Bonecchi R, Savino B, Borroni EM, Mantovani A, Locati M (2010) Chemokine decoy receptors: structure-function and biological properties. Curr Top Microbiol Immunol (in press)

Bruserud Ø, Ryningen A, Olsnes AM, Stordrange L, Øyan A, Kalland KH, Gjertsen BT (2007) Subclassification of patients with acute myelogenous leukemia based on chemokine responsiveness and constitutive chemokine release by their leukemic cells. Haematologica 92:332–341

Calandra G, Bridger G, Fricker S (2010) CXCR4 in clinical hematology. Curr Top Microbiol Immunol (in press)

Christopherson K, Hromas R (2001) Chemokine regulation of normal and pathologic immune responses. Stem Cells 19:388–396

Comerford I, Nibbs RJ (2005) Post-translational control of chemokines: a role for decoy receptors? Immunol Lett 96:163–174

Dimberg A (2010) Chemokines in angiogenesis. Curr Top Microbiol Immunol (in press)

Engelhardt BE, Crowe JE (2010) Homing in on acute graft-versus-host disease: Tissue-specific T regulatory and Th17 cells. Curr Top Microbiol Immunol (in press)

Ersvaer E, Hampson P, Hatfield K, Ulvestad E, Wendelbo O, Lord JM, Gjertsen BT, Bruserud Ø (2007) T cells remaining after intensive chemotherapy for acute myelogenous leukemia show a broad cytokine release profile including high levels of interferon-gamma that can be further increased by a novel protein kinase C agonist PEP005. Cancer Immunol Immunother 56:913–925

Fernandez EJ, Lolis E (2002) Structure, function, and inhibition of chemokines. Annu Rev Pharmacol Toxicol 42:469–499

Hatfield KJ, Olsnes AM, Gjertsen BT, Bruserud Ø (2005) Antiangiogenic therapy in acute myelogenous leukemia: targeting of vascular endothelial growth factor and interleukin 8 as possible antileukemic strategies. Curr Cancer Drug Targets 5:229–248

Jaffe ES, Harris NL, Stein H, Vardiman JW (2001) Tumors of the haematopoietic and lymphoid tissues. IARC, Lyon

Johnson Z, Power CA, Weiss C, Rintelen F, Ji H, Ruckle T, Camps M, Wells TN, Schwarz MK, Proudfoot AE, Rommel C (2004) Chemokine inhibition – why, when, where, which and how? Biochem Soc Trans 32:366–377

Kittan NA, Hildebrandt GC (2010) The chemokine system – a possible therapeutic target in acute graft versus host disease. Curr Top Microbiol Immunol (in press)

Kittang AM, Hatfield K, Sand KE, Reikvam H, Bruserud Ø (2010) The chemokine network in acute myelogenous leukemia: molecular mechanisms involved in leukemogenesis and their therapeutic implications. Curr Top Microbiol Immunol (in press)

Kunwar PS, Starz-Gaiano M, Bainton RJ, Heberlein U, Lehmann R (2003) Tre1, a G protein-coupled receptor, directs transepithelial migration of Drosophila germ cells. PLoS Biol 1:E80

Laurence AD (2006) Location, movement and survival: the role of chemokines in haematopoiesis and malignancy. Br J Haematol 132:255–267

Leonard EJ (1996) Plasma chemokine and chemokine-autoantibody complexes in health and disease. Methods 10:150–157

Liseth K, Sjo M, Paulsen K, Bruserud Ø, Ersvaer E (2010) Early pre-engraftment functional *in vitro* responsiveness of T lymphocytes in allotransplanted acute leukemia patients: proliferation and release of a broad profile of cytokines, possibly predictive of graft versus host disease. Eur Cytokine Netw 21:40–49

Löffler J, Ok M, Oliver Morton C, Mezger M, Einsele H (2010) Genetic polymorphisms in the cytokine and chemokine system – their possible importance in allogeneic stem cell transplantation. Curr Top Microbiol Immunol (in press)

Lokshin AE, Winans M, Landsittel D, Marrangoni AM, Velikokhatnaya L, Modugno F, Nolen BM, Gorelik E (2006) Circulating IL8 and anti-IL8 autoantibodies in patients with ovarian cancer. Gynecol Oncol 102:244–251

Maghazachi AA (2010) Role of chemokines in the biology of natural killer cells. Curr Top Microbiol Immunol (in press)

Mahabaleshwar H, Boldajipour B, Raz E (2008) Killing the messenger: the role of CXCR7 in regulating primordial germ cell migration. Cell Adh Migr 2:69–70

Molyneaux KA, Zinszner H, Kunwar PS, Schaible K, Stebler J, Sunshine MJ, O'Brien W, Raz E, Littman D, Wylie C, Lehmann R (2003) The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. Development 130:4279–4286

Moser B, Wolf M, Walz A, Loetscher P (2004) Chemokines: multiple levels of leukocyte migration control. Trends Immunol 25:75–84

Murdoch C, Finn A (2000) Chemokine receptors and their role in inflammation and infectious diseases. Blood 95:3032–3043

Murphy PM, Baggiolini M, Hébert CIF, CA HR, Matsushima K, Miller LH, Oppenheim JJ, Power CA (2000) International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Rev 52:145–176

Olsnes AM, Ersvaer E, Ryningen A, Paulsen K, Hampson P, Lord JM, Gjertsen BT, Kristoffersen EK, Bruserud Ø (2009a) The protein kinase C agonist PEP005 increases NF-kappaB expression, induces differentiation and increases constitutive chemokine release by primary acute myeloid leukaemia cells. Br J Haematol 145:761–774

Olsnes AM, Hatfield KJ, Bruserud Ø (2009b) The chemokine system and its contribution to leukemogenesis and treatment responsiveness in patients with acute myeloid leukemia. J BUON (Suppl) 1:S131–S140

Paczesny S, Hanauer D, Sun Y, Reddy P (2010) New perspectives on the biology of acute GVHD. Bone Marrow Transplant 45:1–11

Reuschenbach M, von Knebel DM, Wentzensen N (2009) A systematic review of humoral immune responses against tumor antigens. Cancer Immunol Immunother 58:1535–1544

Sandset PM (2010) Immunobiology of Heparin-induced thrombocytopenia. Curr Top Microbiol Immunol (in press)

Sergeeva A, Ono Y, Rios R, Molldrem JJ (2008) High titer autoantibodies to GM-CSF in patients with AML, CML and MDS are associated with active disease. Leukemia 22:783–790

Tanaka T, Bai Z, Srinoulprasert Y, Yang B, Hayasaka H, Miyasaka M (2005) Chemokines in tumor progression and metastasis. Cancer Sci 96:317–322

Part II
Experimental Hematology

Chemokine Decoy Receptors: Structure–Function and Biological Properties

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Contents

Abstract Chemokines induce cell migration through the activation of a distinct family of structurally related heterotrimeric G protein-coupled receptors (GPCR). Over the last few years, several receptors in this family that recognize chemokines but do not induce cell migration have been identified. These "atypical" chemokine receptors are unable to activate transduction events that lead directly to cell migration, but appear nonetheless to play a nonredundant role in the control of leukocyte recruitment at inflammatory sites and in tumors by shaping the

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chemoattractant gradient, either by removing, transporting, or concentrating their cognate ligands.

Abbreviations

GPCR	G-protein-coupled receptors
CCL	CC chemokine ligand
CXCL	CXC chemokine ligand
CCR	CC chemokine receptor
CXCR	CXC chemokine receptor
DARC	Duffy antigen receptor for chemokines
HCV	Hepatitis C virus
LPS	Lipopolysaccharide
MMP-9	Metallo protease 9

1 The Chemokine Universe

Cell migration is a key element in the ontogenesis of lymphoid tissues in normal and pathological conditions, in the patrolling of body compartments by leukocytes, and in the activation and orientation of innate and adaptive immunity. Different classes of soluble mediators can elicit directional migration of leukocytes. The main mediators of leukocyte trafficking are molecules that interact with rhodopsin-like, seven transmembrane domain, G-protein-coupled receptors (GPCR) and include bacteria-derived formyl-peptides, the complement fragments C5a and C3a, bioactive lipids (sphingosine, leukotrienes, and platelet-activating factor), and chemokines. Chemokines, whose name has in fact been derived by the condensation of *chemo*tactic cyto*kines*, are functionally related small secreted proteins structurally characterized by a conserved protein structure called chemokine scaffold, which is strictly dependent on the presence of two conserved disulfide bonds connecting cysteine residues. CC chemokines, which have the first two cysteine residues in adjacent position, and CXC chemokines, which have cysteine residues separated by a single intervening amino acid, account altogether for the large majority of molecules (25 and 15, respectively). The C subfamily, whose members have a single cysteine residue in the amino-terminus, and CX3C chemokines, with three residues separating the cysteine tandem, account only for a small minority of molecules (2 and 1, respectively) (Allen et al. 2007).

The structure-based classification of chemokines is reflected in the classification of chemokine receptors, which display in most cases significant ligand promiscuity among members of a defined subfamily, but are strictly restricted to members of

that given subfamily. Thus, the ten CC chemokine receptors (CCR1 to 10) and the six CXC chemokine receptors (CXCR1 to 6) recognize only CC and CXC chemokines, respectively. Similarly, the only receptors for C and CX3C chemokines (XCR1 and CX3CR1, respectively) are restricted to their respective ligands. All chemokine receptors are GPCR constituted by single polypeptide chain with three extracellular and three intracellular loops, an acidic amino-terminal extracellular domain involved in ligand binding, and a serine/threonine-rich intracellular carboxy-terminal domain. The external interface contributes to the ligand-recognition specificity, while conserved transmembrane sequences, the cytoplasmic loops, and the carboxy-terminal domain are involved in receptor signaling and internalization (Murphy et al. 2000).

The chemokine system is highly promiscuous, presumably to provide flexibility and specificity in leukocyte trafficking, and pleiotropic, with a given chemokine acting on different leukocyte populations to coordinate the recruitment of different but functionally related cells (Mantovani 1999). In general, polymorphonuclear neutrophils (PMN) are major target of ELR^{+}-CXC chemokines acting through CXCR1 and CXCR2; monocytes are mainly recruited by CC chemokines acting through CCR1, CCR2, and CCR5; Th1 and natural killer (NK) cells, major players of type 1 inflammation, are mainly responsive to ELR^{-}-CXC chemokines through CXCR3 and to CX3CL1 acting through CX3CR1; Th2 and eosinophils, associated to type 2 inflammatory responses, are attracted through the action of CCR3 and CCR4 agonists.

It is important to realize that though chemokines' major function is the coordination of leukocyte recruitment in physiologic and pathologic conditions, they also mediate other biological activities, including regulation of cell differentiation and proliferation, survival, and senescence. This is of particular relevance considering that chemokine receptors are expressed in several normal and malignant non-leukocyte cell types (Bonecchi et al. 2009; Charo and Ransohoff 2006).

2 Regulation of the Chemokine System

Chemokine's biological activities are regulated at several levels. At the ligand level, chemokines may be classified according to their production in homeostatic (i.e., produced constitutively) and inflammatory (i.e., produced in response to inflammatory or immunological stimuli) conditions (Mantovani 1999). Chemokines are also target of post-translational modifications, which influence their functional properties, including processing at the amino- and carboxy-terminus by proteases (Proost et al. 2006) and cytrullination (Proost et al. 2008).

The action of different chemokines can also be controlled at the receptor level. Chemokine receptor function has been shown to be context-dependent. Under concomitant exposure to pro- and anti-inflammatory stimuli, such as lipopolysaccharide (LPS) and IL-10, inflammatory chemokine receptors (such as CCR2) can undergo uncoupling from G proteins and maintain the ability to

internalize and degrade the ligand, both *in vitro* and *in vivo* (D'Amico et al. 2000). Under these conditions, chemokine receptors in fact act as "false receptors," in that they are structurally identical to signaling receptors but behave as decoys. Indeed, chemokine receptors mediate significant ligand sequestration as part of their normal signaling function, as demonstrated by the increase in chemokine levels observed in chemokine receptors knock-out animals (Cardona et al. 2008).

Finally, a nonredundant role in tuning chemokine's biological properties is mediated by "atypical" chemokine receptors (Mantovani et al. 2006), which are considered chemokine decoy receptors being unable to directly mediate cell migration, but tune signaling receptors' activity by clearance, transport, or presentation of the ligand.

3 Chemokine Decoy Receptors

Chemokine decoy receptors recognize distinct and complementary sets of ligands and are strategically expressed in different cellular contexts (Fig. 1). On the basis of the absence of evidence of signaling properties, these receptors were initially called "silent" (Mantovani et al. 2001). More recent evidence has shown that, despite being unable to activate conventional signaling events, they can activate poorly characterized signaling pathways that lead to ligand internalization, degradation, or transport (Mantovani et al. 2006). Detailed structure–function analysis of this

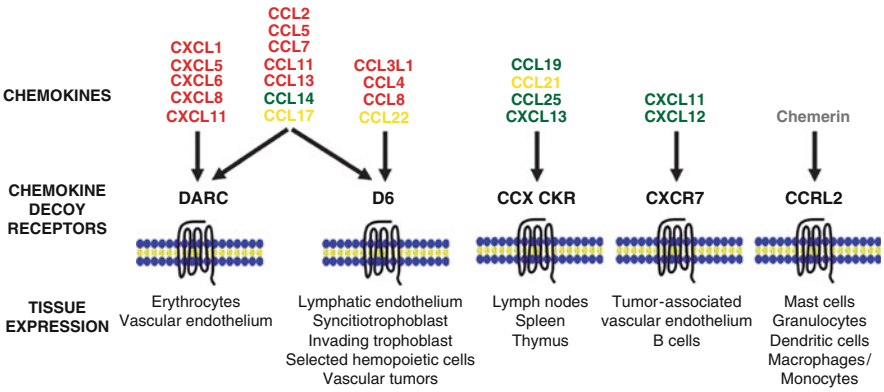

Fig. 1 Chemokine decoy receptors ligand specificity and tissue distribution. D6 and DARC are mainly expressed on the endothelium (lymphatic and vascular, respectively); DARC is also expressed by erythrocytes, and some evidence of D6 expression by leukocytes has also been provided. CCX-CKR is expressed by various tissues. CXCR7 is expressed by lymphocytes and by tumor-associated vascular endothelium. CCRL2 is expressed by myeloid-derived leukocytes. Chemokines are color-coded as pro-inflammatory (*red*), homeostatic (*green*), and those with mixed function (*yellow*)

receptor subfamily is not available, but it is interesting to note that structural determinants supporting $G_{\alpha i}$ activation, a key signaling event in cell migration, are not conserved in these receptors. Other general characteristics that distinguish this class of receptors from other chemokine receptors are unusual expression patterns and broad binding profiles.

The subfamily of chemokine decoy receptors includes D6 (Bonini et al. 1997; Nibbs et al. 1997b), Duffy Antigen Receptor for Chemokines (DARC) (Horuk et al. 1993), CCRL2 (Fan et al. 1998), CCX CKR (Gosling et al. 2000), and CXCR7 (Boldajipour et al. 2008).

3.1 D6

D6 has been the first atypical chemokine receptor functionally identified as a decoy (Fra et al. 2003). Cloned from placenta (Bonini et al. 1997) and hematopoietic stem cells (Nibbs et al. 1997b), it is located in the CCR cluster within the 3p21.3 region of the human genome (Maho et al. 1999). D6 binds most of the inflammatory CC chemokines (agonists of CCR1 through CCR5, see Fig. 1). Nevertheless, D6 has some binding selectivity, in that it does not recognize homeostatic CC chemokines (Nibbs et al. 1997b) and, among inflammatory CC chemokines, it degrades the active forms of CCL22 and CCL14 but not their amino-terminal CD26-processed inactive forms (Bonecchi et al. 2004; Savino et al. 2009). D6 is expressed at high levels by endothelial cells of lymphatic afferent vessels in the skin, gut, and lungs (Nibbs et al. 2001), and in the placenta, where it is present on invading trophoblast cells and on the apical side of syncytiotrophoblast cells (Martinez de la Torre et al. 2007). D6 is also expressed at very low levels by circulating leukocytes (McKimmie and Graham 2006). Following initial results to the contrary (Nibbs et al. 1997a), it is now clear that neither the human nor the murine D6 sustain signaling and functional activities that are typically observed after chemokine receptor triggering by ligand binding, such as calcium fluxes and chemotaxis (Fra et al. 2003; Martinez de la Torre et al. 2005). In all cells tested so far, including the physiologically relevant milieu of lymphatic endothelium (Fra et al. 2003) and trophoblast cells (Martinez de la Torre et al. 2007), D6 does not mediate chemokine transfer through the cell monolayer and instead mediates chemokine degradation. $D6^{-/-}$ mice have been generated, and the data obtained in different animal models are consistent with the role of D6 as a chemokine scavenger *in vivo*. $D6^{-/-}$ mice develop exacerbated inflammatory responses in different experimental diseases sustained by increased levels of inflammatory CC chemokines detected both locally and in draining lymph nodes, which may also result in some conditions in a defective-specific immune response and inflammation-driven tumor promotion (see below). In conclusion, *in vitro* and *in vivo* data strongly support a decoy function for D6, which controls tissue inflammation by acting as a chemokine scavenger on lymphatic vessels.

3.2 DARC

Originally described as the erythrocyte receptor for malaria parasites (Miller et al. 1976), DARC was later identified as the erythrocyte receptor for the chemokine CXCL8 (Horuk et al. 1993). It was subsequently demonstrated that DARC has a promiscuous chemokine-binding profile, interacting with 11 inflammatory chemokines of both the CXC and the CC subfamilies but not with homeostatic chemokines (Gardner et al. 2004), and among CXC chemokines, DARC selectively binds angiogenic ELR^+ chemokines (CXCL1, CXCL3, CXCL5, CXCL6, and CXCL8) but not angiostatic ELR^- chemokines (CXCL9 and CXCL10). The Fy gene that encodes DARC is located outside the CCR and CXCR clusters in the 1q22–q23 region of chromosome 1 (Pruenster and Rot 2006) and has likely evolved independently from other chemokine receptors. Indeed, at the structural level, DARC shows only a minor sequence homology with other chemokine receptors (40% similarity) and lacks structural determinant required for G-protein coupling (Chaudhuri et al. 1993). Consistently with this, after chemokine binding, it does not support ligand-induced signaling or migration (Neote et al. 1994), although cells expressing DARC can internalize the ligand (Peiper et al. 1995).

DARC expression in red blood cells decreases during maturation, being higher on reticulocytes than on older cells in peripheral blood (Liu et al. 2010). DARC function is also necessary for osteoclast differentiation (Edderkaoui et al. 2007). Most individuals of African descent (>95% Africans in malaria endemic regions, 70% of African-Americans) lack expression of DARC by erythrocytes, which was presumably the result of a selective advantage provided by resistance against certain forms of parasite infection (Miller et al. 1976). The lack of DARC in these individuals is the principal genetic determinant for the benign ethnic leukopenia (primarily neutropenia) (Reich et al. 2009), suggesting that DARC function is necessary for a correct hematopoiesis.

Irrespective of ethnicity and expression by erythrocytes, DARC is expressed by endothelial cells (Peiper et al. 1995), suggesting that this molecule has a role in vascular biology. DARC-expressing endothelial cells line postcapillary venules and veins in many organs, including high endothelial venules in lymph nodes. Venular expression of DARC has been reported in diverse normal tissues, such as skin, kidney, lung, brain, thyroid, and spleen, as well as in inflamed tissues, such as the rheumatoid joint synovium, psoriatic skin, various kidney diseases, and lungs with suppurative pneumonia (Hadley and Peiper 1997; Rot 2005).

DARC appears to regulate chemokine bioavailability and, consequently, leukocyte recruitment through two distinct mechanisms: when expressed in endothelial cells, it sustains the abluminal to luminal transcytosis of tissue-derived chemokines and their subsequent presentation to circulating leukocytes (Pruenster et al. 2009); when expressed in erythrocytes, it acts as a sink of circulating chemokines.

3.3 CCRL2

CCRL2 is located in the CCR cluster on chromosome 3p21–23 (Samson et al. 1996) and shares over 40% amino-acid identity with CC chemokine receptors. Like other decoy receptors, it lacks a conserved DRY motif (Fan et al. 1998). This receptor is expressed by monocytes, neutrophils, and DCs, and, in all cell types, it is highly upregulated by LPS-driven stimulation (Migeotte et al. 2002). One study has reported functional activities (chemotaxis and calcium fluxes) after CCRL2 engagement by CCL2, CCL5, CCL7, and CCL8, although no evidence for a direct ligand–receptor interaction was provided (Biber et al. 2003). Using CCRL2-transfected cells, we and others have failed in confirming CCRL2 recognition of these or other CC and CXC chemokines (Bonecchi et al. unpublished data). CCRL2 has been recently shown to bind the chemoattractant protein chemerin with high affinity (Zabel et al. 2008), but unlike the other known chemerin receptor, ChemR23 (Wittamer et al. 2003), it neither induce cell migration nor is it internalized after ligand engagement. Using truncated forms of chemerin, the authors demonstrated that CCRL2 binds the N-terminal domain of chemerin, a portion of the molecule that is not involved in binding and activation of ChemR23. Furthermore, CCRL2-expressing cells preloaded with chemerin induced functional responses in cells transfected with ChemR23, indicating that chemerin is still functional after CCRL2 binding. These results have lead to the proposal of CCRL2 as a receptor presenting its chemoattractant ligand to functional receptors.

3.4 CCX-CKR

The chemokine receptor CCX-CKR is located in the CCR cluster at position 3q22.1. It is widely expressed in several tissues, T cells, and immature dendritic cells (DC). It binds the CC chemokines CCL19 and CCL21, and weakly binds also CXCL13 (Gosling et al. 2000). As other decoy receptors, CCX-CKR presents modifications just after the DRY motif in the second intracellular loop and does not transduce conventional signaling activity after ligand engagement (Mantovani et al. 2001; Townson and Nibbs 2002). Conversely, cells transfected with CCX-CKR degrade CCL19 with very high efficiency (Comerford et al. 2006). Binding their functional receptors, the ligands of CCX-CKR mediate trafficking of naïve T cells, DC, B cells, and follicular helper T cells to and within lymphoid organs, and have a major role in the ontogeny of lymphoid organs and extranodal lymphoid tissues, which characterize chronic autoimmune disorders, such as rheumatoid arthritis and multiple sclerosis (Muller and Lipp 2003). Indeed, although CCL19, CCL21, and CXCL13 are homeostatic chemokines secreted constitutively by DC and monocytes, the production of CCL19 and CCL21 is also augmented by inflammatory signals and CXCL13 expression is induced by the anti-inflammatory cytokine IL-10 (Perrier et al. 2004; Sallusto et al. 1999).

Thus, it is tempting to speculate that CCX-CKR may play a role in the homeostatic and, perhaps more importantly, in the regulated trafficking of lymphocytes and DC in inflammatory and autoimmune conditions in which lymphoid neogenesis occurs. Information from gene-modified mice will be required to put this hypothesis to a test.

3.5 CXCR7

The CXCR7 chemokine receptor, previously known as RDC1, possesses high sequence similarity with other known chemokine receptors, and its gene is located on chromosome 2 in close proximity to the *CXCR1*, *CXCR2*, and *CXCR4* genes (Infantino et al. 2006) in the human genome. CXCR7 binds the chemokines CXCL12 and CXCL11 (Balabanian et al. 2005), and conflicting results on its ability to induce conventional signaling have been published. It was proposed that CXCR7 is functional only when it dimerizes with other chemokine receptor partners such as CXCR4, enhancing (Hartmann et al. 2008; Sierro et al. 2007) or inhibiting CXCL12-induced signaling (Levoye et al. 2009), but recent publications demonstrate that CXCR7 in several cell types is a signaling receptor as evidenced by phosphorylation of MAPKp42/44 (Hartmann et al. 2008) or Akt (Wang et al. 2008). In this respect, it is interesting to note that important functional signatures of signaling chemokine receptor, such as a DRY motif at the boundary of third transmembrane helix and the second intracellular loop, a CxNPxxY sequence in the seventh transmembrane domain, and four conserved cysteine residues in the extracellular segments, are present in CXCR7.

CXCR7 is poorly expressed on normal somatic cells, but it is elevated on transformed cells and during embryonic development in both human and murine tissues (Thelen and Thelen 2008). During development, CXCR7 is expressed on emerging blood vessels in mice, and $CXCR7^{-/-}$ mice are born with ventricular septal defects and semilunar heart valve malformation that lead to perinatal lethality while their hematopoiesis is normal (Sierro et al. 2007). In zebrafish, CXCR7 expression has been detected in somatic cells, and a major function of CXCR7 in this context appears to be to internalize and sequester CXCL12, thus enhancing the dynamics of CXCL12 concentration changes required for proper migration of primordial germ cells (Boldajipour et al. 2008). In the hematopoietic system, CXCR7 has been reported to be expressed on PMN, monocytes, and B cells, while its expression on T lymphocytes is still debated (Balabanian et al. 2005; Hartmann et al. 2008; Infantino et al. 2006; Sierro et al. 2007). Now it is clear that, interfering with CXCL12 activity, CXCR7 plays a nonredundant role in development and in tumors (see below). However, the mechanism of action of CXCR7 needs to be further elucidated to understand if it acts as a CXCL12 scavenger or it interferes with CXCR4 signaling through heterodimerization or competition with signaling molecules (Maksym et al. 2009).

4 Chemokine Decoy Receptors Mechanism of Action

A major role in the biological properties of chemokine decoy receptors relies on their intracellular trafficking properties, which drive continuous chemokine uptake, transport, or concentration.

4.1 Receptor Internalization

Chemokine decoy receptors have been described to internalize through both clathrin-coated pits and caveolae (Fig. 2). The clathrin-mediated pathway has been demonstrated for D6 through a mechanism that is dynamin I-, Rab5- (Bonecchi et al. 2008), and β-arrestin-dependent (Galliera et al. 2004), and for CXCR7 (Borroni unpublished observations). Conversely, overexpression of wild-type caveolin-1 strongly suggests that CCX-CKR endocytosis uses caveolae (Comerford et al. 2006) through a pathway that, while requiring dynamin, does not need β-arrestins- or clathrin-coated pits. DARC is also targeted into caveolae after being internalized into polarized cells (Pruenster et al. 2009).

Like the chemokine receptors CXCR3 and CXCR4 and the viral chemokine receptors US28 and ORF74, D6 and CCX-CKR undergo constitutive ligand-independent

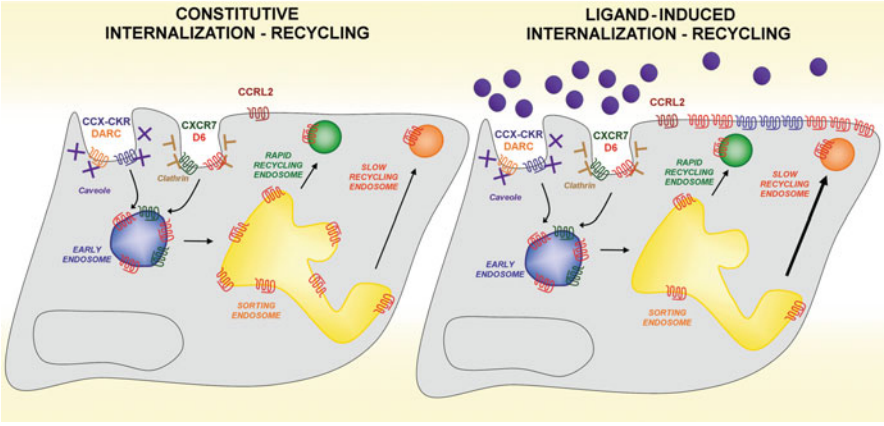

Fig. 2 Chemokine decoy receptors trafficking. D6 and CXCR7 internalize through clathrin-coated pits while DARC and CCX-CKR internalize through caveolae. D6 and CCX-CKR undergo a constitutive internalization followed by recycling in the absence of ligand (*left*). CCRL2 is not internalized even after ligand engagement. After being internalized, receptors may be recycled to the plasma membrane through the rapid (Rab4) and slow (Rab11) recycling endosomes. Detailed information about intracellular pathways are available only for D6 that after being internalized into clathrin-coated pits vesicles are transported to Rab5-positive early endosomes through a dynamin-dependent process. After chemokine engagement, D6 and CCX-CKR upregulate their expression on the cell membrane (*right*)

internalization (Comerford et al. 2006; Galliera et al. 2004). Owing to constitutive internalization, D6 is mainly located in intracellular endosomes and barely detectable on cell surface (Blackburn et al. 2004). Also, CXCR7 has been found predominantly in intracellular compartments colocalized with LAT, a lymphocyte signaling adaptor enriched in the inner leaflet of the plasma membrane and partially colocalized with the early endosomal marker EEA1 (Hartmann et al. 2008). CXCR7 is internalized after CXCL12 or CXCL11 binding in lymphocytes (Balabanian et al. 2005). Conversely, CCRL2 is predominantly expressed on cell surface and a minor pool is present within the cytoplasm. This receptor is not internalized either in the absence or in the presence of the ligand (Zabel et al. 2008).

4.2 Recycling

D6 is constitutively associated with both early (Rab4/5) and recycling endosomes (Rab11) (Bonecchi et al. 2008) but not with lysosomes (Weber et al. 2004). Differently from signaling chemokine receptors, after chemokine engagement, D6 does not decrease its membrane expression but optimizes its degradative activity by increasing its expression on cell surface through a Rab11-dependent mechanism (Bonecchi et al. 2008). Once internalized, the chemokine dissociates from the receptor, and it is targeted to degradation while the receptor is recycled back to the plasma membrane through both rapid and slow recycling pathways, with mechanisms that are strictly dependent on cytoskeleton dynamics (Borroni unpublished observation). A similar ligand-dependent upregulation has been described for CCX-CKR (Comerford et al. 2006), though in this case the redistribution mechanism was not elucidated. Conversely, CXCR7, which is mainly detected in early endosomes (Hartmann et al. 2008), decreased its membrane expression after chemokine stimulation. Thus, it is tempting to speculate that some signal events activated upon ligand engagement might be a prominent sign for receptor cycling events rather than the sign of the typical activation of a chemokine receptor.

4.3 Structural Motifs and Trafficking Adapters

Conventional chemokine receptors use ligand-driven signals to direct occupied receptors to the endocytic machinery through the interaction with β-arrestins, a response accompanied by reduced surface receptor levels and desensitization of remaining surface receptors to further stimulation (Shenoy and Lefkowitz 2003). As mentioned, chemokine decoy receptors have dispensed with signaling due to the presence of altered structural determinants. Nevertheless, association with β-arrestins seems to play a major role in receptor internalization and recycling (Galliera et al. 2004). CXCR7 interacts with β-arrestin in basal conditions, and CXCL11 or

CXCL12 engagement significantly enhanced this interaction that is maintained on endosomes and other intracellular vesicular compartments. CXCR7 interaction with β-arrestin 2 is necessary for CXCL12 uptake from the extracellular space (Luker et al. 2009). The association of D6 with β-arrestin in the absence of ligand is still debated. Galliera et al. proposed that, as for CXCR7, D6 retains the ability to associate β-arrestin 1 and 2 in basal condition (Galliera et al. 2004), and this interaction is required for its constitutive internalization. Conversely, McCulloch et al. demonstrated that relocalization of β-arrestins is not required for D6 internalization but the receptor uses both β-arrestins (McCulloch et al. 2008). Despite the differences, both groups agree that D6 has the potential to constitutively drive the relocalization of β-arrestins within the cytoplasm through a mechanism that is still unknown.

5 Chemokine Decoy Receptors in Action

5.1 Role in Inflammation

Despite the fact that chemokine decoy receptors are structurally unable to support cell migration, it is now well established that they play a nonredundant role in inflammatory responses. They actively participate in the formation of chemotactic gradients removing, transcytating or concentrating chemokines, controlling leukocyte extravasation from the blood vessels to the inflamed tissue, and leukocyte traffic to lymph nodes (Fig. 3). Attenuation of the severity of inflammation by means of chemokine scavenging was demonstrated *in vivo* for D6 by the use of various animal models in several organs. Compared to wild-type mice, $D6^{-/-}$ mice developed exaggerated inflammation, characterized by aberrant leukocyte infiltration and neovascularization due to increased levels of inflammatory CC chemokines, in the skin after phorbol ester application (Jamieson et al. 2005) or complete Freund's adjuvant subcutaneous injection (Martinez de la Torre et al. 2005). In placenta, D6 expressed by syncytiotrophoblast cells reduced inflammation-induced fetal loss in mice (Martinez de la Torre et al. 2007), and loss in D6 immunoreactivity was observed in arresting vs. viable littermate attachment sites in porcine uterus (Wessels et al. 2007). D6 controls inflammation also in the liver, as demonstrated by murine model of acute injury by toxic agents (Berres et al. 2009). In agreement with this murine experimental system, a significant correlation was found between two single nucleotide polymorphisms and liver inflammation in a cohort of HCV-infected patients, even if the functional relevance of these D6 variants was not investigated (Wiederholt et al. 2008). Concerning colon inflammation, conflicting results were published. Bordon et al. using the dextran sodium sulfate-induced model of colitis have found that $D6^{-/-}$ mice are protected. Unexpectedly, they found that this protection is not due to differences in chemokine levels but due to enhanced production of IL-17A secreted by gamma delta T cells in the lamina

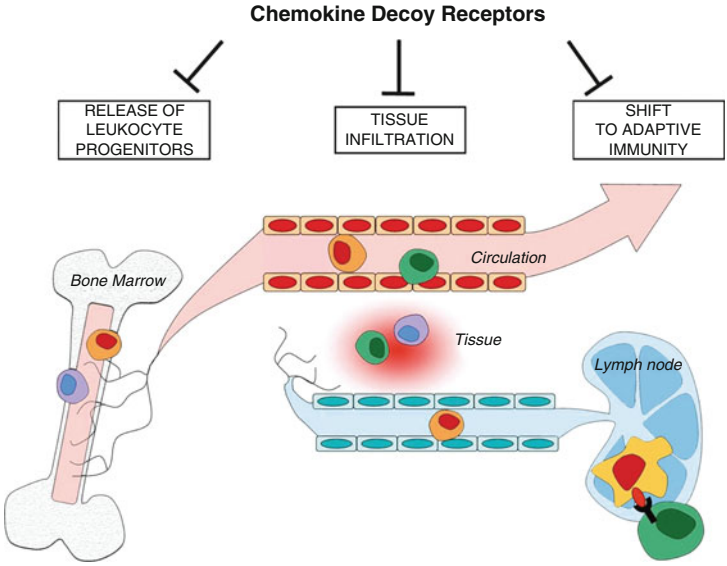

Fig. 3 Chemokine decoy receptors functions in peripheral tissues. Chemokine decoy receptors expressed on blood and lymphatic vessels cooperate in a coordinated action for the control of local inflammatory reactions and adaptive immunity negatively acting on bone marrow leukocyte recruitment, leukocyte extravasation, and trafficking to lymph nodes

propria of $D6^{-/-}$ compared to wild-type mice (Bordon et al. 2009). On the contrary, using the same experimental system, Vetrano et al. found that $D6^{-/-}$ mice display higher levels of several pro-inflammatory chemokines compared to wild-type mice, resulting in increased inflammation. By the use of bone marrow cells' adoptive transfer, they have demonstrated that the protective effect is exerted by D6 expressed by stromal/lymphatic cells (Vetrano et al. 2010). In spite of drawing opposite conclusions from the murine model, both groups found that D6 is expressed in the resting colon predominantly by stromal cells and it is up-regulated in colitic mice and in human colon samples of inflammatory bowel disease patients. D6 was also found abundantly expressed by lymphatic endothelial cells in the lung (Nibbs et al. 2001), and in an allergen-induced airway disease model, $D6^{-/-}$ mice showed increased inflammation compared to wild-type mice (Whitehead et al. 2007). Similarly, $D6^{-/-}$ mice challenged with intranasal administration of low doses of Mycobacterium tuberculosis rapidly die because of a strong local and systemic inflammatory response that give rise to liver and kidney damage (Di Liberto et al. 2008). Interestingly, in certain conditions, the uncontrolled local inflammation observed in $D6^{-/-}$ mice has been shown to impair the development of an appropriate specific immune response. In an encephalomyelitis model based on subcutaneous immunization with the myelin oligodendrocyte glycoprotein-derived peptide 35–55 in complete Freund's adjuvant, the absence of D6 led to an increased tissue inflammation, with local "trapping" of $CD11c^{+}$ dendritic-like

cells causing a blunted adaptive immune response (T-cell proliferation and IFN-γ production) and protection from disease development. However, $D6^{-/-}$ mice showed increased susceptibility to disease when the impairment in adaptive immune response was by-passed by effector lymphocytes' adoptive transfer (Liu et al. 2006).

While the role of D6 as a chemokine scavenger and negative regulator of inflammation is well assessed, the role of DARC in inflammation is still a matter of debate, possibly because this receptor may exert different functions in different cellular contexts. It is well assessed that DARC expressed on erythrocytes modulates chemokine bioavailability by acting as a chemokine scavenger (Darbonne et al. 1991) and as a long-term reservoir of chemokines that prevents their loss from blood into distant organs and tissues (Schnabel et al. 2010). In agreement with this, in a murine model of lung inflammation, DARC expressed by erythrocytes limits lung injury, controlling the distribution and presentation of chemokines that bind CXCR2 (Reutershan et al. 2009), and chemokines disappear from the circulation more rapidly when injected into $DARC^{-/-}$ mice as compared to wild-type animals (Darbonne et al. 1991; Hadley and Peiper 1997). Furthermore, using a systemic endotoxemia model followed by erythrocytes transfusion, it was demonstrated that expression of DARC by red cells reduce lung inflammation (Mangalmurti et al. 2009). Conversely, DARC over-expression on endothelial cells in a transgenic mouse model resulted in increased leukocyte extravasation *in vivo* (Horton et al. 2007), and DARC expression on cell monolayer results in enhanced chemokine-induced leukocyte transmigration *in vitro*. However, the proinflammatory role of DARC as a chemokine transporter is still lacking strong genetic evidence, and the use of $DARC^{-/-}$ mice lacking expression on both erythrocytes and endothelial cells has given rise to conflicting results. LPS treatment resulted in increased neutrophil infiltrate in $DARC^{-/-}$ mice (Dawson et al. 2000), while a different group reported opposite results in a similar experimental setting (Luo et al. 2000). In models of acute renal failure, $DARC^{-/-}$ mice have better renal function than the wild-type littermates due to reduced PMN infiltrate (Zarbock et al. 2007), while in models of prolonged renal inflammation, $DARC^{-/-}$ have increased inflammation at early time points and similar renal injury at later time points (Vielhauer et al. 2009). Finally, Duffy negative individuals (with $DARC^{-}$erythrocytes and $DARC^{+}$ endothelial cells) have a lower mean white blood cell and PMN count (Reich et al. 2009) that correlated with a survival advantage in HIV-infected persons (Kulkarni et al. 2009), and have delayed graft function and increased graft failure following kidney transplantation (Mange et al. 2004), suggesting that DARC expressed by erythrocytes may be protective for kidney inflammation.

The role of CCRL2 in inflammation has been investigated in an atopic allergy model using $CCRL2^{-/-}$ mice, which showed reduced inflammation compared to wild-type littermates when sensitized with a low dose of antigen-specific IgE (Zabel et al. 2008). Experiments using mast cell-deficient mice engrafted with either wild-type or CCRL2-null mast cells showed that the defect was due to the lack of CCRL2 expression by mast cells. These data indicate that CCRL2 expressed on mast cells play a proinflammatory role, possibly acting as glycosaminoglycans

(Proudfoot 2006) in the concentration and presentation of ligands to conventional signal-transducing receptors.

5.2 Role in Tumor Biology

Chemokines are a key component of cancer-related inflammation and are downstream of genetic events that cause neoplastic transformation and affect tumor progression in multiple pathways (Mantovani et al. 2010). Expression of chemokine decoy receptors in the tumor might have profound consequences on their biology, affecting the amounts of intra-tumoral chemokines. Here the preliminary data available in the tumor biology context for DARC, D6, and CXCR7 are reported.

DARC binds both angiogenic ELR$^+$-CXC chemokines, important in tumor angiogenesis, and inflammatory CC chemokines that recruit immune cells that sustain tumor growth. DARC is expressed by several human tumors, including erythroleukemia (Horuk et al. 1994), glioblastoma (Desbaillets et al. 1997), hemangiosarcoma (Tang et al. 1998), and breast carcinoma, in which a negative relationship was found between DARC expression and lymph nodes metastasis, estrogen receptor status, and poor survival (Wang et al. 2006). Experimental tumor models using tumor cell lines over-expressing DARC have shown increased tumor necrosis and decreased angiogenesis and metastasis, associated with decreased CCL2 and MMP-9 levels in the implanted tumors (Addison et al. 2004). Similarly, when melanoma tumor cell lines were injected in transgenic mice over-expressing DARC in endothelial cells, their growth was inhibited (Horton et al. 2007). Explanted tumors displayed enhanced leukocyte infiltration but reduced vascularization, while over-expression of CXCR2 had the reverse effect on tumor angiogenesis and growth. Since prostate cancer has a significantly higher incidence in African-Americans, which in great majority lack DARC erythroid expression (Lentsch 2002), and because clinical data indicate that angiogenic ELR$^+$-CXC chemokines contribute to the pathogenesis of prostate cancer, the role of DARC in this tumor has been the object of intense investigation (Waugh et al. 2008). In a mouse model of spontaneous prostate cancer, the absence of DARC did not modify tumor incidence but was correlated with enhanced tumor growth and levels of angiogenic chemokines (Shen et al. 2006), suggesting that DARC clears angiogenic CXC chemokines from the prostate tumor microcirculation. The molecular mechanism by which DARC exerts its protective effect on tumor growth has been suggested to be mediated by a DARC interactor, the KAI1/CD82 tetraspanin protein (Bandyopadhyay et al. 2006). KAI1/CD82 is down-regulated in several tumors, and its down-modulation in epithelial cells is associated with poor prognosis and metastatic progression, revealing that it is a metastasis-suppressor protein (Hemler 2005). Melanoma cell lines transfected with KAI1/CD82 have a reduced ability to metastatize to the lung when injected in wild-type animals but not DARC$^{-/-}$ mice. Interaction with endothelial DARC transmits a senescence signal

to cancer cells expressing KAI1/CD82, whereas cells that lack KAI1 can proliferate, potentially giving rise to metastases (Bandyopadhyay et al. 2006). Collectively, these data indicate that DARC expression on tumor cells or on endothelial cells plays a negative role in tumor progression: in the first case, through the control of angiogenic and inflammatory chemokines, and in the second, transmitting a senescence signal to tumor cells through interaction with KAI1/CD82.

In line with its recognized role of D6 in the control of the inflammatory responses, recent data show that $D6^{-/-}$ mice have increased susceptibility to tumor development in a phorbol ester-induced skin carcinogenesis model (Nibbs et al. 2007) and in the azoxymethane/sodium dodecylsulphate model of colon cancer (Vetrano et al. 2010), suggesting its relevance also in inflammation-induced tumorigenesis. In both models, a significant increase in chemokine levels and inflammatory cell infiltration was demonstrated. Moreover, transgenic D6 expression in keratinocytes confers significant protection from phorbol ester-induced papilloma formation (Nibbs et al. 2007). D6 is also expressed by large granular lymphocyte leukemia cells (Daibata et al. 2004), malignant vascular tumors (Nibbs et al. 2001), Kaposi's sarcoma spindle cells (our unpublished observation), choriocarcinoma cell lines (Martinez de la Torre et al. 2007), and breast cancer cells (Wu et al. 2008). In human breast, cancer D6 expression is inversely correlated with clinical stages and lymph nodes metastasis, but positively with disease-free survival rate in patients (Wu et al. 2008). Over-expression of D6 in breast cancer cell lines resulted in decreased CC chemokine levels, vessel density, tumor-associated macrophage recruitment, and metastasis. In a similar manner, over-expression of D6 in a Kaposi sarcoma cell line resulted in decreased growth when cells were xenografted in nude mice (Savino et al. manuscript in preparation). These observations indicate that D6 expressed by tumor cells or lymphatic vessels of tumor stroma acts as a tumor suppressor gene by negative regulation of chemokine availability.

CXCR7 is frequently expressed by transformed cells and not their normal counterparts (Wang et al. 2008). $CXCR7^{+}$ cells show *in vitro* increase cell survival and adhesiveness, suggesting a constitutive signaling activity of this receptor (Burns et al. 2006), and CXCR7 over-expression in prostate cancer cells resulted in transcriptional modifications in molecules involved in adhesion (cadherin-11 and CD44) and angiogenesis (CXCL8 and VEGF) (Wang et al. 2008). In human specimens, CXCR7 was found in breast tumoral cells, as well as in tumor-associated blood vessels (Miao et al. 2007) and in Kaposi's sarcoma-associated HHV-8-infected endothelial cells (Raggo et al. 2005). CXCR7 was also found expressed by prostate carcinoma, where its expression correlated with tumor aggressiveness (Wang et al. 2008), and in non-small cell lung carcinoma, being more expressed in patients with post-operative metastatic recurrence (Iwakiri et al. 2009). From a molecular point of view, the CXCR7 gene has been found to be rearranged in lipomas (Broberg et al. 2002; Miao et al. 2007) and to be a direct target of Hypermethylated in Cancer 1 (H1C1), a tumor suppressor gene early inactivated during tumorigenesis (Van Rechem et al. 2009). Finally, in experimental models, treatment of tumor-bearing mice with a selective CXCR7 antagonist or RNA interference for CXCR7 resulted in smaller and poorly organized masses without any vascularization (Burns et al. 2006).

Collectively, *in vitro* and *in vivo* data indicate that, unlike DARC and D6, CXCR7 behaves as a tumor-promoting gene, whose expression induces genes involved in cell adhesion and proliferation and enhanced tumorigenesis.

6 Concluding Remarks

Initially described as "silent" receptors by virtue of their inability to directly induce cell migration, chemokine decoy receptors are now emerging as a new family of molecules with heterogeneous structural and biochemical properties fulfilling the common scope to shape chemokine gradients. Mainly expressed by non-hematopoietic cell types, these receptors transport, remove, or concentrate complementary sets of chemokines, allowing the creation, maintenance, and regulation in time of chemokine gradients in the tissue. Thus, they play a complementary function to canonical chemokine receptors, which are required to recognize the gradient and direct leukocyte recruitment. The use of knock out mice has demonstrated that chemokine decoy receptors have a non-redundant function in inflammation regulating leukocyte extravasation from the blood vessels to the inflamed tissue and their traffic to lymph nodes (Fig. 3). A role in leukocyte bone marrow mobilization is very likely because they target chemokines previously reported to have a myelosuppressive and/or mobilizing effect on hematopoietic stem cells. Though detailed structure–function analysis are not available yet, emerging evidences also suggest that chemokine decoy receptors are not "silent," but they activate G protein-independent signaling pathways, which control their internalization and intracellular trafficking required to fulfill their biological activities.

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References

Addison CL, Belperio JA, Burdick MD, Strieter RM (2004) Overexpression of the duffy antigen receptor for chemokines (DARC) by NSCLC tumor cells results in increased tumor necrosis. BMC Cancer 4:28

Allen SJ, Crown SE, Handel TM (2007) Chemokine: receptor structure, interactions, and antagonism. Annu Rev Immunol 25:787–820

Balabanian K, Lagane B, Infantino S, Chow KY, Harriague J, Moepps B, Arenzana-Seisdedos F, Thelen M, Bachelerie F (2005) The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. J Biol Chem 280:35760–35766

Bandyopadhyay S, Zhan R, Chaudhuri A, Watabe M, Pai SK, Hirota S, Hosobe S, Tsukada T, Miura K, Takano Y, Saito K, Pauza ME, Hayashi S, Wang Y, Mohinta S, Mashimo T, Iiizumi M,

Furuta E, Watabe K (2006) Interaction of KAI1 on tumor cells with DARC on vascular endothelium leads to metastasis suppression. Nat Med 12:933–938

Berres ML, Trautwein C, Zaldivar MM, Schmitz P, Pauels K, Lira SA, Tacke F, Wasmuth HE (2009) The chemokine scavenging receptor D6 limits acute toxic liver injury *in vivo*. Biol Chem 390:1039–1045

Biber K, Zuurman MW, Homan H, Boddeke HW (2003) Expression of L-CCR in HEK 293 cells reveals functional responses to CCL2, CCL5, CCL7, and CCL8. J Leukoc Biol 74:243–251

Blackburn PE, Simpson CV, Nibbs RJ, O'Hara M, Booth R, Poulos J, Isaacs NW, Graham GJ (2004) Purification and biochemical characterization of the D6 chemokine receptor. Biochem J 379:263–272

Boldajipour B, Mahabaleshwar H, Kardash E, Reichman-Fried M, Blaser H, Minina S, Wilson D, Xu Q, Raz E (2008) Control of chemokine-guided cell migration by ligand sequestration. Cell 132:463–473

Bonecchi R, Locati M, Galliera E, Vulcano M, Sironi M, Fra AM, Gobbi M, Vecchi A, Sozzani S, Haribabu B, Van Damme J, Mantovani A (2004) Differential recognition and scavenging of native and truncated macrophage-derived chemokine (macrophage-derived chemokine/CC chemokine ligand 22) by the D6 decoy receptor. J Immunol 172:4972–4976

Bonecchi R, Borroni EM, Anselmo A, Doni A, Savino B, Mirolo M, Fabbri M, Jala VR, Haribabu B, Mantovani A, Locati M (2008) Regulation of D6 chemokine scavenging activity by ligand- and Rab11-dependent surface up-regulation. Blood 112:493–503

Bonecchi R, Galliera E, Borroni EM, Corsi MM, Locati M, Mantovani A (2009) Chemokines and chemokine receptors: an overview. Front Biosci 14:540–551

Bonini JA, Martin SK, Dralyuk F, Roe MW, Philipson LH, Steiner DF (1997) Cloning, expression, and chromosomal mapping of a novel human CC-chemokine receptor (CCR10) that displays high-affinity binding for MCP-1 and MCP-3. DNA Cell Biol 16:1249–1256

Bordon Y, Hansell CA, Sester DP, Clarke M, Mowat AM, Nibbs RJ (2009) The atypical chemokine receptor D6 contributes to the development of experimental colitis. J Immunol 182:5032–5040

Broberg K, Zhang M, Strombeck B, Isaksson M, Nilsson M, Mertens F, Mandahl N, Panagopoulos I (2002) Fusion of RDC1 with HMGA2 in lipomas as the result of chromosome aberrations involving 2q35-37 and 12q13-15. Int J Oncol 21:321–326

Burns JM, Summers BC, Wang Y, Melikian A, Berahovich R, Miao Z, Penfold ME, Sunshine MJ, Littman DR, Kuo CJ, Wei K, McMaster BE, Wright K, Howard MC, Schall TJ (2006) A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. J Exp Med 203:2201–2213

Cardona AE, Sasse ME, Liu L, Cardona SM, Mizutani M, Savarin C, Hu T, Ransohoff RM (2008) Scavenging roles of chemokine receptors: chemokine receptor deficiency is associated with increased levels of ligand in circulation and tissues. Blood 112:256–263

Charo IF, Ransohoff RM (2006) The many roles of chemokines and chemokine receptors in inflammation. N Engl J Med 354:610–621

Chaudhuri A, Polyakova J, Zbrzezna V, Williams K, Gulati S, Pogo AO (1993) Cloning of glycoprotein D cDNA, which encodes the major subunit of the Duffy blood group system and the receptor for the Plasmodium vivax malaria parasite. Proc Natl Acad Sci USA 90:10793–10797

Comerford I, Milasta S, Morrow V, Milligan G, Nibbs R (2006) The chemokine receptor CCX-CKR mediates effective scavenging of CCL19 *in vitro*. Eur J Immunol 36:1904–1916

Daibata M, Matsuo Y, Machida H, Taguchi T, Ohtsuki Y, Taguchi H (2004) Differential gene-expression profiling in the leukemia cell lines derived from indolent and aggressive phases of CD56+ T-cell large granular lymphocyte leukemia. Int J Cancer 108:845–851

D'Amico G, Frascaroli G, Bianchi G, Transidico P, Doni A, Vecchi A, Sozzani S, Allavena P, Mantovani A (2000) Uncoupling of inflammatory chemokine receptors by IL-10: generation of functional decoys. Nat Immunol 1:387–391

Darbonne WC, Rice GC, Mohler MA, Apple T, Hebert CA, Valente AJ, Baker JB (1991) Red blood cells are a sink for interleukin 8, a leukocyte chemotaxin. J Clin Invest 88:1362–1369

Dawson TC, Lentsch AB, Wang Z, Cowhig JE, Rot A, Maeda N, Peiper SC (2000) Exaggerated response to endotoxin in mice lacking the Duffy antigen/receptor for chemokines (DARC). Blood 96:1681–1684

Desbaillets I, Diserens AC, Tribolet N, Hamou MF, Van Meir EG (1997) Upregulation of interleukin 8 by oxygen-deprived cells in glioblastoma suggests a role in leukocyte activation, chemotaxis, and angiogenesis. J Exp Med 186:1201–1212

Di Liberto D, Locati M, Caccamo N, Vecchi A, Meraviglia S, Salerno A, Sireci G, Nebuloni M, Caceres N, Cardona PJ, Dieli F, Mantovani A (2008) Role of the chemokine decoy receptor D6 in balancing inflammation, immune activation, and antimicrobial resistance in Mycobacterium tuberculosis infection. J Exp Med 205:2075–2084

Edderkaoui B, Baylink DJ, Beamer WG, Wergedal JE, Porte R, Chaudhuri A, Mohan S (2007) Identification of mouse Duffy antigen receptor for chemokines (Darc) as a BMD QTL gene. Genome Res 17:577–585

Fan P, Kyaw H, Su K, Zeng Z, Augustus M, Carter KC, Li Y (1998) Cloning and characterization of a novel human chemokine receptor. Biochem Biophys Res Commun 243:264–268

Fra AM, Locati M, Otero K, Sironi M, Signorelli P, Massardi ML, Gobbi M, Vecchi A, Sozzani S, Mantovani A (2003) Cutting edge: scavenging of inflammatory CC chemokines by the promiscuous putatively silent chemokine receptor D6. J Immunol 170:2279–2282

Galliera E, Jala VR, Trent JO, Bonecchi R, Signorelli P, Lefkowitz RJ, Mantovani A, Locati M, Haribabu B (2004) beta-Arrestin-dependent constitutive internalization of the human chemokine decoy receptor D6. J Biol Chem 279:25590–25597

Gardner L, Patterson AM, Ashton BA, Stone MA, Middleton J (2004) The human Duffy antigen binds selected inflammatory but not homeostatic chemokines. Biochem Biophys Res Commun 321:306–312

Gosling J, Dairaghi DJ, Wang Y, Hanley M, Talbot D, Miao Z, Schall TJ (2000) Cutting edge: identification of a novel chemokine receptor that binds dendritic cell- and T cell-active chemokines including ELC, SLC, and TECK. J Immunol 164:2851–2856

Hadley TJ, Peiper SC (1997) From malaria to chemokine receptor: the emerging physiologic role of the Duffy blood group antigen. Blood 89:3077–3091

Hartmann TN, Grabovsky V, Pasvolsky R, Shulman Z, Buss EC, Spiegel A, Nagler A, Lapidot T, Thelen M, Alon R (2008) A crosstalk between intracellular CXCR7 and CXCR4 involved in rapid CXCL12-triggered integrin activation but not in chemokine-triggered motility of human T lymphocytes and CD34+ cells. J Leukoc Biol 84:1130–1140

Hemler ME (2005) Tetraspanin functions and associated microdomains. Nat Rev Mol Cell Biol 6:801–811

Horton LW, Yu Y, Zaja-Milatovic S, Strieter RM, Richmond A (2007) Opposing roles of murine duffy antigen receptor for chemokine and murine CXC chemokine receptor-2 receptors in murine melanoma tumor growth. Cancer Res 67:9791–9799

Horuk R, Chitnis CE, Darbonne WC, Colby TJ, Rybicki A, Hadley TJ, Miller LH (1993) A receptor for the malarial parasite Plasmodium vivax: the erythrocyte chemokine receptor. Science 261:1182–1184

Horuk R, Wang ZX, Peiper SC, Hesselgesser J (1994) Identification and characterization of a promiscuous chemokine-binding protein in a human erythroleukemic cell line. J Biol Chem 269:17730–17733

Infantino S, Moepps B, Thelen M (2006) Expression and regulation of the orphan receptor RDC1 and its putative ligand in human dendritic and B cells. J Immunol 176:2197–2207

Iwakiri S, Mino N, Takahashi T, Sonobe M, Nagai S, Okubo K, Wada H, Date H, Miyahara R (2009) Higher expression of chemokine receptor CXCR7 is linked to early and metastatic recurrence in pathological stage I nonsmall cell lung cancer. Cancer 115:2580–2593

Jamieson T, Cook DN, Nibbs RJ, Rot A, Nixon C, McLean P, Alcami A, Lira SA, Wiekowski M, Graham GJ (2005) The chemokine receptor D6 limits the inflammatory response *in vivo*. Nat Immunol 6:403–411

Kulkarni H, Marconi VC, He W, Landrum ML, Okulicz JF, Delmar J, Kazandjian D, Castiblanco J, Ahuja SS, Wright EJ, Weiss RA, Clark RA, Dolan MJ, Ahuja SK (2009) The Duffy-null state is associated with a survival advantage in leukopenic HIV-infected persons of African ancestry. Blood 114:2783–2792

Lentsch AB (2002) The Duffy antigen/receptor for chemokines (DARC) and prostate cancer. A role as clear as black and white? FASEB J 16:1093–1095

Levoye A, Balabanian K, Baleux F, Bachelerie F, Lagane B (2009) CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. Blood 113:6085–6093

Liu L, Graham GJ, Damodaran A, Hu T, Lira SA, Sasse M, Canasto-Chibuque C, Cook DN, Ransohoff RM (2006) Cutting edge: the silent chemokine receptor D6 is required for generating T cell responses that mediate experimental autoimmune encephalomyelitis. J Immunol 177:17–21

Liu J, Guo X, Mohandas N, Chasis JA, An X (2010) Membrane remodeling during reticulocyte maturation. Blood 115:2021–2027

Luker KE, Gupta M, Steele JM, Foerster BR, Luker GD (2009) Imaging ligand-dependent activation of CXCR7. Neoplasia 11:1022–1035

Luo H, Chaudhuri A, Zbrzezna V, He Y, Pogo AO (2000) Deletion of the murine Duffy gene (Dfy) reveals that the Duffy receptor is functionally redundant. Mol Cell Biol 20:3097–3101

Maho A, Bensimon A, Vassart G, Parmentier M (1999) Mapping of the CCXCR1, CX3CR1, CCBP2 and CCR9 genes to the CCR cluster within the 3p21.3 region of the human genome. Cytogenet Cell Genet 87:265–268

Maksym RB, Tarnowski M, Grymula K, Tarnowska J, Wysoczynski M, Liu R, Czerny B, Ratajczak J, Kucia M, Ratajczak MZ (2009) The role of stromal-derived factor-1–CXCR7 axis in development and cancer. Eur J Pharmacol 625:31–40

Mangalmurti NS, Xiong Z, Hulver M, Ranganathan M, Liu XH, Oriss T, Fitzpatrick M, Rubin M, Triulzi D, Choi A, Lee JS (2009) Loss of red cell chemokine scavenging promotes transfusion-related lung inflammation. Blood 113:1158–1166

Mange KC, Prak EL, Kamoun M, Du Y, Goodman N, Danoff T, Hoy T, Newman M, Joffe MM, Feldman HI (2004) Duffy antigen receptor and genetic susceptibility of African Americans to acute rejection and delayed function. Kidney Int 66:1187–1192

Mantovani A (1999) The chemokine system: redundancy for robust outputs. Immunol Today 20:254–257

Mantovani A, Locati M, Vecchi A, Sozzani S, Allavena P (2001) Decoy receptors: a strategy to regulate inflammatory cytokines and chemokines. Trends Immunol 22:328–336

Mantovani A, Bonecchi R, Locati M (2006) Tuning inflammation and immunity by chemokine sequestration: decoys and more. Nat Rev Immunol 6:907–918

Mantovani A, Savino B, Locati M, Zammataro L, Allavena P, Bonecchi R (2010) The chemokine system in cancer biology and therapy. Cytokine Growth Factor Rev 21:27–39

Martinez de la Torre Y, Locati M, Buracchi C, Dupor J, Cook DN, Bonecchi R, Nebuloni M, Rukavina D, Vago L, Vecchi A, Lira SA, Mantovani A (2005) Increased inflammation in mice deficient for the chemokine decoy receptor D6. Eur J Immunol 35:1342–1346

Martinez de la Torre Y, Buracchi C, Borroni EM, Dupor J, Bonecchi R, Nebuloni M, Pasqualini F, Doni A, Lauri E, Agostinis C, Bulla R, Cook DN, Haribabu B, Meroni P, Rukavina D, Vago L, Tedesco F, Vecchi A, Lira SA, Locati M, Mantovani A (2007) Protection against inflammation- and autoantibody-caused fetal loss by the chemokine decoy receptor D6. Proc Natl Acad Sci USA 104:2319–2324

McCulloch CV, Morrow V, Milasta S, Comerford I, Milligan G, Graham GJ, Isaacs NW, Nibbs RJ (2008) Multiple roles for the C-terminal tail of the chemokine scavenger D6. J Biol Chem 283:7972–7982

McKimmie CS, Graham GJ (2006) Leucocyte expression of the chemokine scavenger D6. Biochem Soc Trans 34:1002–1004

Miao Z, Luker KE, Summers BC, Berahovich R, Bhojani MS, Rehemtulla A, Kleer CG, Essner JJ, Nasevicius A, Luker GD, Howard MC, Schall TJ (2007) CXCR7 (RDC1) promotes breast and lung tumor growth *in vivo* and is expressed on tumor-associated vasculature. Proc Natl Acad Sci USA 104:15735–15740

Migeotte I, Franssen JD, Goriely S, Willems F, Parmentier M (2002) Distribution and regulation of expression of the putative human chemokine receptor HCR in leukocyte populations. Eur J Immunol 32:494–501

Miller LH, Mason SJ, Clyde DF, McGinniss MH (1976) The resistance factor to Plasmodium vivax in blacks. The Duffy-blood-group genotype, FyFy. N Engl J Med 295:302–304

Muller G, Lipp M (2003) Concerted action of the chemokine and lymphotoxin system in secondary lymphoid-organ development. Curr Opin Immunol 15:217–224

Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, Power CA (2000) International union of pharmacology. XXII Nomenclature for chemokine receptors Pharmacol Rev 52:145–176

Neote K, Mak JY, Kolakowski LF Jr, Schall TJ (1994) Functional and biochemical analysis of the cloned Duffy antigen: identity with the red blood cell chemokine receptor. Blood 84:44–52

Nibbs RJ, Wylie SM, Pragnell IB, Graham GJ (1997a) Cloning and characterization of a novel murine beta chemokine receptor, D6. Comparison to three other related macrophage inflammatory protein-1alpha receptors, CCR-1, CCR-3, and CCR-5. J Biol Chem 272:12495–12504

Nibbs RJ, Wylie SM, Yang J, Landau NR, Graham GJ (1997b) Cloning and characterization of a novel promiscuous human beta-chemokine receptor D6. J Biol Chem 272:32078–32083

Nibbs RJ, Kriehuber E, Ponath PD, Parent D, Qin S, Campbell JD, Henderson A, Kerjaschki D, Maurer D, Graham GJ, Rot A (2001) The beta-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. Am J Pathol 158:867–877

Nibbs RJ, Gilchrist DS, King V, Ferra A, Forrow S, Hunter KD, Graham GJ (2007) The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumors. J Clin Invest 117:1884–1892

Peiper SC, Wang ZX, Neote K, Martin AW, Showell HJ, Conklyn MJ, Ogborne K, Hadley TJ, Lu ZH, Hesselgesser J, Horuk R (1995) The Duffy antigen/receptor for chemokines (DARC) is expressed in endothelial cells of Duffy negative individuals who lack the erythrocyte receptor. J Exp Med 181:1311–1317

Perrier P, Martinez FO, Locati M, Bianchi G, Nebuloni M, Vago G, Bazzoni F, Sozzani S, Allavena P, Mantovani A (2004) Distinct transcriptional programs activated by interleukin-10 with or without lipopolysaccharide in dendritic cells: induction of the B cell-activating chemokine, CXC chemokine ligand 13. J Immunol 172:7031–7042

Proost P, Struyf S, Van Damme J (2006) Natural post-translational modifications of chemokines. Biochem Soc Trans 34:997–1001

Proost P, Loos T, Mortier A, Schutyser E, Gouwy M, Noppen S, Dillen C, Ronsse I, Conings R, Struyf S, Opdenakker G, Maudgal PC, Van Damme J (2008) Citrullination of CXCL8 by peptidylarginine deiminase alters receptor usage, prevents proteolysis, and dampens tissue inflammation. J Exp Med 205:2085–2097

Proudfoot AE (2006) The biological relevance of chemokine-proteoglycan interactions. Biochem Soc Trans 34:422–426

Pruenster M, Rot A (2006) Throwing light on DARC. Biochem Soc Trans 34:1005–1008

Pruenster M, Mudde L, Bombosi P, Dimitrova S, Zsak M, Middleton J, Richmond A, Graham GJ, Segerer S, Nibbs RJ, Rot A (2009) The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. Nat Immunol 10:101–108

Raggo C, Ruhl R, McAllister S, Koon H, Dezube BJ, Fruh K, Moses AV (2005) Novel cellular genes essential for transformation of endothelial cells by Kaposi's sarcoma-associated herpesvirus. Cancer Res 65:5084–5095

Reich D, Nalls MA, Kao WH, Akylbekova EL, Tandon A, Patterson N, Mullikin J, Hsueh WC, Cheng CY, Coresh J, Boerwinkle E, Li M, Waliszewska A, Neubauer J, Li R, Leak TS, Ekunwe L, Files JC, Hardy CL, Zmuda JM, Taylor HA, Ziv E, Harris TB, Wilson JG (2009) Reduced neutrophil count in people of African descent is due to a regulatory variant in the Duffy antigen receptor for chemokines gene. PLoS Genet 5:e1000360

Reutershan J, Harry B, Chang D, Bagby GJ, Ley K (2009) DARC on RBC limits lung injury by balancing compartmental distribution of CXC chemokines. Eur J Immunol 39:1597–1607

Rot A (2005) Contribution of Duffy antigen to chemokine function. Cytokine Growth Factor Rev 16:687–694

Sallusto F, Palermo B, Lenig D, Miettinen M, Matikainen S, Julkunen I, Forster R, Burgstahler R, Lipp M, Lanzavecchia A (1999) Distinct patterns and kinetics of chemokine production regulate dendritic cell function. Eur J Immunol 29:1617–1625

Samson M, Soularue P, Vassart G, Parmentier M (1996) The genes encoding the human CC-chemokine receptors CC-CKR1 to CC-CKR5 (CMKBR1-CMKBR5) are clustered in the p21.3-p24 region of chromosome 3. Genomics 36:522–526

Savino B, Borroni EM, Torres NM, Proost P, Struyf S, Mortier A, Mantovani A, Locati M, Bonecchi R (2009) Recognition versus adaptive up-regulation and degradation of CC chemokines by the chemokine decoy receptor D6 are determined by their N-terminal sequence. J Biol Chem 284:26207–26215

Schnabel RB, Baumert J, Barbalic M, Dupuis J, Ellinor PT, Durda P, Dehghan A, Bis JC, Illig T, Morrison AC, Jenny NS, Keaney JF, Jr., Gieger C, Tilley C, Yamamoto JF, Khuseyinova N, Heiss G, Doyle M, Blankenberg S, Herder C, Walston JD, Zhu Y, Vasan RS, Klopp N, Boerwinkle E, Larson MG, Psaty BM, Peters A, Ballantyne CM, Witteman JC, Hoogeveen RC, Benjamin EJ, Koenig W, Tracy RP (2010) Duffy antigen receptor for chemokines (Darc) polymorphism regulates circulating concentrations of monocyte chemoattractant protein-1 and other inflammatory mediators. Blood prepublished online December 29, 2009; doi 10.1182/blood-2009-05-221382

Shen H, Schuster R, Stringer KF, Waltz SE, Lentsch AB (2006) The Duffy antigen/receptor for chemokines (DARC) regulates prostate tumor growth. Faseb J 20:59–64

Shenoy SK, Lefkowitz RJ (2003) Multifaceted roles of beta-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling. Biochem J 375:503–515

Sierro F, Biben C, Martinez-Munoz L, Mellado M, Ransohoff RM, Li M, Woehl B, Leung H, Groom J, Batten M, Harvey RP, Martinez AC, Mackay CR, Mackay F (2007) Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. Proc Natl Acad Sci USA 104:14759–14764

Tang T, Owen JD, Du J, Walker CL, Richmond A (1998) Molecular cloning and characterization of a mouse gene with homology to the Duffy-antigen receptor for chemokines. DNA Seq 9:129–143

Thelen M, Thelen S (2008) CXCR7, CXCR4 and CXCL12: an eccentric trio? J Neuroimmunol 198:9–13

Townson JR, Nibbs RJ (2002) Characterization of mouse CCX-CKR, a receptor for the lymphocyte-attracting chemokines TECK/mCCL25, SLC/mCCL21 and MIP-3beta/mCCL19: comparison to human CCX-CKR. Eur J Immunol 32:1230–1241

Van Rechem C, Rood BR, Touka M, Pinte S, Jenal M, Guerardel C, Ramsey K, Monte D, Begue A, Tschan MP, Stephan DA, Leprince D (2009) Scavenger chemokine (CXC motif) receptor 7 (CXCR7) is a direct target gene of HIC1 (hypermethylated in cancer 1). J Biol Chem 284:20927–20935

Vetrano S, Borroni EM, Sarukhan A, Savino B, Bonecchi R, Correale C, Arena V, Fantini M, Roncalli M, Malesci A, Mantovani A, Locati M, Danese S (2010) The lymphatic system controls intestinal inflammation and inflammation-associated colon cancer through the chemokine decoy receptor D6. Gut 59:197–206

Vielhauer V, Allam R, Lindenmeyer MT, Cohen CD, Draganovici D, Mandelbaum J, Eltrich N, Nelson PJ, Anders HJ, Pruenster M, Rot A, Schlondorff D, Segerer S (2009) Efficient renal

recruitment of macrophages and T cells in mice lacking the duffy antigen/receptor for chemokines. Am J Pathol 175:119–131

Wang J, Ou ZL, Hou YF, Luo JM, Shen ZZ, Ding J, Shao ZM (2006) Enhanced expression of Duffy antigen receptor for chemokines by breast cancer cells attenuates growth and metastasis potential. Oncogene 25:7201–7211

Wang J, Shiozawa Y, Wang J, Wang Y, Jung Y, Pienta KJ, Mehra R, Loberg R, Taichman RS (2008) The role of CXCR7/RDC1 as a chemokine receptor for CXCL12/SDF-1 in prostate cancer. J Biol Chem 283:4283–4294

Waugh DJ, Wilson C, Seaton A, Maxwell PJ (2008) Multi-faceted roles for CXC-chemokines in prostate cancer progression. Front Biosci 13:4595–4604

Weber M, Blair E, Simpson CV, O'Hara M, Blackburn PE, Rot A, Graham GJ, Nibbs RJ (2004) The chemokine receptor D6 constitutively traffics to and from the cell surface to internalize and degrade chemokines. Mol Biol Cell 15:2492–2508

Wessels JM, Linton NF, Croy BA, Tayade C (2007) A review of molecular contrasts between arresting and viable porcine attachment sites. Am J Reprod Immunol 58:470–480

Whitehead GS, Wang T, DeGraff LM, Card JW, Lira SA, Graham GJ, Cook DN (2007) The chemokine receptor D6 has opposing effects on allergic inflammation and airway reactivity. Am J Respir Crit Care Med 175:243–249

Wiederholt T, von Westernhagen M, Zaldivar MM, Berres ML, Schmitz P, Hellerbrand C, Muller T, Berg T, Trautwein C, Wasmuth HE (2008) Genetic variations of the chemokine scavenger receptor D6 are associated with liver inflammation in chronic hepatitis C. Hum Immunol 69:861–866

Wittamer V, Franssen JD, Vulcano M, Mirjolet JF, Le Poul E, Migeotte I, Brezillon S, Tyldesley R, Blanpain C, Detheux M, Mantovani A, Sozzani S, Vassart G, Parmentier M, Communi D (2003) Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids. J Exp Med 198:977–985

Wu FY, Ou ZL, Feng LY, Luo JM, Wang LP, Shen ZZ, Shao ZM (2008) Chemokine decoy receptor d6 plays a negative role in human breast cancer. Mol Cancer Res 6:1276–1288

Zabel BA, Nakae S, Zuniga L, Kim JY, Ohyama T, Alt C, Pan J, Suto H, Soler D, Allen SJ, Handel TM, Song CH, Galli SJ, Butcher EC (2008) Mast cell-expressed orphan receptor CCRL2 binds chemerin and is required for optimal induction of IgE-mediated passive cutaneous anaphylaxis. J Exp Med 205:2207–2220

Zarbock A, Schmolke M, Bockhorn SG, Scharte M, Buschmann K, Ley K, Singbartl K (2007) The Duffy antigen receptor for chemokines in acute renal failure: A facilitator of renal chemokine presentation. Crit Care Med 35:2156–2163

Role of Chemokines in the Biology of Natural Killer Cells

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Contents

Abstract Natural killer (NK) cells represent a major subpopulation of lymphocytes. These cells have effector functions as they recognize and kill transformed cells as well as microbially infected cells. In addition, alloreactive NK cells have been successfully used to treat patients with acute myeloid leukemia and other hematological malignancies. NK cells are also endowed with immunoregulatory functions since they secrete cytokines such as IFN-γ, which favor the development of T helper 1 (Th1) cells, and chemokines such as CCL3/MIP-1α and CCL4/MIP-1β, which recruit various inflammatory cells into sites of inflammation. In human blood, NK cells are divided into $CD56^{bright}$ $CD16^{dim}$ and $CD56^{dim}$ $CD16^{bright}$ subsets. These subsets have different phenotypic expression and may have different

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functions; the former subset is more immunoregulatory and the latter is more cytolytic. The $CD56^{bright}CD16^{dim}$ NK cells home into tissues such as the peripheral lymph nodes (LNs) under physiological conditions because they express the LN homing receptor CCR7 and they respond to CCL19/MIP-3β and CCL21/SLC chemokines. They also distribute into adenoid tissues or decidual uterus following the CXCR3/CXCL10 or CXCR4/CXCL12 axis. On the other hand, both NK cell subsets migrate into inflammatory sites, with more $CD56^{dim}CD16^{bright}$ NK cells distributing into inflamed liver and lungs. CCR5/CCL5 axis plays an important role in the accumulation of NK cells in virally infected sites as well as during parasitic infections. $CD56^{bright}CD16^{dim}$ cells also migrate into autoimmune sites such as inflamed synovial fluids in patients having rheumatoid arthritis facilitated by the CCR5/CCL3/CCL4/CCL5 axis, whereas they distribute into inflamed brains aided by the CX_3CR1/CX_3CL1 axis. On the other hand, $CD56^{dim}CD16^{bright}$ NK cells accumulate in the liver of patients with primary biliary disease aided by the CXCR1/CXCL8 axis. However, the types of chemokines that contribute to their accumulation in target organs during graft vs. host (GvH) disease are not known. Further, chemokines activate NK cells to become highly cytolytic cells known as CC chemokine-activated killer (CHAK) cells that kill tumor cells. In summary, chemokines whether secreted in an autocrine or paracrine fashion regulate various biological functions of NK cells. Depending on the tissue and the chemokine secreted, NK cells may ameliorate the disease such as their roles in combating tumors or virally infected cells, and their therapeutic potentials in treating leukemias and other hematological malignancies, as well as reducing the incidence of GvH disease. In contrast, they may exacerbate the disease by damaging the affected tissues through direct cytotoxicity or by the release of multiple inflammatory cytokines and chemokines. Examples are their deleterious roles in autoimmune diseases such as rheumatoid arthritis and primary biliary cirrhosis.

1 Natural Killer Cells

Natural killer (NK) cells kill virally infected cells and transformed cells (Maghazachi and Al-Aoukaty 1998). In addition, they possess immunoregulatory activities by secreting multiple cytokines and chemokines (Young and Ortaldo 2006). They also interact with dendritic cells and shape both the innate and adaptive immune responses (Moretta 2002). Human NK cells represent less than 1% of peripheral blood cells but comprise about 10–15% of total blood lymphoid cells. In the blood circulation, human NK cells are classified into two major subsets, those that express high CD56 but low or no CD16 (known as $CD56^{bright}CD16^{dim}$), and those that express high CD16 and low CD56 (known as $CD56^{dim}CD16^{bright}$). $CD56^{dim}CD16^{bright}$ NK cells represent the majority of blood NK cells – about 80–90%, whereas $CD56^{bright}CD16^{dim}$ cells represent a minority of blood NK cells – about 10–20%. $CD56^{dim}CD16^{bright}$ cells are highly cytolytic against target cells such as tumor cells and immature dendritic cells but secrete cytokines with less

intensity than the $CD56^{bright}CD16^{dim}$ cells. Phenotypically, $CD56^{dim}CD16^{bright}$ NK cells are CD94/NKG2^{+}, killer Ig-like receptors (KIR^{+}), natural cytotoxicity $(NC)^{+}$, and perforin^{+}, whereas $CD56^{bright}CD16^{dim}$ NK cells are CD94/NKG2^{+}, $KIR^{low\ or\ -}$, NC^{low}, and perforin^{-} (Chiesa et al. 2003; Moretta 2002).

Upon activation with IL-2, most if not all NK cells up-regulate the expression of CD56 molecule on their surfaces (Agaugue et al. 2008; Loza and Perussia 2004; Maghazachi 2005a). Hence, the distinction among these two subsets based on the density of this molecule becomes a misleading paradigm. In this regard, it is important to remember that it is the activated NK cells that kill and destroy transformed cells. Also, during infection, the milieu is overwhelmed with high concentrations of cytokines and chemokines secreted as a result of infection. These mediators ought to activate NK cells; hence, it is important to study activated NK cells that lyse both abnormal cells as well as microbially infected cells, and which secrete mediators that regulate both innate and adaptive immune cells.

1.1 NK Cell Distribution into Various Tissues

Although NK cells are mainly present in the blood circulation, these cells migrate into inflammatory sites, particularly upon infection. In addition, they migrate toward tumor growth sites to recognize and destroy tumor cells. Early experiments using nonactivated (Rolstad et al. 1986), or activated rat NK cells (Maghazachi and Fitzgibbon 1990), revealed that these cells distribute first into the lungs and redistribute into the liver and spleen, with minimal, if any, accumulation in other organs such as the peripheral lymph nodes (LNs). Later work described two major families of molecules that are involved in the migration of these cells into various sites, including tumor growth sites; these are chemokines and lysophospholipids (Maghazachi 2003, 2005b). This article will discuss the effects of chemokines on NK cell biology; however, the readers are encouraged to read earlier excellent review articles regarding this subject, such as the one written by Taub (1999) or Robertson (2002).

1.2 Role of NK Cells in Hemopoietic Stem Cell Transplantation

NK cells express both inhibitory and activating receptors. The inhibitory receptors are collectively known as killer inhibitory receptors (KIR; part of the killer Ig-like receptors), which recognize ligands belonging to MHC class I molecules. NK cells also lyse target cells that have lost MHC-class I molecules, a phenomenon known as "missing-self" (Ljunggren and Karre 1990). The nature of these receptors is beyond the scope of this article, but they have been extensively described in various review articles, for example Long (1999) or Moretta et al. (2001), among many others. Briefly, each NK cell expresses at least one inhibitory receptor, ensuring that under

normal conditions NK cells are inhibited upon ligating self-MHC molecules, which guards against autoimmunity, but can also sense the lack of MHC molecules on transformed cells or cells infected with some viruses. The expression of these receptors was exploited for bone marrow transplantation (BMT) or stem cell transplantation (SCT) procedures. In some cancer patients, BMT is a standard protocol used to eradicate tumor growth and to populate the host with stem cells. However, in allogeneic transplantation, the major problem that occurs as a result of this form of therapy is graft vs. host (GvH) disease. This is caused by T cells of the donor grafts that attack the host cells when stimulated with host antigen presenting cells such as dendritic cells. So the benefit of attacking tumor cells by donor T cells is compromised by the detrimental effect of GvH disease, which is a great set-back in allogeneic transplantation therapy.

Early experiments showed that administration of IL-2-activated NK cells into tumor-bearing animals just after allogeneic BMT is successful in eradicating the tumor, as well as in reducing the incidence of GvH (Asai et al. 1998). The same authors suggested that TGF-β1 is involved in this activity of NK cells, since administration of antibody to this cytokine abrogates the suppressive effect exerted by NK cells on GvH disease. However, other mechanisms might also be involved, since it is clear from this study that NK cells should be transferred early after BMT (Asai et al. 1998), suggesting that the steps of sensitizing donor T cells in the BMT might have been impeded due to the administration of NK cells. Later work showed that a mismatch between MHC class I molecules and KIR among donors and recipients is highly beneficial in treating acute myeloid leukemia (AML). Patients with AML receiving KIR/MHC mismatched in the donor/recipient combinations have more than 60% survival rate when compared to less than 5% survival in patients not receiving alloreactive NK cells (Ruggeri et al. 2002). In this disease, donor alloreactive NK cells perform several functions: (1) they participate in killing the leukemic cells of the host, (2) they lyse the host dendritic cells that provide stimulation to the donor T cells responsible for GvH disease, and (3) they may also kill the host T cells responsible for host vs. graft (HvG) disease (Velardi et al. 2002, 2009; Voutsadakis 2003). Hence, AML patients receiving alloreactive NK cells not only have minimal load of leukemic cells but are also free of GvH disease. Taken together, these observations suggest that the utilization of alloreactive NK cells in hematopoietic SCT may hold a promise in treating hematological malignancies, and in particular those with leukemias, provided that this procedure is optimized to select alloreactive NK cell clones from the bulk of NK cells administered, taking in consideration the donor-to-donor variability.

1.3 NK Cells in Tumor Immunology

The immune system plays a central role in the immunosurveillance of spontaneous tumors as it recognizes and destroys transformed cells. Dunn et al. (2002) suggested that this pressure exerted on the immune system may select tumor cells that are able

to resist immune defenses. Efficient antitumor immunotherapies can therefore be envisioned as a way to interfere with the host/tumor equilibrium by boosting immune defense or by decreasing the immunosuppressive effect of tumors. One such approach is to boost the immune system by either injecting cytokines into the host or by activating NK cells *in vitro* before administering them into autologous patients. Although activated NK cells have been used to treat cancer patients, the outcome is not impressive (reviewed in Maghazachi and Al-Aoukaty 1998). Two important reasons for the lack of success of this form of therapy are the severe adverse effects of IL-2 (Kammula et al. 1998), and the failure of adaptively transferred cells to reach the sites of tumor growth, indicating that they do not distribute efficiently into malignant tissues (Villegas et al. 2002). However, cytokines such as IL-2 induces a marked accumulation of NK cells inside tumor nodules (Albertsson et al. 2003). Hence, the accumulation of NK cells in tumor growth sites seems to be closely related to NK cell activation stage, as continuous administration of IL-2 may lead to an increase in NK cell numbers within a tumor up to 48 h after the injection of this cytokine (Hokland et al. 1999). Activated NK cells are retained in lung tissues within minutes after intravenous injection, but are rapidly cleared, and few redistribute into the liver and spleen post administration (Maghazachi and Fitzgibbon 1990). In contrast, they accumulate in the lung tumors over time after intratumoral injection, leading to more than 15 times doubling the concentration of activated NK cells 24 h postintratumoral injection (Basse et al. 1991a, b). Nonetheless, it is not clear how these cells reach the tumors. Only few IL-2-activated NK cells are found in the circulation short after injection, and low numbers reach the tumors downstream from the lungs, which might be related to the rigidity of these cells (Sasaki et al. 1989).

To pass through vascular basement membranes and approach cancer cells, NK cells need matrix degrading proteases, and among these are the matrix metalloproteinases (MMPs) believed to be important molecules. Multiple members of this family of proteases are expressed in NK cells (Kim et al. 2000). An interesting finding by Goda et al. (2006) demonstrated that the chemokine CXCL12/SDF-1α induces the expression of matrix degrading enzymes on NK cell surfaces, which facilitate the degradation of collagen type I of extracellular matrix proteins (ECM), hence promoting NK cell invasion into tissues. This finding extends earlier results, showing that CXCL12/SDF-1α stimulates the adhesion of NK cells to VCAM-1 and ICAM-1 (Franitza et al. 2004).

2 Chemokines

Chemokines are important in allergic disorders, autoimmune diseases, and ischemia by orchestrating the infiltration of leukocytes. In addition, they play essential roles in linking the innate and adaptive immune responses (Luster 2002) and in host–pathogen interactions (Chensue 2000). They have low molecular weights and are divided into four subfamilies based on the position of the cystein (C) residue in

the amino terminal end of the molecules; these are known as CXC or α, CC or β, C or γ, and CX_3C or δ chemokines. In addition to their classification based on structures, chemokines and their receptors are classified based on their functions. Those that are up-regulated during inflammation and under pathological conditions are known as inflammatory chemokines or inflammatory chemokine receptors, whereas those that perform house-keeping functions and are involved in the circulation and homing of cells under physiological conditions are known as constitutive chemokines or constitutive chemokine receptors (Baggiolini 2001; Maghazachi 2003). For a list of chemokines and their receptors please see Chensue (2000), Murphy et al. (2000), Bruserud and Kittang (2010), and Olsnes et al. (2009).

2.1 Expression of Receptors for Chemokines in NK Cells

The differential expression of chemokine receptors in NK cells determines the recruitment of different subsets to different tissues. Treatment with cytokines such as IL-2, IL-4, IL-12, and IL-18 found at inflammatory sites influences the chemokine receptor repertoire on NK cells, which may target them to different inflammatory sites, as well as toward the peripheral LNs (Agaugue et al. 2008). Here, they become in contact with dendritic cells, release cytokines such as IFN-γ that shifts the immune system toward T helper 1 (Th1) response. They also secrete chemokines such as CCL3/MIP-1α and CCL4/MIP-1β that may recruit other inflammatory cell types (Inngjerdingen et al. 2001; Taub et al. 1995).

Inngjerdingen et al. (2001) reported that nonactivated NK cells express receptors for the constitutive chemokine receptors CXCR4 and CCR7, as determined by messenger RNA and by surface protein analyses. There was a low expression of the inflammatory chemokine receptors CXCR3 and CCR1 in the nonactivated NK cells. CX_3CR1 is expressed in $CD16^{bright}$, $CD16^{dim}$, and activated NK cells. Campbell et al. (2001) divided NK cells into two subtypes; the first subset includes $CD16^{+}$ cells expressing CXCR1, CXCR3, CXCR4, and CX_3CR1 but not CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR9, and CXCR5. These cells respond to CXCL8/IL-8, CXCL10/IP-10, and CX_3CL1/Fraktalkine. The second subset includes $CD16^{-}$ NK cells, which express high CCR7 but low CXCR3 and CXCR4. These cells respond to CCL19/MIP-3β. Another study showed that about 66% of $CD56^{bright}CD16^{dim}$ and less than 5% of $CD56^{dim}CD16^{bright}$ freshly isolated NK cells express the LN homing receptor CCR7. Also, CCR4, CCR6, and CXCR6 known to facilitate migration toward the skin are expressed more on $CD56^{bright}CD16^{dim}$ than on $CD56^{dim}CD16^{bright}$ cells. However, both subtypes express CCR1 and CCR5 known for inflammatory properties, whereas CCR9 and CXCR5 are expressed only on minor subsets of NK cells (Berahovich et al. 2006). Upon activation with IL-2, NK cells maintain the expression of the constitutive chemokine receptors CXCR4 and CCR7, but similar to T cells there is increased expression of the inflammatory chemokine receptors

CXCR1, CXCR3, CCR1, CCR2, CCR3, CCR6, CCR8, as well as CX_3CR1 in these activated NK cells (Inngjerdingen et al. 2001).

2.2 *Effect of Chemokines on the In Vitro NK Cell Chemotaxis*

The first study reporting the effect of chemokines on NK cells demonstrated that the CXC chemokine CXCL8/IL-8 induces chemokinesis of IL-2-activated NK cells (Sebok et al. 1993). A year later, it was reported that members of the CC chemokines, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES, are chemoattractants for NK cells (Maghazachi et al. 1994). At a similar time, Allavena et al. (1994) reported that CCL2/MCP-1 is a chemoattractant for activated NK cells. Later work showed that CXCL10/IP-10 (Maghazachi et al. 1997; Taub et al. 1995), CXCL12/SDF-1α (Maghazachi 1997), CCL1/I-309 (Inngjerdingen et al. 2000, 2001), CCL2/MCP-1, CCL7/MCP-3, CCL8/MCP-2 (Allavena et al. 1994; Loetscher et al. 1996; Taub et al. 1995), CCL17/TARC (Inngjerdingen et al. 2000), CCL19/MIP-3β (Al-Aoukaty et al. 1998; Kim et al. 1999), CCL20/MIP-3α (Al-Aoukaty et al. 1998), CCL21/SLC (Kim et al. 1999), CCL22/MDC (Godiska et al. 1997; Inngjerdingen et al. 2000), XCL1/Lymphotactin (Bianchi et al. 1996; Hedrick et al. 1997; Maghazachi et al. 1997), and CX_3CL1/Fractalkine (Al-Aoukaty et al. 1998; Fraticelli et al. 2001; Imai et al. 1997) induce the *in vitro* chemotaxis of human NK cells.

2.3 *Effect of Chemokines on the In Situ Accumulation of NK Cells Under Resting Conditions*

2.3.1 Trafficking into the Peripheral LNs

Because $CD56^{bright}CD16^{dim}$ cells express CCR7, a LN homing receptor (Baekkevold et al. 2001), along with their expression of L-selectin adhesion molecule, it was suggested that these cells might home into this organ under normal conditions. Consequently, between 1 and 5% of human $CD56^{bright}CD16^{dim}$ NK cells are found in human peripheral LNs (Fehniger et al. 2003). In contrast to spleen and blood NK cells, which are $CD56^{dim}CD16^{bright}$ and have cytolytic functions, NK cells found in the LNs and tonsils express $CD56^{bright}CD16^{dim}$ and are endowed with IFN-γ secretion (Ferlazzo et al. 2004). However, upon activation with IL-2, these cells become cytolytic. Further study demonstrated that NK cells exposed to IL-18 but not IL-12 are prone to up-regulate CCR7, and consequently migrate into the LNs (Agaugue et al. 2008). Watt et al. (2008) reported that NK cells and in particular $CD27^{high}$ are recruited into dendritic cells draining LNs in IFN-γ-dependent mechanism, suggesting that CXCR3/CXCL10 may be involved since IFN-γ

induces the production of CXCL10/IP-10 chemokine. Taken together, these observations suggest that chemokines contribute to the accumulation of NK cells in secondary lymphoid tissue such as the LNs under both resting and inflammatory conditions.

2.3.2 Localization of NK Cells in the Bone Marrow and Their Egress from This Organ

Before the cells reach the circulation, they must come from housing organs, but it is not clear how this stage of NK cell trafficking is controlled. The bone marrow (BM) is the most important organ in which developments of NK cells take place (Yokoyama et al. 2004). Chemokines may also play a role in NK cell localization in the BM as well as in their egress from this organ. Wald et al. (2006) reported that the IFN-γ/CXCR3/CXCL9 axis is important for the recruitment of NK cells from storage organs such as the BM into the blood circulation. Bernardini et al. (2008) showed that BM immature NK cells express CXCR4 much higher than BM mature NK cells, suggesting a degeneration in CXCR4 receptor expression upon NK cell maturation in the BM. Although CXCR4 is important for the retention of NK cells in this organ, it is observed that mature NK cells that are ready to egress the BM respond chemotactically to CXCR4 ligand, that is, CXCL12/SDF-1α (Bernardini et al. 2008). In addition, BM mature NK cells express CXCR3 and CCR1 and respond to CXCL10/IP-10 and CCL3/MIP-1α, the respective ligands for these receptors. Collectively, these observations indicate that CXCR3, CXCR4, and perhaps CCR1 are important for the egress of NK cells from the BM into the blood circulation.

2.3.3 Trafficking into Decidual and Adenoid Tissues

Uterine NK cells are present in intravascular and perivascular regions of the decidua and are abundant in the mucosal tissues of maternal uterus during early gestation. NK cells are recruited into the uterus from the blood circulation, but this recruitment is independent of CCR2 or CCR5 (Chantakru et al. 2001), suggesting that other chemokine receptors might be involved in this process. It was reported that CXCR4 is expressed on human uterine $CD56^{bright}$ NK cells, which is the major receptor involved in recruiting uterine NK cells into the uterus during early pregnancy where CXCL12/SDF-1α is released by trophoblasts that invade the decidua (Hanna et al. 2003). However, CXCR3/CXCL10 axis is also important for trafficking of NK cells into this site (Van den Heuvel, et al. 2005). Recently, it was observed that, in addition to the above pathways, the CX_3CR1/CX_3CL1 axis may also play a role in recruiting peripheral blood $CD56^{bright}CD16^{dim}$ NK cells into decidual tissues (Carlino et al. 2008).

Similar to uterine NK cells, adenoids $CD56^{bright}CD16^{dim}$ NK cells express CXCR4 (Mizrahi et al. 2007). These cells migrate towards CXCL12/SDF-1α both

in vitro and plausibly in situ since the endothelial cells of the adenoid blood vessels and the epithelial cells of adenoids express this chemokine (Mizrahi et al. 2007).

2.4 Role of Chemokines/Chemokine Receptors in the Sojourn of NK Cells at the Sites of Inflammation

2.4.1 Trafficking into Sites of Tumor Growth

Earlier work showed that NK cells are recruited into tumor growth sites in which tumor cells lack the expression of MHC class I molecules (Glas et al. 2000). This recruitment is facilitated by the release of IFN-γ, suggesting that chemokines such as CXCL9/MIG, CXCL10/IP-10, and CXCL11/I-TAC induced by IFN-γ may play a role in recruiting NK cells toward tumor growth sites. This migration is essential in order for NK cells to eradicate cancer cells. Therapy of neuroblastoma in which the microenvironment is enriched with CX_3CL1/Fractalkine (using a syngeneic model genetically engineered to secrete this chemokine) is successful in these mice as a result of targeted IL-2 therapy. This form of therapy recruits cytolytic cells such as T cells and NK cells towards this microenvironment (Zeng et al. 2007). The role of CX_3CR1/CX_3CL1 axis in NK cell eradication of tumor metastases was also examined by Robinson et al. (2003), who reported that such axis facilitates the binding of NK cells to activated endothelial tissues, which leads to the eventual eradiation of YAC-1 tumor cells localized in the lungs of injected mice.

Administration of adenovirus encoding CCL27/CTAK into OV-HM tumor results in the accumulation of NK cells expressing CCR10 at these sites. However, this does not lead to significant tumor eradication unless IL-12 is also administered intratumorally, suggesting that a combination of cytokines and chemokines may affect the regression of tumor growth via the recruitment and eventual activation of NK cells (Gao et al. 2009). Another study demonstrated that subcutaneous injection of CCL3/MIP-1α in conjunction with IL-2 enhances a protective antineoplastic response against pre-established lymphoblastic disease. This vaccination strategy recruits NK cells towards the tumor sites, resulting in activating these cells and eradicating the tumors (Zibert et al. 2004). Murine breast cancer cell line expressing CCL19/MIP-3β is also rejected by the host due to the secretion of this chemokine that attracts NK cells towards the growth site of breast cancer (Braun et al. 2000). Also transfection of CCL2/MCP-1 gene in lung cancer cells leads to the recruitment and activation of $CD56^{bright}$ NK cells, followed by decreased survival of the lung cancer cells (Nokihara et al. 2000). It is of a great interest that chemokines are not only chemoattractants for NK cells, but in fact they activate these cells to become potent antitumor effector cells. NK cells activated with CCL2/MCP-1, CCL3/MIP-1α, or CCL5/RANTES are designated as CC chemokine-activated NK cells or CHAK cells, which have been shown to exert robust cytolytic activity against tumor cells in

in vitro cytotoxicity assay (Maghazachi et al. 1996). However, the role of CHAK cells in eradicating tumors in situ has not yet been explored.

2.4.2 Trafficking into Sites of Infections

Infection with murine cytomegalovirus (MCMV) results in the accumulation of NK cells in the liver and spleen of infected mice as a consequence to the release of CCL3/MIP-1α, which promotes increased inflammation and decreased susceptibility to the infection with this virus (Salazar-Mather et al. 2002). Interestingly, mouse NK cells secrete XCL1/Lymphotactin, CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES after MCMV infection (Dorner et al. 2004).

Similarly, NK cells isolated from HIV-1 infected individuals produce significant amounts of the chemokines CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES either constitutively or after activation, which inhibit replication of this virus (Oliva et al. 1998). These results are supported by other investigators who reported that the same chemokines are released from NK cells isolated from normal or HIV-1 positive donors (Fehniger et al. 1998). The supernatants collected from these cells exert suppressive activity against HIV-1 replication *in vitro*. Hence, activated NK cells are the major source of CC chemokines both *in vivo* and *in vitro*. In fact, IL-2-activated NK cells highly secrete the CC chemokines CCL3/MIP-1α and CCL4/MIP-1β, which exceed the amount of IFN-γ released by these cells (Rolin et al. 2009). Therefore, it is surprising that IFN-γ is considered the prototype cytokine secreted by these cells. Collectively, these observations suggest that NK cells amplify the inflammatory response by recruiting cells that robustly respond to various chemokines, such as neutrophils, macrophages, and T cells (paracrine effect), or by activating NK cells themselves (autocrine effect).

The expression of CCR5 is up-regulated on NK cells isolated from patients with HIV viremia, which may partly explain the defects of these cells in those patients (Kottilil et al. 2004). The frequency of $CD56^{bright}CCR5^{+}$ NK cells is increased in HIV progressor patients, which is reversed by HAART therapy, suggesting that the expression of CCR5 on NK cells is highly important for HIV disease progression (Jiang et al. 2008). CCR5 is also important for guiding NK cells towards *Toxoplasma gondii* infected tissues (Khan et al. 2006). Without the influx of NK cells, tissues from CCR5 negative mice have increased parasitic infection corroborated with reduced IFN-γ and chemokines secretion. Khan et al. (2006) also speculated that NK cells lacking the expression of CCR5 are unable to migrate into any infected tissue.

Recent study showed that mast cells infected with the RNA retrovirus secrete large quantities of CXCL8/IL-8, and thereafter recruit $CD56^{bright}$ NK cells into the sites of infection (Burke et al. 2008). These results advocate a novel cascade network among mast cells/chemokines/NK cells, which may contribute to eradicating the viral infection.

2.4.3 Trafficking into the Skin and Other Inflammatory Sites

CCR8 may be involved in the migration of NK cells towards the skin, since cutaneous NK cells that express $CD56^{bright}CD16^{dim}$ phenotype highly express CCR8 but lack the expression of CCR7 (Ebert et al. 2006). In psoriatic patients, the plaques are invaded with $CD56^{bright}CD16^{dim}$ NK cells expressing CXCR3 or CCR5, which respond to CXCL10/IP-10 or CCL5/RANTES, respectively (Ottaviani et al. 2006). Hence, CCR8, CXCR3, and CCR5 are important for trafficking of NK cells into inflammatory skin, depending on the type of chemokine secreted by skin cells.

Hanna et al. (2005) reported that, in patients with TAP-2 deficiency, chronically activated NK cells in the lungs up-regulate the expression of CCR2 and are recruited towards CCL2/MCP-1, resulting in granuloma lesion formation. Granuloma formation is considered a part of the defense mechanism since the cells involved in granulomatous inflammation are phagocytic macrophages. During phagocytosis, these cells undergo structural changes to form nodular collections of epithelioid cells. Similar to macrophages, the induction of CCR2 on NK cells may target these cells into inflammatory sites and implicate them in granuloma formation, with subsequent propagation of fibrosis and autoimmunity (Hanna et al. 2005). Further, CCR2 is vital in recruiting NK cells towards the lungs of immunocompromised patients infected with invasive aspergillosis, and that depletion of NK cells in these patients results in greater than twofold rate of mortality (Morrison et al. 2003). Pulmonary granuloma formation is also facilitated by NK cells expressing CCR1 that accumulate at these sites. Shang et al. (2000) demonstrated that these NK cells may shift the cytokine balance at pulmonary granuloma from Th1 to Th2 type of response. Hence, CCR1 knockout mice have less of IL-2 and IFN-γ but more of IL-5 and IL-13 released in the affected lungs (Shang et al. 2000).

2.5 Role of Chemokines in the Accumulation of NK Cells at Autoimmune Sites

2.5.1 Trafficking into the Synovial Fluids

Dalbeth et al. (2004) reported that $CD56^{bright}CD16^{dim}$ cells accumulate in inflamed synovial fluid of patients with rheumatoid arthritis. They suggested that this accumulation may be related to the expression of CCR5 on the surface of these cells, leading to their recruitment towards the concentration gradients of CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES released in inflamed joints. Also, ChemR23 might play a role in recruiting $CD56^{dim}CD16^{bright}$ NK cells towards the synovial fluid of patients with rheumatoid arthritis, as well as other inflammatory tissues of patients with oral lichen planus, since chemrin, the ligand for ChemR23, is produced in large quantities at these sites (Parolini et al. 2007).

2.5.2 Trafficking into Inflamed Liver

Primary biliary cirrhosis is an autoimmune disease of the liver characterized by the presence of antimitochondrial antibodies and progressive destruction of the bile canaliculi within the liver, leading to cholestasis and consequently, scarring, fibrosis, and cirrhosis. In an interesting study, Chuang et al. (2006) reported that the number of NK cells are increased in the liver and blood of patients with primary biliary cirrhosis. They also reported that NK cells are recruited into inflamed sites by the CXCR1/CXCL8 axis (Chuang et al. 2006). These results demonstrate that NK cells may contribute to the pathogenesis of this autoimmune disease.

Also, it was reported that CCR1 is important for trafficking of NK cells into inflammatory liver after concanavalin A-induced hepatitis (Wald et al. 2006). Although this is not an autoimmune disease, nevertheless, the results consistently indicate that NK cells and in particular $CD56^{dim}$ $CD16^{bright}$ migrate into inflamed liver, exacerbating the damage of the liver tissues due to the cytolytic activity of these cells, and their ability to secrete multiple inflammatory cytokines and chemokines.

2.5.3 Trafficking into Inflamed Brains

In CX_3CR1 deficient mice, NK cell recruitment into the central nervous system (CNS) of animals with experimental autoimmune encephalomyelitis (EAE) is impaired, corroborating with increased severity of the disease (Huang et al. 2006). IL-15 down-regulates the expression of CX_3CR1 on $CD56^{bright}CD16^{dim}$ NK cells, which may affect their distribution into inflammatory sites (Sechler et al. 2004). IL-15 also down-regulates the expression of ChemR23 on $CD56^{dim}CD16^{bright}$ cells and prevents them from migrating into sites of inflammatory reactions in which chemrin accumulates (Parolini et al. 2007). These results suggest that IL-15, which is a maturation cytokine for NK cells, may also inhibit the distribution of NK cells into tissues suffering from autoimmune reactions.

What could be the function of NK cells at autoimmune sites in diseases such as EAE/multiple sclerosis (MS)? Conflicting reports have shown that NK cells might either ameliorate EAE or exacerbate the disease. Depletion of NK cells before immunization of sensitive mice with myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) peptide results in clinically more severe relapsing EAE (Zhang et al. 1997). In CX_3CR1 deficient mice mentioned earlier, NK cell recruitment into the cerebrospinal fluid (CSF) of animals with EAE is impaired, corroborating with sever disease incidence (Huang et al. 2006). However, these results contradict others, showing that NK cells exacerbate rather than ameliorate EAE. Pagenstecher et al. (2000) demonstrated that IL-12 released by astrocytes promotes the spontaneous development of NK cells that enhance Th1 activity and cytokine secretion. Further, increased IL-18 production after the primary injection of $MOG_{35–55}$ leads to increased production of IFN-γ secreted by NK cells, which promotes autoreactive Th1 responses, whereas an impaired capacity of NK cells to secrete IFN-γ is

found to be the major mechanism underlying resistance to EAE (Shi et al. 2000). We recently reported that Copaxone (glatiramer acetate; GA), a drug used to treat MS patients, when administered into mice suffering from EAE ameliorates the EAE clinical scores corroborated with isolating NK cells that lyse both immature and mature dendritic cells (Al-Falahi et al. 2009). Also we reported that NK cells exposed to GA *in vitro* lyse both immature and mature dendritic cells, regardless whether these cells are isolated from autologous or allogeneic donors (Sand et al. 2009). Hence, GA by activating NK cells may shut down the Th1 axis pathway perhaps by ridding the system of monocyte-derived dendritic cells that activate autoreactive Th1 cells. The fact that NK cells exposed to GA kill both immature and mature dendritic cells ensures that no antigen presentation would be available to autoreactive Th1 cells. Therefore, it can be suggested that one mechanism of GA amelioration of EAE/MS might be due to activating NK cells that may contribute to reducing the incidence of relapse and increasing the period of remission in MS patients.

2.6 Trafficking into Target Organs of GvH Disease

In addition to the release of inflammatory cytokines and chemokines, GvH disease is characterized by potentiating the activity of antigen-presenting cells and by the infiltration of immune cells into the sites affected by this disease. Not much is known about the effect of chemokines on NK cells during GvH disease. The effects on T cells has, however, been investigated. In an important study, Mapara et al. (2006) showed that target organs of GvH disease, such as the liver, skin, and gastrointestinal (GI) tract, up-regulate the expression of chemokines after mismatched allogeneic BMT in mice that have been conditioned by myeloablative regiments. These authors reported that CCL1/I-309, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES mRNA are expressed in the liver of GvH disease-affected animals, five and seven days post-transplantation. Similarly, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, and CCL11/Eotaxin mRNA are up-regulated in the colon of allogeneic recipients. On the protein level, CXCL10/IP-10, CCL2/MCP-1, CCL3/MIP-1α, and CCL5/RANTES are increased in the serum of animals with GvH disease, whereas CXCL10/IP-10, CCL2/MCP-1, CCL3/ MIP-1α, and CCL5/RANTES are highly increased in colon tissues between 3 and 6 days after BMT. Interestingly, chemokine levels are increased in the gut before the infiltration of T cells into this target organ, suggesting that chemokines play important roles in recruiting inflammatory T cells into target organs of GvH disease. As mentioned earlier, NK cells respond chemotactically to CCL1/I-309, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, and CXCL10/IP-10. Therefore, it is highly plausible that these cells are recruited into target tissues of GvH disease due to the inflammatory processes taking place at these sites, and consequently, the release of inflammatory chemokines. Supporting this concept is the finding that NK cells infiltrate the IP-$10^{+/+}$ allograft rapidly after MHC

mismatch transplantation in mice (Hancock et al. 2001). This occurs as a consequence to the release of CXCL10/IP-10 by endothelial cells, which promotes the initial recruitment of NK cells into the allograft (Hancock et al. 2001).

A recent report showed a complex chemokine/chemokine receptors up-regulation taking place during acute GvH disease (Bouazzaoui et al. 2009). These authors reported increased CXCR3/CCL9/CCL10/CCL11 expression in GvH target organs. They also demonstrated that CXCR6/CXCL16 and CCR5/CCL3 are important for the recruitment of T cells into the intestine at later time points, whereas CXCR2/CXCL1 axis may contribute to hepatic tissue injury. In addition, the XCR1/XCL1 axis may play a role in promoting the inflammatory response observed during GvH disease (Bouazzaoui et al. 2009). As mentioned, NK cells highly express CXCR3 and XCR1 and respond to CXCL10/IP-10 and XCL1/Lymphotactin. These cells also infiltrate inflamed liver and skin as described earlier.

Interestingly, methylprednisolone, a drug used to treat GvH disease in patients undergoing hemopoietic SCT, when cultured with hemopoietic CD34 precursor cells induces these cells to become immature NK cells. This drug also induces the maturation of NK cells since they start to express the NK cytotoxicity receptor NKp46 as well as NKG2D and DNAM-1 (Vitale et al. 2008). They also become highly cytolytic, which may partly explain the efficacy of this drug in reducing GvH disease. In contrast, methylprednisolone inhibits the immunoregulatory activity of NK cells, since incubating hemopoietic $CD34^{+}$ cells with this drug abrogates their ability to secrete CXCL8/IL-8. Hence, this drug may reduce the inflammatory property of NK cells during GvH disease.

3 Summary and Concluding Remarks

It is clear that NK cells must leave the blood circulation and extravasate into various tissues to fight the invaders and at the same time interact with cells of both arms of the immune system, that is, innate immune cells such as dendritic cells and adaptive immune cells such as T cells. Importantly, NK cells have selected multiple pathways to populate various tissues. First, NK cells must leave the storage sites where they are generated (e.g., bone marrow) and enter the blood circulation. This process is facilitated by their expression of chemokine receptors CXCR3, CXCR4, and perhaps CCR1. In the blood circulation, they express markers such as CD56 and CD16, along with others. How this happens is not clear, although it is accepted at a face value that there are at least two subsets of NK cells in the blood circulation characterized by the density of CD56 and CD16 markers on their surfaces. These are designated as $CD56^{dim}CD16^{bright}$ and $CD56^{bright}CD16^{dim}$ that must make the critical decision of where to go next. The consensus is that the $CD56^{dim}CD16^{bright}$ subset migrates into tissues under homoeostatic conditions (this term is used in a cavalier way since all humans have been exposed to environmental insults and all cell types must have been activated at one point in their history. It is easier to study the actual resting conditions in pathogen-free animals, but the relevance of these

animals to humans is conjectural, at best). It appears that the trafficking of NK cells under these conditions is regulated by specific sets of chemokines/chemokine receptors. It is plausible that $CD56^{bright}CD16^{dim}$ distribute into noninflamed peripheral LNs because they express the chemokine receptor CCR7 and respond to CCL19 and CCL21 present in the high endothelial venules of the peripheral LNs. On the other hand, CXCR4/CXCL12 axis is important for their homing into adenoids tissues or decidual uterus, albeit uterine NK cells may also utilize CXCR3/CXCL10 or CX_3CR1/CX_3CL1 axis for accumulation in the decidual uterus.

Depending on the site and the chemokine secreted, it seems that $CD56^{dim}$ $CD16^{bright}$ extravasate into inflamed liver or lungs. The ChemR23/Chemrin axis may guide $CD56^{dim}CD16^{bright}$ NK cells into the skin (and perhaps other tissues) during active oral lichen planus disease. The CXCR3/CXCL10 and CCR1/CCL3/CCL4/CCL5 are important for the accumulation of $CD56^{dim}CD16^{bright}$ in inflamed liver. On the other hand, $CD56^{bright}CD16^{dim}$ NK cells distribute into inflamed skin guided by CXCR3/CXCL10, CCR5/CCL5, or CCR8/CCL17 axis. There is a consensus that CCR5/CCL3/CCL4/CCL5 axis is most important for guiding activated NK cells towards virally infected tissues such as HIV-infected sites or CMV-infected cells. CCR5 is also exquisite in their accumulation at sites of parasitic infections such as those infected with *T. Gondii*. There are also reports showing that CCR2/CCL2 axis contributes to the trafficking of NK cells into the lungs of TAP-2 patients or pulmonary granuloma formation, but the subtype of NK cells distributing into the lungs has not been described. One might assume that, similar to the liver, it is the $CD56^{dim}CD16^{bright}$ cells that may be endowed with this property.

The other site where NK cells accumulate are tissues affected with autoimmune diseases. In the synovial fluids of patients with rheumatoid arthritis, it is the CCR5/CCL3/CCL4/CCL5 axis that is important for recruiting $CD56^{bright}CD16^{dim}$ NK cells into inflamed joints. Published reports indicate that the CX_3CR1/CX_3CL1 axis is responsible for recruiting these cells into inflamed brain and CSF of EAE mice, which could also be true for MS patients, whereas the CXCR1/CXCL8 axis is important for trafficking of $CD56^{dim}CD16^{bright}$ NK cells into the liver of primary biliary cirrhosis patients. Figure 1 summarizes these findings.

It should also be stressed that NK cells play essential role in reducing GvH disease resulting from allogeneic transplantation procedures, but the factors that affect their distribution into GvH target organs such as the liver, skin, and GI tract are not know. Because the inflammatory chemokines CCL1/I-309, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, and CXCL10/IP-10 are highly increased in these sites, it would not be surprising to demonstrate that these chemokines may recruit NK cells into GvH disease target organs.

Although it has been more than 30 years since the migration of NK cells is established, this aspect of NK cell biology is still unresolved. Therefore, it is pertinent to continue investigating how NK cells traffick into various tissues. This field should yield highly important information that can be utilized to build rational protocols to treat diseases such as cancer, autoimmune diseases, inflammatory diseases, hematological diseases, and infectious diseases.

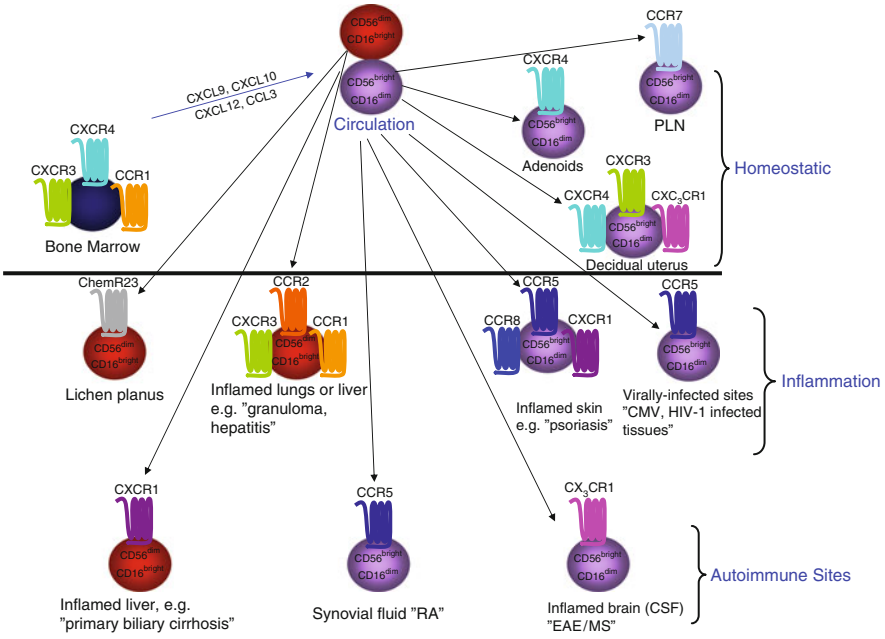

Fig. 1 Summary of current knowledge regarding the accumulation of NK cells at various sites. First, NK cells must egress the storage organs such as the bone marrow and enter the blood circulation guided by CXCR3/CCL9/CCL10, CXCR4/CXCL12, and/or CCR1/CCL3 axis. From the blood, NK cells extravasate into the peripheral lymph nodes (LNs), decidual uterus or adenoids under homeostatic conditions. However, they must also distribute into the lungs, liver, kidneys, to name a few, under similar conditions, but reports concerning such distribution are lacking. Trafficking under homeostatic conditions is dominated by cells that are $CD56^{bright}CD16^{dim}$. On the other hand, both $CD56^{bright}CD16^{dim}$ and $CD56^{dim}CD16^{bright}$ migrate into inflammatory sites. CCR5 is important for the accumulation of NK cells at sites of viral infections. The third site of accumulation is inside tissues affected by autoimmune reactions. The published reports about this subject indicate that both $CD56^{bright}CD16^{dim}$ and $CD56^{dim}CD16^{bright}$ NK cells distribute into these sites. Of note: all chemokine receptors are seven transmembrane spanning domains or G protein-coupled receptors

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References

Agaugue S, Marcenaro E, Ferranti B, Moretta L, Moretta A (2008) Human natural killer cells exposed to IL-2, IL-12, IL-18 or IL-4 differently modulate priming of naïve T cells by monocyte-derived dendritic cells. Blood 112:1776–1783

Al-Aoukaty A, Rolstad B, Giaid A, Maghazachi AA (1998) MIP-3α MIP-3β and fractalkine induce the locomotion and the mobilization of intracellular calcium, and activate the hetero-trimeric G proteins in human natural killer cells. Immunology 95:618–624

Albertsson PA, Basse PH, Hokland M, Goldfarb RH, Nagelkerke JF, Nannmark U, Kuppen PJ (2003) NK cells and the tumour microenvironment: implications for NK-cell function and anti-tumour activity. Trends Immunol 24:603–609

Al-Falahi Y, Sand KL, Knudsen E, Rolin J, Maghazachi AA (2009) Splenic natural killer cell activity in two models of experimental neurodegenerative diseases. J Cell Mol Med 13:2693–2703

Allavena P, Bianchi G, Zhou D, van Damme J, Jílek P, Sozzani S, Mantovani A (1994) Induction of natural killer cell migration by monocyte chemotactic protein-1, -2, and -3. Eur J Immunol 24:3233–3236

Asai O, Longo DL, Tian ZG, Hornung RL, Taub DD, Ruscetti FW, Murphy WJ (1998) Suppression of graft-versus-host disease and amplification of graft-versus-tumor effects by activated natural killer cells after allogeneic bone marrow transplantation. J Clin Invest 101:1835–1842

Baekkevold ES, Yamanaka T, Palframan RT, Carlsen HS, Reinholt FP, von Andrian UH, Brandtzaeg P, Haraldsen G (2001) The CCR7 ligand ELC (CCL19) is transcytosed in high endothelial venules and mediates T cell recruitment. J Exp Med 193:1105–1111

Baggiolini M (2001) Chemokines in pathology and medicine. J Intern Med 250:91–104

Basse PH, Herberman RB, Nannmark U (1991a) Accumulation of adoptively transferred adherent, lymphokine-activated killer cells in murine metastases. J Exp Med 174:479–488

Basse PH, Nannmark U, Johansson BR, Herberman RB, Goldfarb RH (1991b) Establishment of cell-to-cell contact by adoptively transferred adherent lymphokine-activated killer cells with metastatic murine melanoma cells. J Natl Cancer Inst 83:944–950

Berahovich RD, Lai NL, Wei Z, Lanier LL, Schall TJ (2006) Evidence for NK cell subsets based on chemokines receptor expression. J Immunol 177:7833–7840

Bernardini G, Sciumè G, Bosisio D, Morrone S, Sozzani S, Santoni A (2008) CCL3 and CXCL12 regulate trafficking of mouse bone marrow NK cell subsets. Blood 111:3626–3634

Bianchi G, Sozzani S, Zlotnik A, Mantovani A, Allavena P (1996) Migratory response of human natural killer cells to lymphotactin. Eur J Immunol 26:3238–3241

Bouazzaoui A, Spacenko E, Mueller G, Miklos S, Huber E, Holler E, Andreesen R, Hildebrandt GC (2009) Chemokine and chemokine receptor expression analysis in target organs of acute graft-versus-host disease. Genes Immun (Epub ahead of print)

Braun SE, Chen K, Foster RG, Kim CH, Hromas R, Kaplan MH, Broxmeyer HE, Cornetta K (2000) The CC chemokine CK beta-11/MIP-3 beta/ELC/Exodus 3 mediates tumor rejection of murine breast cancer cells through NK cells. J Immunol 164:4025–4031

Bruserud Ø, Kittang AO (2010) Chemokines in experimental and clinical hematology. Curr Top Microbiol Immunol (in press)

Burke SM, Issekutz TB, Mohan K, Lee PW, Shmulevitz M, Marshall JS (2008) Human mast cell activation with virus-associated stimuli leads to the selective chemotaxis of natural killer cells by a CXCL8-dependent mechanism. Blood 111:5467–5476

Campbell JJ, Qin S, Unutmaz D, Soler D, Murphy KE, Hodge MR, Wu L, Butcher EC (2001) Unique subpopulations of $CD56^{+}$ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. J Immunol 166:6477–6482

Carlino C, Stabile H, Morrone S, Bulla R, Soriani A, Agostinis C, Bossi F, Mocci C, Sarazani F, Tedesco F, Santoni A, Gismondi A (2008) Recruitment of circulating NK cells through decidual tissues: a possibile mechanism controlling NK cell accumulation in the uterus during early pregnancy. Blood 111:3108–3115

Chantakru S, Kuziel WA, Maeda N, Croy BA (2001) A study on the density and distribution of uterine natural killer cells at mid pregnancy in mice genetically-ablated for CCR2, CCR 5 and the CCR5 receptor ligand, MIP-1α. J Reprod Immunol 49:33–47

Chensue SW (2000) Molecular machinations: chemokine signals in host–pathogen interactions. Clin Microbiol Rev 14:821–835

Chiesa MD, Vitale M, Carlomagno S, Ferlazzo G, Moretta L, Moretta A (2003) The natural killer cell-mediated killing of autologous dendritic cells is confined to a cell subset expressing CD94/NKG2A, but lacking inhibitory killer Ig-like receptors. Eur J Immunol 33:1657–1666

Chuang Y-H, Lian Z-X, Tsuneyama K, Chiang BL, Ansari AA, Coppel RL, Gershwin ME (2006) Increased killing activity and decreased cytokine production in NK cells in patients with primari biliary cirrhosis. J Autoimmun 26:232–240

Dalbeth N, Gundle R, Davies RJ, Lee YC, McMichael AJ, Callan MF (2004) $CD56^{bright}$ NK cells are enriched at inflammatory sites and can engage with monocytes in a reciprocal program of activation. J Immunol 173:6418–6426

Dorner BG, Smith HR, French AR, Kim S, Poursine-Laurent J, Beckman DL, Pingel JT, Kroczek RA, Yokoyama WM (2004) Coordinate expression of cytokines and chemokines by NK cells during murine cytomegalovirus infection. J Immunol 172:3119–3131

Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 3:991–998

Ebert LM, Meuter S, Moser B (2006) Homing and function of human skin γδ T cells and NK cells: relevance for tumor surveillance. J Immunol 176:4331–4336

Fehniger TA, Herbein G, Yu H, Para MI, Bernstein ZP, O'Brien WA, Caligiuri MA (1998) Natural killer cells from $HIV\text{-}1^{+}$ patients produce C-C chemokines and inhibit HIV-1 infection. J Immunol 161:6433–6438

Fehniger TA, Cooper MA, Nuovo GJ, Cella M, Facchetti F, Colonna M, Caligiuri MA (2003) $CD56^{bright}$ natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. Blood 101: 3052–3057

Ferlazzo G, Thomas D, Lin S-L, Goodman K, Morandi B, Muller WA, Moretta A, Münz C (2004) The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic. J Immunol 172:1455–1462

Franitza S, Grabovsky V, Wald O, Weiss I, Beider K, Dagan M, Darash-Yahana M, Nagler A, Brocke S, Galun E, Alon R, Peled A (2004) Differential usage of VLA-4 and CXCR4 by $CD3^{+}CD56^{+}$ NKT cells and $CD56^{+}CD16^{+}$ NK cells regulates their interaction with endothelial cells. Eur J Immunol 34:1–9

Fraticelli P, Sironi M, Bianchi G, D'Ambrosio D, Albanesi C, Stoppacciaro A, Chieppa M, Allavena P, Ruco L, Girolomoni G, Sinigaglia F, Vecchi A, Mantovani A (2001) Fractalkine (CX3CL1) as an amplification circuit of polarized Th1 responses. J Clin Invest 107:1173–1181

Gao J-Q, Tsuda Y, Han M, Xu DH, Kanagawa N, Hatanaka Y, Tani Y, Mizuguchi H, Tsutsumi Y, Mayumi T, Okada N, Nakagawa S (2009) NK cells are migrated and indispensabile in the anti-tumor activity induced by CCL27 gene therapy. Cancer Immunol Immunother 58:291–299

Glas R, Franksson L, Une C, Eloranta ML, Ohlén C, Orn A, Kärre K (2000) Recruitment and activation of natural killer (NK) cells *in vivo* determined by the target cell phenotype: an adaptive component of NK cell-mediated responses. J Exp Med 191:129–138

Goda S, Inoue H, Umehara H, Miyaji M, Nagano Y, Harakawa N, Imai H, Lee P, Macarthy JB, Ikeo T, Domae N, Shimizu Y, Iida J (2006) Matrix metalloproteinase-1 produced by human CXCL12-stimulated natural killer cells. Am J Pathol 169:445–458

Godiska R, Chantry D, Raport CJ, Sozzani S, Allavena P, Leviten D, Mantovani A, Gray PW (1997) Human macrophage-derived chemokine (MDC), a novel chemoattractant for monocytes, monocyte-derived dendritic cells, and natural killer cells. J Exp Med 185:1595–1604

Hancock WW, Gao W, Csizmadia V, Faia KL, Shemmeri N, Luster AD (2001) Donor-derived IP-10 initiates development of acute allograft rejection. J Exp Med 193:975–980

Hanna J, Wald O, Wohl-Goldman D, Prus D, Markel G, Gazit R, Katz G, Haimov-Kochman R, Fujii N, Yagel S, Peled A, Mandelboim O (2003) CXCL12 expression by invasive trophoblasts induces the specific migration of $CD16^{-}$ human natural killer cells. Blood 102:1569–1577

Hanna J, Mussaffi H, Steuer G (2005) Functional aberrant expression of CCR2 receptor on chronically activated NK cells in patients with TAP-2 deficiency. Blood 106:3465–3474

Hedrick JA, Saylor V, Figueroa D, Mizoue L, Xu Y, Menon S, Abrams J, Handel T, Zlotnik A (1997) Lymphotactin is produced by NK cells and attracts both NK cells and T cells *in vivo*. J Immunol 158:1533–1540

Hokland M, Kjaergaard J, Kuppen PJ, Nannmark U, Agger R, Hokland P, Basse P (1999) Endogenous and adoptively transferred A-NK and T-LAK cells continuously accumulate within murine metastases up to 48 h after inoculation. In Vivo 13:199–204

Huang D, Shi F-D, Jung S, Pien GC, Wang J, Salazar-Mather TP, He TT, Weaver JT, Ljunggren HG, Biron CA, Littman DR, Ransohoff RM (2006) The neuronal chemokine CX3CL1/fractalkine selectively recruits NK cells that modify experimental autoimmune encephalomyelitis within the central nervous system. FASEB J 20:896–905

Hyakudomi M, Matsubara T, Hyakudomi R, Yamamoto T, Kinugasa S, Yamanoi A, Maruyama R, Tanaka T (2008) Increased expression of fraktaline is correlated with a better prognosis and an increased number of both $CD8^+$ T cells and natural killer cells in gastric adenocarcinoma. Ann Surg Oncol 15:1775–1782

Imai T, Hieshima K, Haskell C, Baba M, Nagira M, Nishimura M, Kakizaki M, Takagi S, Nomiyama H, Schall TJ, Yoshie O (1997) Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. Cell 91:521–530

Inngjerdingen M, Damaj B, Maghazachi AA (2000) Human NK cells express CC chemokine receptors 4 and 8 and respond to thymus and activation-regulated chemokine, macrophage-derived chemokine, and I-309. J Immunol 164:4048–4054

Inngjerdingen M, Damaj B, Maghazachi AA (2001) Expression and regulation of chemokine receptors in human natural killer cells. Blood 97:367–375

Jiang Y, Zhang Z, Diao Y, Jin X, Shi W, Geng W, Dai D, Zhang M, Han X, Liu J, Wang Y, Shang H (2008) Expression of chemokines receptors on natural killer cells in HIV-infected individuals. Cell Immunol 251:19–24

Kammula US, White DE, Rosenberg SA (1998) Trends in the safety of high dose bolus interleukin-2 administration in patients with metastatic cancer. Cancer 83:797–805

Khan IA, Thomas SY, Moretto MM, Lee FS, Islam SA, Combe C, Schwartzman JD, Luster AD (2006) CCR5 is essential for NK cell trafficking and host survival following *Toxoplasma gondii* infection. PLoS Pathogens 2:484–500

Kim CH, Pelus LM, Appelbaum E, Johanson K, Anzai N, Broxmeyer HE (1999) CCR7 ligands, SLC/6Ckine/Exodus2/TCA4 and CKβ-11/MIP-3β/ELC, are chemoattractants for $CD56^+CD16^-$ NK cells and late stage lymphoid progenitors. Cell Immunol 193:226–235

Kim MH, Kitson RP, Albertsson P, Nannmark U, Basse PH, Kuppen PJ, Hokland ME, Goldfarb RH (2000) Secreted and membrane-associated matrix metalloproteinases of IL-2-activated NK cells and their inhibitors. J Immunol 164:5883–5889

Kottilil S, Shin K, Planta M, McLaughlin M, Hallahan CW, Ghany M, Chun TW, Sneller MC, Fauci AS (2004) Expression of chemokine and inhibitory receptors on natural killer cells: effect of immune activation and HIV viremia. J Infect Dis 189:1193–1198

Ljunggren HG, Karre K (1990) In search of the "missing self". MHC molecules and NK cell recognition. Immunol Today 11:237–244

Loetscher P, Seitz M, Clark-Lewis I, Baggiolini M, Moser B (1996) Activation of NK cells by CC chemokines. Chemotaxis, Ca2+ mobilization, and enzyme release. J Immunol 156:322–327

Long EO (1999) Regulation of immune responses through inhibitory receptors. Annu Rev Immunol 199:875–904

Loza MJ, Perussia B (2004) The IL-12 signature: NK cell terminal $CD56^{+high}$ stage and effector functions. J Immunol 172:88–96

Luster AD (2002) The role of chemokines in linking innate and adaptive immunity. Curr Opin Immunol 14:29–35

Maghazachi AA (1997) Role of the heterotrimeric G proteins in stromal-derived factor-1α-induced natural killer cell chemotaxis and calcium mobilization. Biochem Biophys Res Commun 236:270–274

Maghazachi AA (2003) G protein-coupled receptors in natural killer cells. J Leukoc Biol 74:16–24

Maghazachi AA (2005a) Compartmentalization of human natural killer cells. Mol Immunol 42:523–529

Maghazachi AA (2005b) Insights into seven and single transmembrane-spanning domain receptors and their signalling pathways in human natural killer cells. Pharmacol Rev 57:339–357
Maghazachi AA, Al-Aoukaty A (1998) Chemokines activate natural killer cells through heterotrimeric G-proteins: implications for the treatment of AIDS and cancer. FASEB J 12:913–924
Maghazachi AA, Fitzgibbon L (1990) Fate of intravenously administered rat lymphokine-activated killer cells labeled with different markers. Cancer Immunol Immunother 31:139–145
Maghazachi AA, Al-Aoukaty A, Schall TJ (1994) C-C chemokines induce the chemotaxis of NK and IL-2-activated NK cells. Role for G proteins. J Immunol 153:4969–4977
Maghazachi AA, Al-Aoukaty A, Schall TJ (1996) CC chemokines induce the generation of killer cells from $CD56^{+}$ cells. Eur J Immunol 26:315–319
Maghazachi AA, Skålhegg BS, Rolstad B, Al-Aoukaty A (1997) Interferon-inducible protein-10 and lymphotactin induce the chemotaxis and mobilization of intracellular calcium in natural killer cells through pertussis toxin-sensitive and -insensitive heterotrimeric G-proteins. FASEB J 11:765–774
Mapara MY, Leng C, Kim YM, Bronson R, Lokshin A, Luster A, Meukoc S (2006) Expression of chemokines in GVHD target organs is influenced by conditioning and genetic factors and amplified by GVHR. Biol Blood Marrow Transplant 12:623–634
Mizrahi S, Yefenof E, Gross M, Attal P, Ben Yaakov A, Goldman-Wohl D, Maly B, Stern N, Katz G, Gazit R, Sionov RV, Mandelboim O, Chaushu S (2007) A phenotypic and functional characterization of NK cells in adenoids. J Leukoc Biol 82:1095–1105
Moretta A (2002) Natural killer cells and dendritic cells: rendezvous in abused tissues. Nat Rev Immunol 2:957–964
Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, Biassoni R, Moretta L (2001) Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol 19:197–223
Morrison BE, Park SJ, Mooney LM, Mehrad B (2003) Chemokine-mediated recruitment of NK cells is a critical host defense mechanism in invasive aspergillosis. J Clin Invest 112:1862–1870
Murphy PM, Baggiolini M, Charo IF, Hébert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, Power CA (2000) International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Rev 52:145–176
Nannmark U, Hokland ME, Agger R (2000) Tumor blood supply and tumor localization by adoptively transferred IL-2 activated natural killer cells. In Vivo 14:651–658
Nokihara H, Yanagawa H, Nishioka Y, Yano S, Mukaida N, Matsushima K, Sone S (2000) Natural killer cell-dependent suppression of systemic spread of human lung adenocarcinoma cells by monocyte chemoattractant protein-1 gene transfection in severe combined immunodeficient mice. Cancer Res 60:7002–7007
Oliva A, Kinter AL, Vaccarezza M, Rubbert A, Catanzaro A, Moir S, Monaco J, Ehler L, Mizell S, Jackson R, Li Y, Romano JW, Fauci AS (1998) Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication *in vitro*. J Clin Invest 102:223–231
Olsnes AM, Hatfield KJ, Bruserud Ø (2009) The chemokine system and its contribution to leukemogenesis and treatment responsiveness in patients with acute myeloid leukemia. J BUON 14(Suppl 1):S131–S140
Ottaviani C, Nasorri F, Bedini C, de Pità O, Girolomoni G, Cavani A (2006) $CD56^{bright}CD16^{-}$ NK cells accumulate in psoriatic skin in response to CXCL10 and CCL5 and exacerbate skin inflammation. Eur J Immunol 36:118–128
Pagenstecher A, Lassmann S, Carson MJ, Kincaid CL, Stalder AK, Campbell IL (2000) Astrocyte-targeted expression of IL-12 induces active cellular immune responses in the central nervous system and modulates experimental allergic encephalomyelitis. J Immunol 164:4481–4492
Parolini S, Santoro A, Marcenaro E (2007) The role of chemerin in the colocalization of NK and dendritic cell subsets into inflamed tissues. Blood 109:3625–3632

Robertson MJ (2002) Role of chemokines in the biology of natural killer cells. J Leukoc Biol 71:173–183

Robinson LA, Nataraj C, Thomas DW, Cosby JM, Griffiths R, Bautch VL, Patel DD, Coffman TM (2003) The chemokines CX_3CL1 regulates NK cell activity *in vivo*. Cell Immunol 225:122–130

Rolin J, Sand KL, Knudsen E, Maghazachi AA (2010) FTY720 and SEW2871 reverse the inhibitory effect of S1P on natural killer cell mediated lysis of K562 tumor cells and dendritic cells but not on cytokine release. Cancer Immunol Immunother. 59:575–586

Rolstad B, Herberman RB, Reynolds CW (1986) Natural killer cell activity in the rat. V. The circulation patterns and tissue localization of peripheral blood large granular lymphocytes (LGL). J Immunol 136:2800–2808

Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, Posati S, Rogaia D, Frassoni F, Aversa F, Martelli MF, Velardi A (2002) Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 295:2097–2100

Salazar-Mather TP, Lewis CA, Biron CA (2002) Type I interferons regulate inflammatory cell trafficking and macrophage inflammatory protein 1α delivery to the liver. J Clin Invest 110:321–330

Sand KL, Knudsen E, Rolin J, Al-Falahi Y, Maghazachi AA (2009) Modulation of natural killer cell cytotoxicity and cytokine release by the drug glatiramer acetate. Cell Mol Life Sci 66:1446–1456

Sasaki A, Jain RK, Maghazachi AA, Goldfarb RH, Herberman RB (1989) Low deformability of lymphokine-activated killer cells as a possible determinant of *in vivo* distribution. Cancer Res 49:3742–3746

Sebok K, Woodside D, Al-Aoukaty A, Ho AD, Gluck S, Maghazachi AA (1993) IL-8 induces the locomotion of human IL-2-activated natural killer cells. Involvement of a guanine nucleotide binding (G_o) protein. J Immunol 150:1524–1534

Sechler JM, Barlic J, Grivel J-C, Murphy PM (2004) IL-15 alters expression and function of the chemokines receptor CX3CR1 in human NK cells. Cell Immunol 230:99–108

Shang X, Qiu B, Frait KA, Hu JS, Sonstein J, Curtis JL, Lu B, Gerard C, Chensue SW (2000) Chemokine receptor 1 knockout abrogates natural killer cell recruitment and impairs type-1 cytokines in lymphoid tissue during pulmonary granuloma formation. Am J Pathol 157:2055–2063

Shi F-D, Takeda K, Akira S, Sarvetnick N, Ljunggren HG (2000) IL-18 directs autoreactive T cells and promotes autodestruction in the central nervous system via induction of IFN-γ by NK cells. J Immunol 165:3099–3104

Taub D (1999) Natural killer cell-chemokine interactions. Biologic effects of natural killer cell trafficking and cytolysis. In: Rollin BJ (ed) Chemokines and cancer. Humana Press, NJ

Taub DD, Sayers TJ, Carter CRD, Ortaldo JR (1995) α and β Chemokines induce NK cell migration and enhance NK-mediated cytolysis. J Immunol 155:3877–3888

van den Heuvel MJ, Chantakru S, Xuemei X, Evans SS, Tekpetey F, Mote PA, Clarke CL, Croy BA (2005) Trafficking of circulating pro-NK cells to the decidualizing uterus: regulatory mechanisms in the mouse of human. Immunol Invest 34:273–293

Velardi A, Ruggeri L, Alessandro M, Moretta L (2002) NK cells: a lesson from mismatched hematopoietic transplantation. Trends Immunol 23:438–444

Velardi A, Ruggeri L, Mancusi A, Aversa F, Christiansen FT (2009) Natural killer cell allorecognition of missing self in allogeneic hematopoietic transplantation: a tool for immunotherapy of leukemia. Curr Opin Immunol 21:525–530

Villegas FR, Coca S, Villarrubia VG, Jiménez R, Chillón MJ, Jareño J, Zuil M, Callol L (2002) Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. Lung Cancer 35:23–28

Vitale C, Cottalasso F, Montaldo E, Moretta L, Mingari MC (2008) Methylprednisolone induces preferential and rapid differentiation of $CD34^+$ cord blood precursors toward NK cells. Int Immunol 20:565–575

Voutsadakis IA (2003) NK cells in allogeneic bone marrow transplantation. Cancer Immunol Immunother 52:525–534

Wald O, Weiss ID, Wald H (2006) IFN-γ acts on T cells to induce NK cell mobilization and accumulation in target organs. J Immunol 176:4716–4729

Watt SV, Andrews DA, Takeda K, Smyth MJ, Hayakawa Y (2008) IFN-γ-dependent recruitment of mature $CD27^{high}$ NK cells to lymph nodes primed by dendritic cells. J Immunol 181:5323–5330

Yokoyama WM, Kim S, French AR (2004) The dynamic life of natural killer cells. Annu Rev Immunol 22:405–29

Young HA, Ortaldo J (2006) Cytokines as critical co-stimulatory molecules in modulating the immune response of natural killer cells. Cell Res 16:20–24

Zeng Y, Huebener N, Fest S, Weixler S, Schroeder U, Gaedicke G, Xiang R, Schramm A, Eggert A, Reisfeld RA, Lode HN (2007) Fractalkine (CX3CL1)- and interleukin-2-enriched neuroblastoma microenvironment induces eradication of metastases mediated by T cells and natural killer cells. Cancer Res 67:2331–2338

Zhang B, Yamamura T, Kondo T, Fujiwara M, Tabira T (1997) Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. J Exp Med 186:1677–1687

Zibert A, Balzer S, Souquet M, Quang TH, Paris-Scholz C, Roskrow M, Dilloo D (2004) CCL3/MIP-1alpha is a potent immunostimulator when coexpressed with interleukin-2 or granulocyte-macrophage colony-stimulating factor in a leukemia/lymphoma vaccine. Hum Gene Ther 15:21–34

Chemokines in Angiogenesis

Anna Dimberg

Contents

Abstract Chemokines are a family of small heparin-binding proteins, mostly known for their role in inflammation and immune surveillance, which have emerged as important regulators of angiogenesis. Chemokines influence angiogenesis either through recruitment of pro-angiogenic immune cells and endothelial progenitors to the neo-vascular niche or via direct regulation of endothelial function

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downstream of activation of G-protein coupled chemokine receptors. The dual function of chemokines in regulating immune response and angiogenesis confers a central role in modulating the tissue microenvironment. Therefore, chemokines may constitute attractive targets for therapeutic intervention in several pathological disorders. This review will summarize the current understanding of the role of chemokines in angiogenesis, and give an overview of angiostatic and angiogenic chemokines and their crosstalk with other angiogenic factors.

1 Introduction

Angiogenesis is required for embryonic development and physiological functions, but may also affect the outcome of pathological conditions such as cancer, chronic inflammation, and ischemia. Vascular growth and remodeling is a rare event in adults, with the exception of the female menstrual cycle. However, angiogenesis is readily induced when a need for new vasculature arises, for example, during tissue ischemia or wound healing, through a shift in the balance between endogenous pro- and anti-angiogenic factors. For instance, various oxygen-sensing mechanisms directly induce angiogenesis through stabilization of members of the hypoxia-inducible transcription factor (HIF) family that up-regulate expression of pro-angiogenic molecules, including vascular endothelial growth factor (VEGF) (Fraisl et al. 2009). VEGF is critical for embryonic vascular development through binding to its cognate receptor VEGFR2, and is a central mediator of physiological and pathological angiogenesis and vascular function in the adult (Olsson et al. 2006). Using various *in vitro* and *in vivo* assays modeling angiogenesis (Table 1), several other pro- and anti-angiogenic factors have been identified, which act in concert in the tight regulation of blood vessel formation in health and disease. In addition, non-endothelial cell types participate in the angiogenic process either through secretion of factors or via stabilization of the growing vasculature.

Blood vessel formation may occur through several distinct mechanisms (Adams and Alitalo 2007). In sprouting angiogenesis, pro-angiogenic growth factors activate endothelial cells in pre-existing vessels and stimulate invasion of endothelial cells into the surrounding matrix through expression of proteases. Endothelial cells then proliferate and migrate towards the growth factor gradient as a growing stalk, guided by a specialized tip-cell that probes the microenvironment using multiple filopodial extensions. Lumenized growing stalks, often surrounded by stabilizing pericytes, then make contacts through tip-cell filopodia and fuse, allowing blood flow in the newly formed vasculature. Alternatively, new vessels may form through pillar formation in intussuceptive growth (splitting of vessels) (Makanya et al. 2009) or through non-angiogenic biomechanical extension of the existing vasculature (Kilarski et al. 2009). Finally, circulating endothelial progenitor cells are mobilized during tissue ischemia and may be recruited to hypoxic tissue, and contribute to neovascularization in a process reminiscent of embryonic vasculogenesis (Jujo et al. 2008).

Table 1 Angiogenesis assays used to study chemokine function

Assay	Description
Transfilter assay – modified Boyden chamber	Endothelial cells are seeded on top of a filter containing 8 μm diameter pores, allowing active passage of cells toward a test substance added in the lower chamber.
In vitro "wound healing" assay	A portion of a confluent endothelial monolayer is removed using a scraping tool, and endothelial cells migrating back to reform the monolayer are quantified.
Tubule formation assay	Tubular morphogenesis of endothelial cells is induced by seeding cells onto or into a three-dimensional matrix (usually matrigel, fibrin or collagen) in the presence of an angiogenic substance.
Rat aortic ring assay	*Ex vivo* aortic explants are cultured in a three-dimensional matrix *in vitro*, inducing microvessel outgrowths in response to angiogenic substances.
Chick chorioallantoic membrane (CAM) assay	A test substance is placed onto the CAM, commonly through a window cut in the eggshell. The resulting effects on vascularization in the CAM are scored.
Matrigel plug assay	The test substance is suspended in matrigel and injected subcutaneously in mice, forming a solid plug. Angiogenesis in the plug is determined by analyzing vessel growth or by measuring the hemoglobin content in the plug.
Corneal micropocket model	Pellets releasing the test substance are implanted into corneas of, e.g., rabbits, rats, or mice. Vessel formation is visualized by perfusing the cornea with fluorescent dye or India ink.

Brief description of assays used to study the role of chemokines in angiogenesis. For a complete review and discussion of current angiogenesis assays, see Staton et al. (2009)

2 The Chemokine Network

Chemokines are a large family of 8–12 kDa *chemo*attractant heparin-binding cyto*kine*s that may modulate angiogenesis through several distinct mechanisms (Fig. 1). Secreted chemokines accumulate at sites of inflammation through binding to extracellular matrix components and cell-surface carbohydrates, and are presented on the surface of endothelial cells mediating firm adhesion of leukocytes to the vessel wall (Ley et al. 2007; Thelen and Stein 2008). Thereby, chemokines play a central role in the recruitment of immune cells which, in turn, may secrete angiogenic growth factors (Table 2). Also, chemokines regulate recruitment and retention of endothelial progenitor cells that may directly participate in formation of a new vascular plexus (Petit et al. 2007). Importantly, several chemokines influence angiogenesis directly through binding to G-protein-coupled chemokine receptors expressed on endothelial cells, inducing down-stream signaling events that eventually result in enhanced or inhibited formation of new blood vessels (Keeley et al. 2008). The well-established role of chemokines in leukocyte recruitment has been the subject of several excellent reviews (Ley et al. 2007; Thelen and Stein 2008), and, although clearly very important for angiogenesis in health and disease, will not be extensively discussed here. Instead, this review is focused on

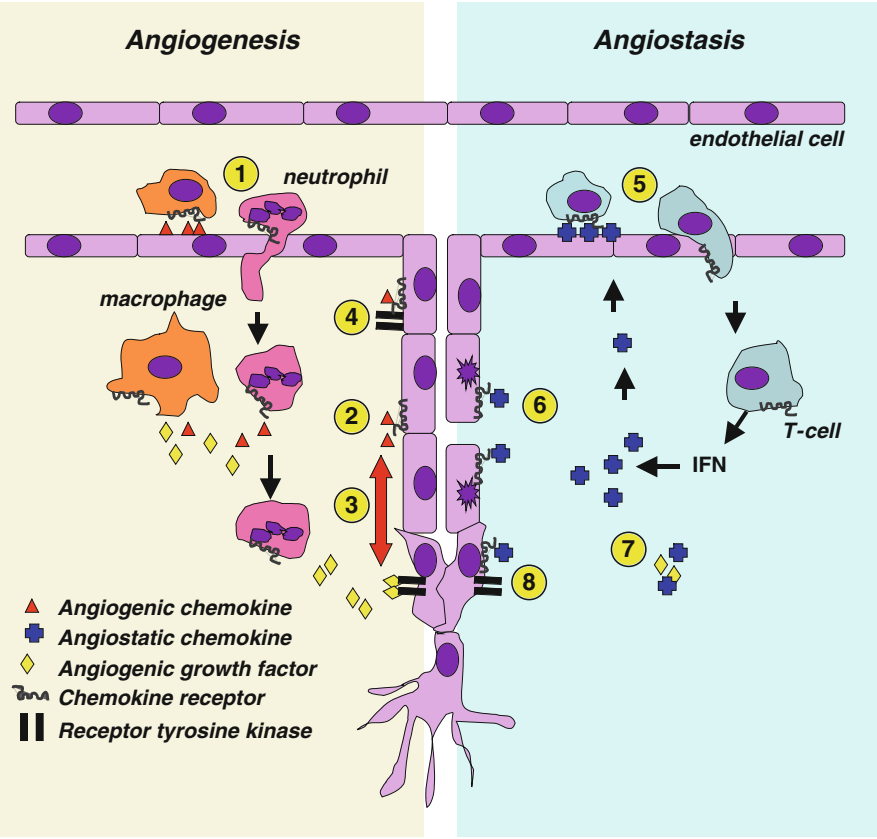

Fig. 1 Mechanisms involved in chemokine-mediated regulation of angiogenesis. Chemokines induce angiogenesis through (1) recruitment of pro-angiogenic hematopoietic cells and progenitors, (2) activation of cognate receptors expressed on endothelial cells inducing chemotaxis and tubular morphogenesis, (3) molecular cross-talk with angiogenic growth factor signaling, and (4) direct interaction between chemokine/chemokine receptor complexes and receptor tyrosine kinase receptors. Angiostatic chemokines inhibit angiogenesis through (5) recruitment of T-cells that in turn induce expression of angiostatic chemokines in a positive feedback loop, (6) binding to cognate receptors expressed on endothelial cells inducing apoptosis or regression of vessels, (7) binding of angiogenic growth factors, and (8) inhibition of receptor tyrosine kinase receptor signaling

the crucial role of chemokines as direct regulators of angiogenesis, and on the potential implication of this function in various pathological conditions.

The human chemokine network involves approximately 20 receptors and 50 ligands, classified according to the spacing of their first cystein residues into four subfamilies designated C, CC, CXC, and CX3C (Taub 2004). Furthermore, the CXC chemokines are divided into two groups depending on the presence or absence of three conserved amino acids (Glu-Leu-Arg; ELR) preceding the first cystein residue in the NH_2-terminal domain. The ELR-motif affects the receptor binding specificity and thereby determines biological function, including the promotion or

Table 2 Factors secreted by myeloid cells that stimulate angiogenesis

Cell type	Angiogenic factor
Macrophages	IL-1β, TNF-α, CXCL8, bFGF, IL-6
Tumor-associated macrophages (TAM)	VEGF, bFGF, TNF-α, IL-1β, CXCL8, COX2, PDGF, VEGF-C, VEGF-D, MMP7, MMP9, MMP12, Sema4D
Dendritic cells	VEGF, TNF-α, CXCL8
Mast cells	VEGF, bFGF, MMP-9, TNF-α, TGFβ, CCL2, CXCL8
Neutrophils	VEGF, CXCL8, CXCL1, Bv8, MMP9
Myeloid-derived suppressor cells	VEGF, bFGF, IL-1β, MMP9, CCL2
Eosinophils	VEGF, bFGF, IL-6, GM-CSF, PDGF, TGFβ, CCL11

Myeloid cells secrete multiple angiogenic molecules, including growth factors, cytokines, chemokines, and matrix metalloproteinases. The table indicates prominent angiogenic factors released by different subsets of myeloid cells, reviewed in Mantovani et al. (2002), Dirkx et al. (2006), Murdoch et al. (2008), Shojaei and Ferrara (2008), Shojaei et al. (2008), Zumsteg and Christofori (2009). Myeloid-derived suppressor cells are a heterogeneous population of immature myeloid progenitor cells that are immunosuppressive (Murdoch et al. 2008; Shojaei and Ferrara 2008; Shojaei et al. 2008)

IL interleukin; *TNF* tumor necrosis factor; *VEGF* vascular endothelial growth factor; *bFGF* basic fibroblast growth factor; *COX2* cyclooxygenase 2; *PDGF* platelet derived growth factor; *MMP* matrix metalloproteinase; *Sema4D* semaphorin4D; *TGF* transforming growth factor

inhibition of angiogenesis (Strieter et al. 1995). Chemokines act through binding to seven-transmembrane G-protein-coupled receptors (GPCR), triggering activation of downstream signaling events typically including release of calcium from intracellular stores (Salanga et al. 2009). Several chemokines can bind multiple chemokine receptors, and many chemokine receptors bind multiple ligands. Adding complexity to the system, chemokines may form homo- or heterodimers or be presented as multimers through binding to glucosaminoglycans, leading to oligomerization of chemokine receptors (Salanga et al. 2009). Interestingly, GPCRs have cross talk with other signaling pathways through multiple mechanisms. This allows chemokine signaling to impinge on, for example, receptor tyrosine kinase pathways, and thereby affect a range of cellular events. As discussed later, many chemokines/chemokine receptor pathways have cross talk with pro-angiogenic VEGF and fibroblast growth factor (FGF) signaling, resulting in differential effects on angiogenesis.

3 Angiostatic Chemokines

Vascular homeostasis requires a strict balance between pro- and anti-angiogenic factors, resulting in restraint of vessel formation denoted angiostasis. Angiogenic stimuli, such as hypoxia, shift the balance of factors towards angiogenesis, initiating formation of vessels. Importantly, during physiological angiogenesis, the process is terminated when adequate vascularization has been achieved, shifting the balance back towards angiostasis. During pathological conditions, such as in tumors or during chronic inflammation, the strict balance between angiogenic and angiostatic (anti-angiogenic) factors is lost, leading to the continued formation of

dysfunctional vessels. Notably, the net effect on angiogenesis is equally dependent on the expression levels of pro- and anti-angiogenic factors.

3.1 ELR-Negative CXCR3-Ligands Are Angiostatic

Interferon (IFN)-inducible ELR-negative CXC-family chemokines are potent angiostatic factors that prevent angiogenesis in response to growth factors and angiogenic chemokines (Balestrieri et al. 2008). Among these, CXCL4/platelet factor 4 (PF4) and CXCL10/interferon-γ inducible protein 10 (IP-10) have been most extensively studied, but angiostatic activity has also been noted for CXCL9/monokine induced by gamma (MIG), CXCL11/interferon inducible T-cell alpha chemoattractant (I-TAC), and the CXCL4 analog CXCL4L1 (Strieter et al. 1995; Maione et al. 1990; Angiolillo et al. 1995; Sato et al. 1990; Romagnani et al. 2001; Struyf et al. 2004). These angiostatic chemokines all bind to CXCR3a and CXCR3b receptors, which are splice variants of the same gene, with the exception of CXCL4 that binds CXCR3b exclusively. CXCR3b expressed on the endothelial cell surface mediates the angiostatic activity of ELR-negative chemokines *in vitro* (Lasagni et al. 2003). Importantly, mice lacking CXCR3 show excessive vessel formation during wound healing, highlighting the importance of ELR-negative CXC-family chemokines in maintaining vascular homeostasis.

Several immune cells express CXCR3 and are consequently recruited and activated by CXCR3 ligands, coupling regulation of immune response with angiostasis. However, CXCL4, CXCL9, CXCL10, and CXCL11 chemokines have been shown to directly inhibit growth factor-induced endothelial chemotaxis and tube formation *in vitro* and angiogenesis in the chick chorioallantoic membrane (CAM) and matrigel plugs in mice *in vivo*, implying that the angiostatic function is, at least in certain contexts, cell autonomous (Strieter et al. 1995; Maione et al. 1990; Angiolillo et al. 1995; Sato et al. 1990; Romagnani et al. 2001; Struyf et al. 2004). Interestingly, it was recently demonstrated that CXCL10 induce dissociation and regression of newly formed vessels during wound healing. Endothelial cord dissociation was induced through a CXCR3-dependent activation of μ-calpain, leading to cleavage of the cytoplasmic tail of β3 integrins followed by endothelial apoptosis (Bodnar et al. 2009). This process was equally induced in the presence of angiogenic factors, suggesting a role in pruning of developing vasculature.

3.2 Regulation of Angiostatic Chemokines: Coupling to Immune Response

There is a reciprocal regulation of IFN-inducible ELR-chemokine expression and induction of Th1-type immune response (Balestrieri et al. 2008). CXCR3 ligands activate interleukin secretion from recruited CXCR3-expressing Th1 T-cells,

natural killer cells, and mononuclear cells, leading to increased production of IFN-γ. IFN-γ induces expression of CXCL9, CXCL10, and CXCL11, which again enhances recruitment and activation of CXCR3-expressing cells. The direct interaction between monocytes and endothelial cells has also been shown to synergistically induce expression of CXCL10 (Kasama et al. 2002).This positive feedback loop may result in "immunoangiostasis," in which Type 1 immune response and inhibition of angiogenesis occur simultaneously. Similarly, interleukin (IL)-12, which is a T-cell stimulating factor, induces secretion of angiostatic chemokines from splenocytes, including CXCL10 and CXCL9 (Strasly et al. 2001). Interestingly, IL-12-induced inhibition of FGF-induced angiogenesis is strictly dependent on CXCL10 (Sgadari et al. 1996).

CXCR3 is expressed in a cell-cycle-dependent manner in cultured microvascular endothelial cells, its expression coinciding with that of cyclin A1, in the S-phase of the cell-cycle (Romagnani et al. 2001). This interesting observation suggests that only actively dividing endothelial cells are able to respond to CXCR3 ligands, directly coupling induction of angiogenesis to its potential inhibition. Supporting this notion, CXCL4 was shown to bind specifically to areas of active angiogenesis *in vivo* (Hansell et al. 1995). Notably, it is not clear to what extent binding to endothelial CXCR3 is required for angiostatic activity of ELR-chemokines *in vivo*. It has been shown that CXCL4 exerts its inhibitory effect on FGF-induced angiogenesis through direct complex formation with bFGF, which inhibits dimerization, binding, and activation of FGFR2 (Perollet et al. 1998). Similar mechanisms are involved in CXCL4-mediated inhibition of VEGF/VEGFR signaling (Gengrinovitch et al. 1995). CXCL4 may also interact with integrins implicated in angiogenesis, and consequently, soluble CXCL4 inhibits integrin-dependent adhesion of endothelial cells (Aidoudi et al. 2008).

3.3 Angiostatic CXCR3-Ligands in Tumor Growth and Angiogenesis

The concept of chemokine-dependent immunoangiostasis proposes that interferon-inducible ELR-chemokines in combination with Th1-type immune cells may synergistically induce tumor regression. In accordance, neutralization of CXCL10 increases growth, metastasis, and endothelial content in non-small cell lung cancer (NSCLC) implanted in SCID mice (Arenberg et al. 1996a). Viral-mediated transduction of CXCL4 cDNA inhibited angiogenesis and growth of intracerebral gliomas in mice, prolonging survival of treated animals (Tanaka et al. 1997). Interestingly, the CXCL4 analog CXCL4L1 was recently shown to be an even more potent inhibitor of growth and metastasis of melanoma through inhibition of angiogenesis (Struyf et al. 2007). These studies collectively suggest that treatment with angiostatic chemokines may be very beneficial for cancer patients and

warrants further research, evaluating this interesting group of chemokines as therapeutic drugs.

4 Angiogenic Chemokines

4.1 ELR-Positive CXC-Family Chemokines Induce Angiogenesis

A subgroup of the CXC family chemokines with an ELR-motif in the N-terminal region has the potential to increase angiogenesis via the recruitment of polymorphonuclear neutrophils into inflamed tissue or through direct modulation of endothelial function. CXCL8/IL-8 was the first discovered chemokine to exhibit angiogenic activity, initially found to induce neo-vascularization *in vivo* using the rabbit corneal pocket model and then identified as a macrophage-derived factor that enhances angiogenesis through induction of proliferation and chemotaxis of endothelial cells (Strieter et al. 1992; Koch et al. 1992). Subsequently, several related ELR-positive chemokines, including CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL7, were found to directly induce endothelial migration *in vitro* and cornea neovascularization *in vivo* (Strieter et al. 1995). Although ELR-positive chemokines mediate angiogenesis in the absence of preceding inflammation in the rat cornea model, CXCL1, CXCL2, and CXCL8 failed to induce angiogenesis in matrigel plugs in neutropenic mice, suggesting that additional neutrophil-derived pro-angiogenic factors are required for efficient vascularization in this setting (Benelli et al. 2002). The importance of the presence of an ELR-motif has been elegantly proven by mutating the ELR-sequence in CXCL8, which completely switched the function of the protein to inhibition of angiogenesis (Strieter et al. 1995). Similarly, the introduction of an ELR-motif converted CXCL9 to a pro-angiogenic protein. There is an important exception to this rule, as discussed later, namely the pro-angiogenic ELR-negative chemokine CXCL12/stromal-derived factor (SDF)-1.

There is ample proof that CXCR2 is the common receptor mediating the pro-angiogenic effects of ELR+ chemokines. Importantly, aside from CXCL8 and CXCL6, which also bind to CXCR1, all angiogenic ELR+ chemokines are exclusive ligands for CXCR2 (Keeley et al. 2008). A functional role of CXCR2 in angiogenesis was convincingly demonstrated through antibody-mediated inhibition of CXCR2, resulting in blocked chemotaxis induced by ELR+ chemokines and perturbed angiogenesis in rat corneal micropockets (Addison et al. 2000). Consistent with this, ELR+ chemokines failed to induce angiogenesis in corneas of CXCR2-deficient mice. There is also a delay in wound healing in mice lacking CXCR2, which was associated with decreased neovascularization of the wound tissue (Devalaraja et al. 2000). However, the inhibition of angiogenesis in this setting may be partially due to perturbed recruitment of neutrophils, precluding conclusions to be drawn regarding a direct function of CXCR2 in neovascularization during wound healing.

4.2 Pro-Angiogenic ELR-Positive Chemokines Are Induced Downstream of NF-kB Activation

CXCR2 ligands have been shown to be pro-angiogenic downstream of several distinct pathways, typically involving activation of NF-κB transcription factors. Chemokines are direct targets for NF-κB signaling pathways, which are central mediators of inflammatory signaling and endothelial activation downstream of tumor necrosis factor (TNF)-α and IL-1β stimulation. For instance, CXCL2 was up-regulated in IL-1β-expressing lewis lung carcinoma cells, and induced tumor angiogenesis through binding to CXCR2 (Saijo et al. 2002). The NF-κB-pathway is also induced during hypoxia downstream of HIF-1a induction. Constitutive activation of HIF-1 in keratinocytes enhanced NF-κB activation and induced transcription of CXCL1, CXCL2, and CXCL3 in a transgenic mouse model (Scortegagna et al. 2008). However, HIF-1-independent up-regulation of CXCL8 in response to hypoxia has also been described, suggesting that multiple pathways may lead to chemokine activation during low oxygen pressure (Mizukami et al. 2005). Tissue injury also induces chemokine expression through alternative pathways. For instance, CXCL1 is induced by thrombin in endothelial cells and tumor cells, and plays a pivotal role in thrombin-induced angiogenesis through up-regulation of pro-angiogenic molecules, including its receptor CXCR2 (Caunt et al. 2006).

CXCL8 interacts with the VEGF-signaling pathway in several ways. Autocrine VEGF stimulation was shown to induce CXCL8 secretion in tumor-associated brain endothelial cells (Charalambous et al. 2005). Conversely, CXCL8 has recently been shown to induce VEGF expression through Carma3/Bcl10/Malt1-dependent activation of NF-κB, leading to autocrine stimulation of VEGFR2 (Martin et al. 2009). A direct interaction between CXCL8 and VEGF-signaling pathways has also been described. Petreaca and co-authors showed that CXCL8 induced phosphorylation of VEGFR2 in a VEGF-independent manner, and that transactivation of VEGFR2 was required for CXCL8-induced endothelial permeability (Petreaca et al. 2007). Treatment with recombinant CXCL8 induced complex formation between CXCR1/CXCR2 and VEGFR2 in a Src-dependent manner, leading to phosphorylation of VEGFR2 and endothelial gap formation downstream of RhoA-activation.

4.3 The CXCR2L/CXCR2 Axis in Tumor Vascularization

Interestingly, oncogenes and growth factors may directly induce chemokines, coupling tumorigenesis to inflammation and angiogenesis. Overexpression of Bcl-2, a pro-survival protein up-regulated in many tumors, induces CXCL1 and CXCL8 in microvascular endothelial cells through activation of NF-κB (Karl et al. 2005). Similarly, EGF-induced up-regulation of Bcl-XL induces sprouting angiogenesis in a CXCR2-dependent fashion, involving an ERK-dependent increase in VEGF and a subsequent rise in CXCL1 and CXCL8 downstream of Bcl-2 (Karl et al. 2007).

CXCL8 stimulation has in turn been shown to increase survival of endothelial cells through up-regulation of Bcl-2 and inhibition of Bax, suggesting the existence of a synergistic feedback loop between chemokines and Bcl-2 family signaling pathways (Li et al. 2003). Semaphorin 3B, previously described as a tumor suppressor, increases metastatic dissemination in experimental tumor models through up-regulation of CXCL8 downstream of neuropilin-1-mediated p38-mitogen-activated protein kinase activation (Rolny et al. 2008). Notably, CXCL8 is a transcriptional target for oncogenic RAS-signaling required for initiation of tumor-associated inflammation and neovascularization in RAS-expressing tumors (Sparmann and Bar-Sagi 2004). In a recent report, p53 was implicated in ID4-mediated stabilization of CXCL1 and CXCL8 mRNA, offering a new level of regulation of chemokine expression by oncogenes (Fontemaggi et al. 2009).

The role of CXCL8 in tumor angiogenesis is undisputed. Aside from its direct role in enhancing survival, chemotaxis, and tubular morphogenesis of endothelial cells, CXCL8 is a potent chemoattractant for monocytes and neutrophils, which in turn provide angiogenic growth factors and MMPs. The important function of CXCL8 in tumor angiogenesis was first suggested by the observation that angiogenic activity of bronchogenic carcinoma tumor cell homogenates was blocked through inhibition of CXCL8 (Smith et al. 1994). Consistent with this, neutralizing antibodies to CXCL8 or its cognate receptor CXCR2 inhibited tumorigenesis in human NSCLC in SCID mice, associated with reduced angiogenic activity and decreased vascular density (Arenberg et al. 1996b; Numasaki et al. 2005). Furthermore, CXCR2-deficient mice show decreased growth of renal cell carcinoma (RENCA), associated with decreased angiogenesis, reduced metastatic dissemination, and increased necrosis (Mestas et al. 2005). Tumor growth, angiogenesis, and metastasis of CXCL8-expressing human melanoma were also reduced in CXCR2-deficient nude mice, in association with decreased neutrophil infiltration, underscoring an important role of host CXCR2 in tumorigenesis (Singh et al. 2009). Transgene expression of CXCR2 in endothelial cells enhanced angiogenesis and tumor growth of melanocytes expressing a functional homologue to human CXCL8/CXCL1, macrophage inflammatory protein (MIP)-1, providing additional evidence for a direct role of CXCR ligands on endothelial cell function *in vivo* (Horton et al. 2007). Conversely, endothelial expression of the chemokine decoy receptor DARC decreased tumor vascular density and growth in the same model, while increasing trafficking of leukocytes.

4.4 CXCL12/CXCR4 Signaling in Recruitment of Pro-Angiogenic Bone Marrow Derived Cells

CXCL12/stromal cell-derived factor (SDF)-1 is an ELR-negative chemokine that acts as a potent inducer of angiogenesis. While angiostatic ELR-negative chemokines bind CXCR3, CXCL12 is a ligand for CXCR4 and the newly discovered

receptor CXCR7 (Petit et al. 2007; Burns et al. 2006). CXCL12 is constitutively expressed in many different cell types, and expression is further induced upon ischemia or tissue injury. In the bone marrow, where the oxygen pressure is lower than in peripheral blood, CXCL12 is constitutively secreted by stromal cells and presented on endothelial cells (Petit et al. 2007; Mendez-Ferrer and Frenette 2007; Burger and Burkle 2007). Thereby, CXCL12 regulates homing and retention of CXCR4-expressing hematopoietic stem and progenitor cells in the marrow.

CXCL12 may enhance angiogenesis through several distinct mechanisms. First, CXCL12 can act directly through CXCR4 expressed on endothelial cells, inducing endothelial chemotaxis, tubular morphogenesis, and endothelial sprouting from rat aortic rings *in vitro* (Gupta et al. 1998; Salcedo et al. 1999; Deshane et al. 2007). Recently, the signaling pathway regulating CXCL12-induced endothelial migration has been elucidated and shown to be dependent on JNK3 activation, which occur downstream of eNOS-induced nitrosylation of MKP7 (Pi et al. 2009). CXCL12/CXCR4 activation is an important mediator of VEGF- and FGF-induced angiogenic programs. Indeed, blocking either CXCL12 or CXCR4 inhibits VEGF and FGF-induced tubular morphogenesis of endothelial cells (Salvucci et al. 2002; Salcedo et al. 2003). Conversely, the CXCL12/CXCR4 signaling pathway has been implicated in regulating the angiogenic switch in prostate cancer through down-regulation of phosphoglycerate kinase, leading to the production of VEGF and CXCL8 and decreasing expression of anti-angiogenic angiostatin (Wang et al. 2007). Second, CXCL12 induces adhesion, chemotaxis, and homing of circulating pro-angiogenic CXCR4+ hemotopoetic cells and endothelial progenitor cells to neo-vascular niches, contributing to angiogenesis in ischemic tissue and tumors (Petit et al. 2007). The recruitment of pro-angiogenic bone marrow-derived cells to wound tissue may be induced by platelet release of CXCL12 in response to vessel injury (Jin et al. 2006). Hypoxic gradients directly induce CXCL12 expression through activation of HIF-1α transcription factors in endothelial cells, leading to mobilization of bone marrow-derived progenitors (Schioppa et al. 2003; Ceradini et al. 2004). Endothelial progenitor cells express CXCR4 and have been suggested to contribute to CXCL12-induced angiogenesis. Indeed, CXCL12 increases the number of circulating endothelial progenitor cells during ischemia, and induce formation of tubular structures of co-injected c-kit+ progenitor cells in matrigel plugs in mice (De Falco et al. 2004). However, the relative contribution of endothelial progenitor cells to angiogenesis in various pathological conditions is still controversial (Ahn and Brown 2009; Pearson 2009). Finally, CXCL12 is important for retention of recruited bone marrow-derived cells close to the developing neovasculature, allowing continuous secretion of angiogenic growth factors during wound healing (Grunewald et al. 2006).

CXCL12 is well established as a potent inducer of tumor growth and metastatic dissemination through induction of angiogenesis (Guleng et al. 2005; Orimo et al. 2005; Singh et al. 2007; Yoon et al. 2007; Li and Ransohoff 2009). However, the pro-angiogenic role of CXCL12 may be dependent on the tumor type, as neutralizing antibodies to CXCL12 blocked tumor growth and metastasis of NSCLC in SCID mice without affecting tumor angiogenesis (Phillips et al. 2003). The molecular

mechanisms that regulate CXCL12/CXCR4-induced angiogenesis *in vivo* are still not fully elucidated, and are likely to be at least partially dependent on recruitment of pro-angiogenic bone marrow-derived cells. Interestingly, mice lacking either CXCL12 or CXCR4 reveal vascular abnormalities, strongly supporting an important role of CXCL12/CXCR4 signaling in embryonic vasculogenesis (Tachibana et al. 1998; Ara et al. 2005). CXCL12 does not induce signaling through CXCR7, but CXCR4/CXCR7 heterodimers enhance CXCL12 signaling (Sierro et al. 2007). This appears to be specifically important in cardiac development, as endothelial-specific deletion of CXCR7 resulted in ventricular septal defects and heart valve malformations (Sierro et al. 2007).

4.5 CCL-Family Chemokines as Inducers of Angiogenesis

The CCL family is the largest chemokine subgroup involved in both inflammatory response during infection and tissue injury, immune surveillance, and lymphocyte homing. Several CCL family members promote an angiogenic program inducing migration, invasion, and tubular morphogenesis of endothelial cells *in vitro*, but it is not clear as to what extent the angiogenic function of these chemokines *in vivo* depend on recruitment of leukocytes. Interestingly, release of CCL-family chemokines from tumor neovessels and recruitment of CCR2- and CCR5-expressing progenitors has been described as a late event in carcinogenesis, correlating with mobilization of circulating endothelial progenitor cells (Spring et al. 2005).

CCL1 participates in the recruitment of monocytes and Th2-cells, and has been shown to induce angiogenesis in the rabbit cornea micropocket and CAM assays (Bernardini et al. 2000). CCL1 binds to CCR8, expressed on endothelial cells, and induces migration, invasion, and tubular morphogenesis *in vitro*. However, CCL1-induced recruitment of pro-angiogenic leukocytes is likely to at least partially contribute to angiogenesis *in vivo*. The eosinophil chemoattractant CCL11/eotaxin has angiogenic effects *in vivo* and induces chemotaxis of endothelial cells *in vitro* through binding to CCR3 (Salcedo et al. 2001). Blood vessel formation *in vivo* in the CAM and matrigel plug assay in mice following CCL11-stimulation was accompanied by infiltration of eosinophils, which may in turn release pro-angiogenic factors. Importantly, the observation of robust endothelial sprouting from rat aortic rings, in the absence of eosinophils, supports a direct pro-angiogenic function of CCL11. Likewise, CCL3 is an indirect inducer of angiogenesis during inflammation through recruitment of macrophages (Barcelos et al. 2009; Wu et al. 2008), but has also been implicated in autocrine stimulation of neovessel proliferation in a murine model of hepatocellular carcinoma through binding to its cognate receptor CCR5 (Ryschich et al. 2006).

CCL15, CCL16, and CCL23 induce migration and tube formation of endothelial cells *in vitro*, and induce neo-vascularization in the CAM assay through binding to CCR1 (Hwang et al. 2004, 2005; Strasly et al. 2004). In the case of CCL15, this effect was partially dependent on CCR3 binding, evidenced by a complete block in

migration requiring treatment with neutralizing antibodies towards both CCR1 and CCR3 (Hwang et al. 2004). Interestingly, CCL16 signaling has cross talk with other pro-angiogenic pathways through increased production of CXCL8 and CCL2, and primes endothelial cells to mitogen signals by VEGF (Strasly et al. 2004). Because of the common binding and activation of CCR1, it is likely that similar pathways are induced by CCL15 and CCL23.

4.5.1 CCL2 in Arteriogenesis and Angiogenesis

CCL2/monocyte chemoattractant protein (MCP)-1 has an important role in arteriogenesis, mainly due to its role in recruitment of monocytes. Monocyte recruitment plays an important role during arteriogenesis, especially during collateral growth in response to vessel occlusion and tissue ischemia. CCL2 has a nonredundant function in monocyte recruitment through binding to its cognate receptor CCR2, evidenced by the impaired ability of CCL2−/− or CCR2−/− mice to recruit monocytes to affected tissues during inflammation (Charo and Taubman 2004).

CCL2 treatment induces chemotaxis of endothelial cells expressing CCR2, increases sprouting of rat aortic rings, and enhances angiogenesis in the CAM assay and in matrigel plugs in mice (Weber et al. 1999; Salcedo et al. 2000). Although the *in vitro* data support a direct role of CCL2 in modulation of endothelial function, the *in vivo* effects of CCL2 on vessel formation may be partially due to recruitment of monocytes secreting pro-angiogenic factors. CCL2-induced angiogenesis involves activation of Ets-1 and MCPIP transcription factors and up-regulation of MTI-MMP, and can be attenuated through blocking of either of these events (Galvez et al. 2005; Stamatovic et al. 2006; Niu et al. 2008).

CCL2 has a functional role in angiogenesis and arteriogenesis downstream of cytokine and growth factor signaling. CCL2 up-regulation is critical for VEGF-induced tubular morphogenesis and permeability (Yamada et al. 2003; Marumo et al. 1999). TGF-β up-regulates CCL2 directly through SMAD-signaling, which leads to angiogenesis and smooth muscle cell migration dependent on CCL2 (Ma et al. 2007). bFGF enhances CCL2 production from mesenchymal cells, which in turn improves arteriogenesis during hind limb ischemia in mice (Fujii et al. 2006).

4.6 CX3CL/CX3CR Axis in Angiogenesis

CX3CL/Fractalkine is the only known member of the CX3C-subfamily of chemokines, and is expressed as a transmembrane protein that can also be cleaved to a soluble variant. CX3CL induces chemotaxis and tubular morphogenesis of endothelial cells and increases sprouting in rat aortic rings *in vitro*, and enhances angiogenesis in the CAM assay, rabbit corneal micropocket neovascularization assay, and mouse matrigel plugs *in vivo* (Volin et al. 2001; Ryu et al. 2008;

You et al. 2007). The pro-angiogenic effects of CX3CL are due to induction of HIF-1α transcription factors, leading to increased VEGF-levels (Ryu et al. 2008). Consequently, CX3CL-induced angiogenesis is blocked by inhibition of VEGFR2. CXC3L levels are increased in vitreous fluid in diabetic retinopathy patients and in synovial fluid from rheumatoid arthritis patient, and may be a target for anti-angiogenic treatment of these diseases (Volin et al. 2001; You et al. 2007). However, CX3CL may induce opposite effects in different microenvironmental settings. While CX3CL injection improved the condition of hind limb ischemia through pro-angiogenic effects, it reduced alkali-induced corneal neovascularization through production of anti-angiogenic factors in CX3CR expressing macrophages (Ryu et al. 2008; Lu et al. 2008).

4.7 KSHV-Encoded and CMV-Encoded Chemokines in Angiogenesis

Virally encoded chemokines and chemokine receptors that induce angiogenesis have been described and may influence human disease. A constitutively active GPCR of Kaposis sarcoma-associated herpesvirus (KSHV), with homology to chemokines receptors, has been shown to induce an angiogenic switch mediated by VEGF (Bais et al. 1998). Conditioned media from transfected NIH3T3 fibroblasts induced HUVEC proliferation and tubular morphogenesis in a VEGF-dependent fashion. The KSHV-encoded chemokines vMIP-11 is a CCR4 agonist that has been shown to stimulate angiogenesis in the CAM assay and attract Th2-cells (Stine et al. 2000). Finally, the CMV-encoded chemokine receptor US28 promotes angiogenesis and tumorigenesis via NFκB-driven VEGF-induction of COX-2 (Maussang et al. 2006, 2009).

5 The Chemokines and Their Receptors as Future Therapeutic Targets

Angiogenesis and inflammation are linked processes that affect the outcome of many pathological conditions. Chemokines are central regulators of both these processes, suggesting that targeting chemokines or chemokine receptors may be beneficial in a wide range of diseases (Ley et al. 2007; Thelen and Stein 2008) (Table 3). Several anti-angiogenic drugs, many of which disrupt VEGF/VEGFR activation, have been approved and are successfully used in the treatment of, for example, age-related macula degeneration and various types of cancer (Olsson et al. 2006; Jain et al. 2006). Combining anti-angiogenic therapy with chemotherapy is beneficial in cancer treatment, presumably due to normalization of the tumor vasculature and increased delivery of drugs into the tumor. However, applying

Table 3 Human chemokines and chemokine receptors involved in angiogenesis

Systematic name	Receptor	Key references
Angiostatic chemokines		
CXCL4, CXCL4L1	CXCR3b	Sato et al. (1990), Lasagni et al. (2003), Struyf et al. (2007), Maione et al. (1991)
CXCL9	CXCR3b	Strieter et al. (1995), Lasagni et al. (2003)
CXCL10	CXCR3b	Angiolillo et al. (1995), Lasagni et al. (2003)
CXCL11	CXCR3b	Strieter et al. (1995), Lasagni et al. (2003)
Angiogenic chemokines		
CXCL1	CXCR2	Strieter et al. (1995), Addison et al. (2000)
CXCL2	CXCR2	Strieter et al. (1995), Addison et al. (2000)
CXCL3	CXCR2	Strieter et al. (1995), Addison et al. (2000)
CXCL5	CXCR2	Strieter et al. (1995), Addison et al. (2000)
CXCL6	CXCR2	Strieter et al. (1995), Addison et al. (2000)
CXCL7	CXCR2	Strieter et al. (1995), Addison et al. (2000)
CXCL8	CXCR2	Strieter et al. (1992), Koch et al. (1992), Addison et al. (2000)
CXCL12	CXCR4, CXCR7	Salcedo et al. (1999), Deshane et al. (2007), Tachibana et al. (1998), Ara et al. (2005), Sierro et al. (2007)
CCL1	CCR8	Bernardini et al. (2000)
CCL2	CCR2	Weber et al. (1999), Salcedo et al. (2000)
CCL3	CCR5	Ryschich et al. (2006)
CCL11	CCR3	Salcedo et al. (2001)
CCL15	CCR1, CCR3	Hwang et al. (2004)
CCL16	CCR1	Strasly et al. (2004)
CCL23	CCR1	Hwang et al. (2005)
CX3CL	CX3CR	Volin et al. (2001), Ryu et al. (2008), You et al. (2007)

Chemokines/chemokine receptors and the key references demonstrating a direct role in angiogenesis are indicated. Additional references are given in the text

anti-angiogenic therapy does not cure the disease. In fact, aggressive anti-angiogenic therapy has recently been associated with increased metastatic dissemination in animal models, although this has yet to be confirmed in human patients (Ebos et al. 2009; Paez-Ribes et al. 2009). Moreover, tumor resistance to anti-angiogenic therapy is still an unresolved clinical problem (Kerbel 2008). One mechanism of refractoriness to anti-VEGF therapy is through recruitment of pro-angiogenic myeloid cells (Shojaei et al. 2007). Drugs targeting chemokines may simultaneously block angiogenesis and inhibit immune cell recruitment, thereby reducing the risk of relapse. Targeting chemokines may also decrease the rate of metastatic dissemination as cancer cells are believed to utilize similar pathways for extravasation as do hematopoietic cells. Indeed, treatment with angiostatic chemokines or inhibition of angiogenic chemokines/chemokine receptors reduces angiogenesis, inhibits tumor growth, and reduces metastasis in many different tumor models. The observation that chemokines frequently have cross talk with several angiogenic factors suggests that combined targeting of chemokines and growth factors may have synergistic effects. Importantly, chemokines have been proposed as therapeutic targets for chronic inflammatory disorders, myocardial ischemia, artherosclerosis, and pulmonary fibrosis, and play an important role during wound healing

(Keeley et al. 2008; Strieter et al. 2007). Strategies to inhibit or augment chemokine signaling may therefore be of general importance, and would potentially be utilized in the clinical treatment of several conditions. However, the central role of chemokines in the immune system and the extensive redundancy of chemokine/chemokine receptor signaling necessitate further investigation regarding the putative benefit in intervening with chemokine signaling in pathological disorders.

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References

Adams RH, Alitalo K (2007) Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol 8:464–478

Addison CL, Daniel TO, Burdick MD, Liu H, Ehlert JE, Xue YY, Buechi L, Walz A, Richmond A, Strieter RM (2000) The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. J Immunol 165:5269–5277

Ahn GO, Brown JM (2009) Role of endothelial progenitors and other bone marrow-derived cells in the development of the tumor vasculature. Angiogenesis 12:159–164

Aidoudi S, Bujakowska K, Kieffer N, Bikfalvi A (2008) The CXC-chemokine CXCL4 interacts with integrins implicated in angiogenesis. PLoS One 3:e2657

Angiolillo AL, Sgadari C, Taub DD, Liao F, Farber JM, Maheshwari S, Kleinman HK, Reaman GH, Tosato G (1995) Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis *in vivo*. J Exp Med 182:155–162

Ara T, Tokoyoda K, Okamoto R, Koni PA, Nagasawa T (2005) The role of CXCL12 in the organ-specific process of artery formation. Blood 105:3155–3161

Arenberg DA, Kunkel SL, Polverini PJ, Morris SB, Burdick MD, Glass MC, Taub DT, Iannettoni MD, Whyte RI, Strieter RM (1996a) Interferon-gamma-inducible protein 10 (IP-10) is an angiostatic factor that inhibits human non-small cell lung cancer (NSCLC) tumorigenesis and spontaneous metastases. J Exp Med 184:981–992

Arenberg DA, Kunkel SL, Polverini PJ, Glass M, Burdick MD, Strieter RM (1996b) Inhibition of interleukin-8 reduces tumorigenesis of human non-small cell lung cancer in SCID mice. J Clin Invest 97:2792–2802

Bais C, Santomasso B, Coso O, Arvanitakis L, Raaka EG, Gutkind JS, Asch AS, Cesarman E, Gershengorn MC, Mesri EA (1998) G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator. Nature 391:86–89

Balestrieri ML, Balestrieri A, Mancini FP, Napoli C (2008) Understanding the immunoangiostatic CXC chemokine network. Cardiovasc Res 78:250–256

Barcelos LS, Coelho AM, Russo RC, Guabiraba R, Souza AL, Bruno-Lima G Jr, Proudfoot AE, Andrade SP, Teixeira MM (2009) Role of the chemokines CCL3/MIP-1 alpha and CCL5/RANTES in sponge-induced inflammatory angiogenesis in mice. Microvasc Res 78:148–154

Benelli R, Morini M, Carrozzino F, Ferrari N, Minghelli S, Santi L, Cassatella M, Noonan DM, Albini A (2002) Neutrophils as a key cellular target for angiostatin: implications for regulation of angiogenesis and inflammation. FASEB J 16:267–269

Bernardini G, Spinetti G, Ribatti D, Camarda G, Morbidelli L, Ziche M, Santoni A, Capogrossi MC, Napolitano M (2000) I-309 binds to and activates endothelial cell functions and acts as an angiogenic molecule *in vivo*. Blood 96:4039–4045

Bodnar RJ, Yates CC, Rodgers ME, Du X, Wells A (2009) IP-10 induces dissociation of newly formed blood vessels. J Cell Sci 122:2064–2077

Burger JA, Burkle A (2007) The CXCR4 chemokine receptor in acute and chronic leukaemia: a marrow homing receptor and potential therapeutic target. Br J Haematol 137:288–296

Burns JM, Summers BC, Wang Y, Melikian A, Berahovich R, Miao Z, Penfold ME, Sunshine MJ, Littman DR, Kuo CJ, Wei K, McMaster BE, Wright K, Howard MC, Schall TJ (2006) A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. J Exp Med 203:2201–2213

Caunt M, Hu L, Tang T, Brooks PC, Ibrahim S, Karpatkin S (2006) Growth-regulated oncogene is pivotal in thrombin-induced angiogenesis. Cancer Res 66:4125–4132

Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, Gurtner GC (2004) Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med 10:858–864

Charalambous C, Pen LB, Su YS, Milan J, Chen TC, Hofman FM (2005) Interleukin-8 differentially regulates migration of tumor-associated and normal human brain endothelial cells. Cancer Res 65:10347–10354

Charo IF, Taubman MB (2004) Chemokines in the pathogenesis of vascular disease. Circ Res 95:858–866

De Falco E, Porcelli D, Torella AR, Straino S, Iachininoto MG, Orlandi A, Truffa S, Biglioli P, Napolitano M, Capogrossi MC, Pesce M (2004) SDF-1 involvement in endothelial phenotype and ischemia-induced recruitment of bone marrow progenitor cells. Blood 104:3472–3482

Deshane J, Chen S, Caballero S, Grochot-Przeczek A, Was H, Li Calzi S, Lach R, Hock TD, Chen B, Hill-Kapturczak N, Siegal GP, Dulak J, Jozkowicz A, Grant MB, Agarwal A (2007) Stromal cell-derived factor 1 promotes angiogenesis via a heme oxygenase 1-dependent mechanism. J Exp Med 204:605–618

Devalaraja RM, Nanney LB, Du J, Qian Q, Yu Y, Devalaraja MN, Richmond A (2000) Delayed wound healing in CXCR2 knockout mice. J Invest Dermatol 115:234–244

Dirkx AE, Oude Egbrink MG, Wagstaff J, Griffioen AW (2006) Monocyte/macrophage infiltration in tumors: modulators of angiogenesis. J Leukoc Biol 80:1183–1196

Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS (2009) Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer Cell 15:232–239

Fontemaggi G, Dell'Orso S, Trisciuoglio D, Shay T, Melucci E, Fazi F, Terrenato I, Mottolese M, Muti P, Domany E, Del Bufalo D, Strano S, Blandino G (2009) The execution of the transcriptional axis mutant p53, E2F1 and ID4 promotes tumor neo-angiogenesis. Nat Struct Mol Biol 16:1086–1093

Fraisl P, Mazzone M, Schmidt T, Carmeliet P (2009) Regulation of angiogenesis by oxygen and metabolism. Dev Cell 16:167–179

Fujii T, Yonemitsu Y, Onimaru M, Tanii M, Nakano T, Egashira K, Takehara T, Inoue M, Hasegawa M, Kuwano H, Sueishi K (2006) Nonendothelial mesenchymal cell-derived MCP-1 is required for FGF-2-mediated therapeutic neovascularization: critical role of the inflammatory/arteriogenic pathway. Arterioscler Thromb Vasc Biol 26:2483–2489

Galvez BG, Genis L, Matias-Roman S, Oblander SA, Tryggvason K, Apte SS, Arroyo AG (2005) Membrane type 1-matrix metalloproteinase is regulated by chemokines monocyte-chemoattractant protein-1/ccl2 and interleukin-8/CXCL8 in endothelial cells during angiogenesis. J Biol Chem 280:1292–1298

Gengrinovitch S, Greenberg SM, Cohen T, Gitay-Goren H, Rockwell P, Maione TE, Levi BZ, Neufeld G (1995) Platelet factor-4 inhibits the mitogenic activity of VEGF121 and VEGF165 using several concurrent mechanisms. J Biol Chem 270:15059–15065

Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S, Chimenti S, Landsman L, Abramovitch R, Keshet E (2006) VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. Cell 124:175–189

Guleng B, Tateishi K, Ohta M, Kanai F, Jazag A, Ijichi H, Tanaka Y, Washida M, Morikane K, Fukushima Y, Yamori T, Tsuruo T, Kawabe T, Miyagishi M, Taira K, Sata M, Omata M (2005) Blockade of the stromal cell-derived factor-1/CXCR4 axis attenuates *in vivo* tumor growth by inhibiting angiogenesis in a vascular endothelial growth factor-independent manner. Cancer Res 65:5864–5871

Gupta SK, Lysko PG, Pillarisetti K, Ohlstein E, Stadel JM (1998) Chemokine receptors in human endothelial cells. Functional expression of CXCR4 and its transcriptional regulation by inflammatory cytokines. J Biol Chem 273:4282–4287

Hansell P, Maione TE, Borgstrom P (1995) Selective binding of platelet factor 4 to regions of active angiogenesis *in vivo*. Am J Physiol 269:H829–836

Horton LW, Yu Y, Zaja-Milatovic S, Strieter RM, Richmond A (2007) Opposing roles of murine duffy antigen receptor for chemokine and murine CXC chemokine receptor-2 receptors in murine melanoma tumor growth. Cancer Res 67:9791–9799

Hwang J, Kim CW, Son KN, Han KY, Lee KH, Kleinman HK, Ko J, Na DS, Kwon BS, Gho YS, Kim J (2004) Angiogenic activity of human CC chemokine CCL15 *in vitro* and *in vivo*. FEBS Lett 570:47–51

Hwang J, Son KN, Kim CW, Ko J, Na DS, Kwon BS, Gho YS, Kim J (2005) Human CC chemokine CCL23, a ligand for CCR1, induces endothelial cell migration and promotes angiogenesis. Cytokine 30:254–263

Jain RK, Duda DG, Clark JW, Loeffler JS (2006) Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nat Clin Pract Oncol 3:24–40

Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, Hooper AT, Amano H, Avecilla ST, Heissig B, Hattori K, Zhang F, Hicklin DJ, Wu Y, Zhu Z, Dunn A, Salari H, Werb Z, Hackett NR, Crystal RG, Lyden D, Rafii S (2006) Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes. Nat Med 12:557–567

Jujo K, Ii M, Losordo DW (2008) Endothelial progenitor cells in neovascularization of infarcted myocardium. J Mol Cell Cardiol 45:530–544

Karl E, Warner K, Zeitlin B, Kaneko T, Wurtzel L, Jin T, Chang J, Wang S, Wang CY, Strieter RM, Nunez G, Polverini PJ, Nor JE (2005) Bcl-2 acts in a proangiogenic signaling pathway through nuclear factor-kappaB and CXC chemokines. Cancer Res 65:5063–5069

Karl E, Zhang Z, Dong Z, Neiva KG, Soengas MS, Koch AE, Polverini PJ, Nunez G, Nor JE (2007) Unidirectional crosstalk between Bcl-xL and Bcl-2 enhances the angiogenic phenotype of endothelial cells. Cell Death Differ 14:1657–1666

Kasama T, Muramatsu M, Kobayashi K, Yajima N, Shiozawa F, Hanaoka R, Miwa Y, Negishi M, Ide H, Adachi M (2002) Interaction of monocytes with vascular endothelial cells synergistically induces interferon gamma-inducible protein 10 expression through activation of specific cell surface molecules and cytokines. Cell Immunol 219:131–139

Keeley EC, Mehrad B, Strieter RM (2008) Chemokines as mediators of neovascularization. Arterioscler Thromb Vasc Biol 28:1928–1936

Kerbel RS (2008) Tumor angiogenesis. N Engl J Med 358:2039–2049

Kilarski WW, Samolov B, Petersson L, Kvanta A, Gerwins P (2009) Biomechanical regulation of blood vessel growth during tissue vascularization. Nat Med 15:657–664

Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SG, Strieter RM (1992) Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science 258:1798–1801

Lasagni L, Francalanci M, Annunziato F, Lazzeri E, Giannini S, Cosmi L, Sagrinati C, Mazzinghi B, Orlando C, Maggi E, Marra F, Romagnani S, Serio M, Romagnani P (2003) An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. J Exp Med 197:1537–1549

Ley K, Laudanna C, Cybulsky MI, Nourshargh S (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol 7:678–689

Li M, Ransohoff RM (2009) The roles of chemokine CXCL12 in embryonic and brain tumor angiogenesis. Semin Cancer Biol 19:111–115

Li A, Dubey S, Varney ML, Dave BJ, Singh RK (2003) IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. J Immunol 170:3369–3376

Lu P, Li L, Kuno K, Wu Y, Baba T, Li YY, Zhang X, Mukaida N (2008) Protective roles of the fractalkine/CX3CL1-CX3CR1 interactions in alkali-induced corneal neovascularization through enhanced antiangiogenic factor expression. J Immunol 180:4283–4291

Ma J, Wang Q, Fei T, Han JD, Chen YG (2007) MCP-1 mediates TGF-beta-induced angiogenesis by stimulating vascular smooth muscle cell migration. Blood 109:987–994

Maione TE, Gray GS, Petro J, Hunt AJ, Donner AL, Bauer SI, Carson HF, Sharpe RJ (1990) Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. Science 247:77–79

Maione TE, Gray GS, Hunt AJ, Sharpe RJ (1991) Inhibition of tumor growth in mice by an analogue of platelet factor 4 that lacks affinity for heparin and retains potent angiostatic activity. Cancer Res 51:2077–2083

Makanya AN, Hlushchuk R, Djonov VG (2009) Intussusceptive angiogenesis and its role in vascular morphogenesis, patterning, and remodeling. Angiogenesis 12:113–123

Mantovani A, Sozzani S, Locati M, Allavena P, Sica A (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 23:549–555

Martin D, Galisteo R, Gutkind JS (2009) CXCL8/IL8 stimulates vascular endothelial growth factor (VEGF) expression and the autocrine activation of VEGFR2 in endothelial cells by activating NFkappaB through the CBM (Carma3/Bcl10/Malt1) complex. J Biol Chem 284:6038–6042

Marumo T, Schini-Kerth VB, Busse R (1999) Vascular endothelial growth factor activates nuclear factor-kappaB and induces monocyte chemoattractant protein-1 in bovine retinal endothelial cells. Diabetes 48:1131–1137

Maussang D, Verzijl D, van Walsum M, Leurs R, Holl J, Pleskoff O, Michel D, van Dongen GA, Smit MJ (2006) Human cytomegalovirus-encoded chemokine receptor US28 promotes tumorigenesis. Proc Natl Acad Sci USA 103:13068–13073

Maussang D, Langemeijer E, Fitzsimons CP, Stigter-van Walsum M, Dijkman R, Borg MK, Slinger E, Schreiber A, Michel D, Tensen CP, van Dongen GA, Leurs R, Smit MJ (2009) The human cytomegalovirus-encoded chemokine receptor US28 promotes angiogenesis and tumor formation via cyclooxygenase-2. Cancer Res 69:2861–2869

Mendez-Ferrer S, Frenette PS (2007) Hematopoietic stem cell trafficking: regulated adhesion and attraction to bone marrow microenvironment. Ann N Y Acad Sci 1116:392–413

Mestas J, Burdick MD, Reckamp K, Pantuck A, Figlin RA, Strieter RM (2005) The role of CXCR2/CXCR2 ligand biological axis in renal cell carcinoma. J Immunol 175:5351–5357

Mizukami Y, Jo WS, Duerr EM, Gala M, Li J, Zhang X, Zimmer MA, Iliopoulos O, Zukerberg LR, Kohgo Y, Lynch MP, Rueda BR, Chung DC (2005) Induction of interleukin-8 preserves the angiogenic response in HIF-1alpha-deficient colon cancer cells. Nat Med 11:992–997

Murdoch C, Muthana M, Coffelt SB, Lewis CE (2008) The role of myeloid cells in the promotion of tumour angiogenesis. Nat Rev Cancer 8:618–631

Niu J, Azfer A, Zhelyabovska O, Fatma S, Kolattukudy PE (2008) Monocyte chemotactic protein (MCP)-1 promotes angiogenesis via a novel transcription factor, MCP-1-induced protein (MCPIP). J Biol Chem 283:14542–14551

Numasaki M, Watanabe M, Suzuki T, Takahashi H, Nakamura A, McAllister F, Hishinuma T, Goto J, Lotze MT, Kolls JK, Sasaki H (2005) IL-17 enhances the net angiogenic activity and *in vivo* growth of human non-small cell lung cancer in SCID mice through promoting CXCR-2-dependent angiogenesis. J Immunol 175:6177–6189

Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L (2006) VEGF receptor signalling – in control of vascular function. Nat Rev Mol Cell Biol 7:359–371

Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA (2005) Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 121:335–348

Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, Inoue M, Bergers G, Hanahan D, Casanovas O (2009) Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell 15:220–231

Pearson JD (2009) Endothelial progenitor cells – hype or hope? J Thromb Haemost 7:255–262

Perollet C, Han ZC, Savona C, Caen JP, Bikfalvi A (1998) Platelet factor 4 modulates fibroblast growth factor 2 (FGF-2) activity and inhibits FGF-2 dimerization. Blood 91:3289–3299

Petit I, Jin D, Rafii S (2007) The SDF-1-CXCR4 signaling pathway: a molecular hub modulating neo-angiogenesis. Trends Immunol 28:299–307

Petreaca ML, Yao M, Liu Y, Defea K, Martins-Green M (2007) Transactivation of vascular endothelial growth factor receptor-2 by interleukin-8 (IL-8/CXCL8) is required for IL-8/CXCL8-induced endothelial permeability. Mol Biol Cell 18:5014–5023

Phillips RJ, Burdick MD, Lutz M, Belperio JA, Keane MP, Strieter RM (2003) The stromal derived factor-1/CXCL12-CXC chemokine receptor 4 biological axis in non-small cell lung cancer metastases. Am J Respir Crit Care Med 167:1676–1686

Pi X, Wu Y, Ferguson JE 3rd, Portbury AL, Patterson C (2009) SDF-1alpha stimulates JNK3 activity via eNOS-dependent nitrosylation of MKP7 to enhance endothelial migration. Proc Natl Acad Sci USA 106:5675–5680

Rolny C, Capparuccia L, Casazza A, Mazzone M, Vallario A, Cignetti A, Medico E, Carmeliet P, Comoglio PM, Tamagnone L (2008) The tumor suppressor semaphorin 3B triggers a prometastatic program mediated by interleukin 8 and the tumor microenvironment. J Exp Med 205:1155–1171

Romagnani P, Annunziato F, Lasagni L, Lazzeri E, Beltrame C, Francalanci M, Uguccioni M, Galli G, Cosmi L, Maurenzig L, Baggiolini M, Maggi E, Romagnani S, Serio M (2001) Cell cycle-dependent expression of CXC chemokine receptor 3 by endothelial cells mediates angiostatic activity. J Clin Invest 107:53–63

Ryschich E, Lizdenis P, Ittrich C, Benner A, Stahl S, Hamann A, Schmidt J, Knolle P, Arnold B, Hammerling GJ, Ganss R (2006) Molecular fingerprinting and autocrine growth regulation of endothelial cells in a murine model of hepatocellular carcinoma. Cancer Res 66:198–211

Ryu J, Lee CW, Hong KH, Shin JA, Lim SH, Park CS, Shim J, Nam KB, Choi KJ, Kim YH, Han KH (2008) Activation of fractalkine/CX3CR1 by vascular endothelial cells induces angiogenesis through VEGF-A/KDR and reverses hindlimb ischaemia. Cardiovasc Res 78:333–340

Saijo Y, Tanaka M, Miki M, Usui K, Suzuki T, Maemondo M, Hong X, Tazawa R, Kikuchi T, Matsushima K, Nukiwa T (2002) Proinflammatory cytokine IL-1 beta promotes tumor growth of Lewis lung carcinoma by induction of angiogenic factors: *in vivo* analysis of tumor-stromal interaction. J Immunol 169:469–475

Salanga CL, O'Hayre M, Handel T (2009) Modulation of chemokine receptor activity through dimerization and crosstalk. Cell Mol Life Sci 66:1370–1386

Salcedo R, Wasserman K, Young HA, Grimm MC, Howard OM, Anver MR, Kleinman HK, Murphy WJ, Oppenheim JJ (1999) Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: In vivo neovascularization induced by stromal-derived factor-1alpha. Am J Pathol 154:1125–1135

Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ, Murphy WJ (2000) Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. Blood 96:34–40

Salcedo R, Young HA, Ponce ML, Ward JM, Kleinman HK, Murphy WJ, Oppenheim JJ (2001) Eotaxin (CCL11) induces *in vivo* angiogenic responses by human CCR3+ endothelial cells. J Immunol 166:7571–7578

Salcedo R, Zhang X, Young HA, Michael N, Wasserman K, Ma WH, Martins-Green M, Murphy WJ, Oppenheim JJ (2003) Angiogenic effects of prostaglandin E2 are mediated by up-regulation of CXCR4 on human microvascular endothelial cells. Blood 102:1966–1977

Salvucci O, Yao L, Villalba S, Sajewicz A, Pittaluga S, Tosato G (2002) Regulation of endothelial cell branching morphogenesis by endogenous chemokine stromal-derived factor-1. Blood 99:2703–2711

Sato Y, Abe M, Takaki R (1990) Platelet factor 4 blocks the binding of basic fibroblast growth factor to the receptor and inhibits the spontaneous migration of vascular endothelial cells. Biochem Biophys Res Commun 172:595–600

Schioppa T, Uranchimeg B, Saccani A, Biswas SK, Doni A, Rapisarda A, Bernasconi S, Saccani S, Nebuloni M, Vago L, Mantovani A, Melillo G, Sica A (2003) Regulation of the chemokine receptor CXCR4 by hypoxia. J Exp Med 198:1391–1402

Scortegagna M, Cataisson C, Martin RJ, Hicklin DJ, Schreiber RD, Yuspa SH, Arbeit JM (2008) HIF-1alpha regulates epithelial inflammation by cell autonomous NFkappaB activation and paracrine stromal remodeling. Blood 111:3343–3354

Sgadari C, Angiolillo AL, Tosato G (1996) Inhibition of angiogenesis by interleukin-12 is mediated by the interferon-inducible protein 10. Blood 87:3877–3882

Shojaei F, Ferrara N (2008) Refractoriness to antivascular endothelial growth factor treatment: role of myeloid cells. Cancer Res 68:5501–5504

Shojaei F, Wu X, Malik AK, Zhong C, Baldwin ME, Schanz S, Fuh G, Gerber HP, Ferrara N (2007) Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. Nat Biotechnol 25:911–920

Shojaei F, Zhong C, Wu X, Yu L, Ferrara N (2008) Role of myeloid cells in tumor angiogenesis and growth. Trends Cell Biol 18:372–378

Sierro F, Biben C, Martinez-Munoz L, Mellado M, Ransohoff RM, Li M, Woehl B, Leung H, Groom J, Batten M, Harvey RP, Martinez AC, Mackay CR, Mackay F (2007) Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. Proc Natl Acad Sci USA 104:14759–14764

Singh S, Sadanandam A, Singh RK (2007) Chemokines in tumor angiogenesis and metastasis. Cancer Metastasis Rev 26:453–467

Singh S, Varney M, Singh RK (2009) Host CXCR2-dependent regulation of melanoma growth, angiogenesis, and experimental lung metastasis. Cancer Res 69:411–415

Smith DR, Polverini PJ, Kunkel SL, Orringer MB, Whyte RI, Burdick MD, Wilke CA, Strieter RM (1994) Inhibition of interleukin 8 attenuates angiogenesis in bronchogenic carcinoma. J Exp Med 179:1409–1415

Sparmann A, Bar-Sagi D (2004) Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. Cancer Cell 6:447–458

Spring H, Schuler T, Arnold B, Hammerling GJ, Ganss R (2005) Chemokines direct endothelial progenitors into tumor neovessels. Proc Natl Acad Sci USA 102:18111–18116

Stamatovic SM, Keep RF, Mostarica-Stojkovic M, Andjelkovic AV (2006) CCL2 regulates angiogenesis via activation of Ets-1 transcription factor. J Immunol 177:2651–2661

Staton CA, Reed MW, Brown NJ (2009) A critical analysis of current *in vitro* and *in vivo* angiogenesis assays. Int J Exp Pathol 90:195–221

Stine JT, Wood C, Hill M, Epp A, Raport CJ, Schweickart VL, Endo Y, Sasaki T, Simmons G, Boshoff C, Clapham P, Chang Y, Moore P, Gray PW, Chantry D (2000) KSHV-encoded CC chemokine vMIP-III is a CCR4 agonist, stimulates angiogenesis, and selectively chemoattracts TH2 cells. Blood 95:1151–1157

Strasly M, Cavallo F, Geuna M, Mitola S, Colombo MP, Forni G, Bussolino F (2001) IL-12 inhibition of endothelial cell functions and angiogenesis depends on lymphocyte-endothelial cell cross-talk. J Immunol 166:3890–3899

Strasly M, Doronzo G, Cappello P, Valdembri D, Arese M, Mitola S, Moore P, Alessandri G, Giovarelli M, Bussolino F (2004) CCL16 activates an angiogenic program in vascular endothelial cells. Blood 103:40–49

Strieter RM, Kunkel SL, Elner VM, Martonyi CL, Koch AE, Polverini PJ, Elner SG (1992) Interleukin-8. A corneal factor that induces neovascularization. Am J Pathol 141:1279–1284

Strieter RM, Polverini PJ, Kunkel SL, Arenberg DA, Burdick MD, Kasper J, Dzuiba J, Van Damme J, Walz A, Marriott D et al (1995) The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. J Biol Chem 270:27348–27357

Strieter RM, Gomperts BN, Keane MP (2007) The role of CXC chemokines in pulmonary fibrosis. J Clin Invest 117:549–556

Struyf S, Burdick MD, Proost P, Van Damme J, Strieter RM (2004) Platelets release CXCL4L1, a nonallelic variant of the chemokine platelet factor-4/CXCL4 and potent inhibitor of angiogenesis. Circ Res 95:855–857

Struyf S, Burdick MD, Peeters E, Van den Broeck K, Dillen C, Proost P, Van Damme J, Strieter RM (2007) Platelet factor-4 variant chemokine CXCL4L1 inhibits melanoma and lung carcinoma growth and metastasis by preventing angiogenesis. Cancer Res 67:5940–5948

Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S, Kishimoto T, Nagasawa T (1998) The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature 393:591–594

Tanaka T, Manome Y, Wen P, Kufe DW, Fine HA (1997) Viral vector-mediated transduction of a modified platelet factor 4 cDNA inhibits angiogenesis and tumor growth. Nat Med 3:437–442

Taub DD (2004) Cytokine, growth factor, and chemokine ligand database. Curr Protoc Immunol Chapter 6: Unit 6 29

Thelen M, Stein JV (2008) How chemokines invite leukocytes to dance. Nat Immunol 9:953–959

Volin MV, Woods JM, Amin MA, Connors MA, Harlow LA, Koch AE (2001) Fractalkine: a novel angiogenic chemokine in rheumatoid arthritis. Am J Pathol 159:1521–1530

Wang J, Dai J, Jung Y, Wei CL, Wang Y, Havens AM, Hogg PJ, Keller ET, Pienta KJ, Nor JE, Wang CY, Taichman RS (2007) A glycolytic mechanism regulating an angiogenic switch in prostate cancer. Cancer Res 67:149–159

Weber KS, Nelson PJ, Grone HJ, Weber C (1999) Expression of CCR2 by endothelial cells: implications for MCP-1 mediated wound injury repair and In vivo inflammatory activation of endothelium. Arterioscler Thromb Vasc Biol 19:2085–2093

Wu Y, Li YY, Matsushima K, Baba T, Mukaida N (2008) CCL3-CCR5 axis regulates intratumoral accumulation of leukocytes and fibroblasts and promotes angiogenesis in murine lung metastasis process. J Immunol 181:6384–6393

Yamada M, Kim S, Egashira K, Takeya M, Ikeda T, Mimura O, Iwao H (2003) Molecular mechanism and role of endothelial monocyte chemoattractant protein-1 induction by vascular endothelial growth factor. Arterioscler Thromb Vasc Biol 23:1996–2001

Yoon Y, Liang Z, Zhang X, Choe M, Zhu A, Cho HT, Shin DM, Goodman MM, Chen ZG, Shim H (2007) CXC chemokine receptor-4 antagonist blocks both growth of primary tumor and metastasis of head and neck cancer in xenograft mouse models. Cancer Res 67:7518–7524

You JJ, Yang CH, Huang JS, Chen MS, Yang CM (2007) Fractalkine, a CX3C chemokine, as a mediator of ocular angiogenesis. Invest Ophthalmol Vis Sci 48:5290–5298

Zumsteg A, Christofori G (2009) Corrupt policemen: inflammatory cells promote tumor angiogenesis. Curr Opin Oncol 21:60–70

Part III
Immunobiology of Allogeneic Stem Cell Transplantation

Genetic Polymorphisms in the Cytokine and Chemokine System: Their Possible Importance in Allogeneic Stem Cell Transplantation

Juergen Loeffler, Michael Ok, Oliver C. Morton, Markus Mezger, and Hermann Einsele

Contents

Abstract Chemokines represent central players of the innate and adaptive immunity and are involved in the regulation of inflammatory events occurring during infectious complications or during graft vs. host disease (GvHD). Patients after allogeneic stem cell transplantation (alloSCT) are at a high risk for the development of acute GvHD or to suffer from fungal infections. Susceptibility to fungal infections and the course of GvHD can be genetically influenced by single nucleotide

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polymorphisms (SNPs), which regulate expression or biological activity of chemokines, and therefore have an impact on the outcome of invasive aspergillosis and GvHD.

High lightened studies of abetting factors for GvHD revealed SNPs in *TNFA*, *IL-6*, *IL-10*, *INF-γ*, *CCL2*, *CCL5* (*RANTES*), *IL-1Ra*, *IL-23R*, *IL-7Ralpha*, *IL-10RB*, and *CCR9* genes as prevalent considerable. Furthermore, additional SNPs were described to be significantly associated with fungal infections (*Aspergillus fumigatus, Candida albicans*), including markers in *CCL3*, *CCL4*, *CCL20*, *CXCL2*, *CXCL8*, *CXCL10*, *CCR1*, and *CCR2*.

This review summarizes the current knowledge about the growing number of genetic markers in chemokine genes and their relevance for patients after alloSCT.

1 General Introduction

Cytokines and chemokines comprise a large family of signaling molecules that are widely used in cellular communication processes. They are relatively small proteins or glycoproteins (8–10 kDa) that circulate in nano- or picomolar concentrations to fulfill versatile functions. Chemokines were first identified in 1977 with the purification of the secreted platelet factor 4 (Wu et al. 1977). Since then, studies have identified more than 50 human chemokines and 20 chemokine receptors (Ruffini et al. 2007).

Originally, cytokines have been divided into lymphokines, interleukins, and chemokines. The further classification of chemokines is based on their structural characteristics, comprising three distinct domains: a highly flexible N-terminal domain, which is constrained by disulfide bonding between the N-terminal cysteine(s); a long loop, which leads into three antiparallel β-pleated sheets; and an α-helix that overlies the sheets (Baggiolini and Loetscher 2000).

Several subfamilies of chemokines can be distinguished: The characteristic of the CXC-chemokine family is that the first two cystein residues are separated by a single amino acid. In contrast, the CC-chemokines show two cystein residues that are direct neighbors (Luster 1998). The genes for CXC chemokines are tightly clustered mainly on chromosome 4, whereas the members of CC chemokines are encoded by genes that are located mainly on chromosome 17 (Naruse et al. 1996).

Chemokines can be also divided into two main subfamilies according to their functionality: inflammatory and homeostatic chemokines. Inflammatory chemokines control the recruitment of leukocytes in inflammation and tissue injury, while homeostatic chemokines navigate leukocytes to and within secondary lymphoid organs in the bone marrow and the thymus (Wagner et al. 2007). In addition, chemokines play important roles in development, hematopoiesis, lymphocyte trafficking, and angiogenesis.

Single nucleotide polymorphisms (SNPs) represent the most frequent genetic variations in the genome that genetically determine how humans develop diseases and respond to pathogens, chemicals, or other agents. There were approximately 15

million SNPs discovered so far, with over 5 million SNPs validated by multiple investigators (Chorley et al. 2008). Interestingly, two individuals differ in approximately 300,000 genetic markers from each other (=1/10,000 base pairs).

During the last decade, it has become obvious that SNPs in the genes encoding for chemokines and chemokine receptors influence the expression, structure, and biological activity of chemokines and chemokine receptors, leading to individual course and outcome of various infectious diseases, autoimmune disorders, as well as therapeutic strategies (Hollegaard and Bidwell 2006).

2 Hematopoietic Stem Cell Transplantation

Hematopoietic stem cell transplantation (HSCT) is an effective therapy for patients having a broad array of hematological malignancies. With the development of non-myeloablative SCT, this form of therapy can be also applied to older patients, and also to those patients with other medical conditions. The mechanism of disease control is due in large part to the immunological effects of the donor graft recognizing allo-antigens, or possibly tumor-specific antigens expressed by the malignant cells, resulting in immunological clearance. However, accompanying allogeneic stem cell transplantation (alloSCT), there is also the risk of an immunological reaction against the patient, termed graft vs. host disease (GvHD) (Zeiser et al. 2006).

Therefore, all HSCT patients receive immunosuppressive agents that impair T-cell functions or receive allografts that have been depleted of T-cells to diminish the risk of GvHD. The "price" for GvHD prevention in allo-SCT patients is high, as immunosuppression greatly impairs immune reconstitution.

Infectious complications (e.g., invasive aspergillosis, IA) as well as the development of GvHD are, apart from the reoccurrence of the malignancies post transplantation, the main determinants for successful HSCT. Thus, polymorphisms within genes that are associated with an individual's capability to build up an immune response to infectious agents, such as genetic markers in cytokine and chemokine genes, are of special interest for their association with HSCT outcome.

2.1 Invasive Aspergillosis

IA is the most tremendous and clinically relevant infection after SCT, mainly caused by the mold *Aspergillus fumigatus* (Stevens 2000). Patients after alloSCT have a higher risk of IA compared to autologous transplant recipients, as immunosuppression is administered with greater intensity (Einsele and Hebart 2002). Most cases of IA occur in the early stage after alloSCT, when neutropenia and mucosal toxicities from conditioning regimens are the major cause for immune defects. However, recently, many cases of IA also occur after neutrophil recovery, such as during immunosuppressive therapy for GvHD.

Morgan et al. presented data about the cumulative incidence of IA after 12 months. Based on a multicenter surveillance, incidence of IA after autologous SCT was 0.5%, 2.3% after alloSCT from a human leukocyte antigen (HLA)-matched related donor, and 3.9% after alloSCT from an unrelated donor (Morgan et al. 2005).

The patient condition after alloSCT is distinct and complex depending on individual hematopoietic reconstitution, donor/patient chimerism, infections, graft vs. host, and graft vs. tumor reactions. Marr and colleagues (Marr et al. 2002) have published data from the Fred Hutchinson Cancer Research Center on risk factors and outcomes among 1,682 patients who received HSCTs between January 1993 and December 1998. These risk factors include host variables (age, underlying disease), transplant variables (stem cell source), and later complications (acute and chronic GvHD [aGvHD and cGvHD], receipt of corticosteroids, secondary neutropenia, cytomegalovirus [CMV] disease, and respiratory virus infection). Very late IA (>6 months after transplantation) was associated with chronic GvHD and CMV disease. These results emphasize the post-engraftment timing of IA; risk factor analyzes verify previously recognized risk factors (receipt of corticosteroids and neutropenia), uncover the roles of lymphopenia and viral infections, and underline the relevance of GvHD as an important risk factor.

In addition to these general risk factors, susceptibility of alloSCT patients to IA is influenced by genetic markers. The first part of this review (paragraph 3) focuses on the relevance of SNPs in chemokine genes for infections with *A. fumigatus*.

3 Chemokine Response During Fungal Infections

Chemokines are central players for triggering innate and adaptive immune responses. They are often released by immune cells that have encountered a pathogen, thereby activating and recruiting further immune cells to increase the immune response to the pathogen. Transcriptome analysis of the interaction between *A. fumigatus* and immature dendritic cells (Mezger et al. 2008a) and monocytes (Loeffler et al. 2009; Cortez et al. 2006) showed differential regulation of a variety of chemokine genes, including CCL3, CCL4, CCL5, CCL20, and CXCL10 in iDC and CXCL2, CXCL8, CCL2, and CCL4 in monocytes. The chemokines can induce the migration of effector memory Th1 cells (Gafa et al. 2007). In an *in vitro* study, the *A. fumigatus* antigen Aspf1 caused increased expression of CXCL10 and CCL20 (Ok et al. 2009), which are important regulators for the immune response to *A. fumigatus*, as well as to *Candida albicans* (Kim et al. 2005).

During infections with *A. fumigatus* and *C. albicans*, the recruitment of neutrophils to the site of infection is essential for the clearance of the fungi. In addition, macrophages are the primary immune effector cells involved in the killing of *Cryptococcus neoformans* and *Pneumocystis* (Traynor and Huffnagle 2001).

Knockout deletions of chemokine receptors (CCR1 and CCR2) in mice underline the importance of the chemokine system for efficient clearance of *A. fumigatus*, *C. neoformans*, and *Histoplasma capsulatum* (Szymczak and Deepe 2009), whereas a deletion of CCL3 caused increase in susceptibility to *C. neoformans* (Esche et al. 2005).

3.1 Association of Polymorphisms in Chemokine Genes with Invasive Fungal Infection

SNPs in immune-related genes have been identified to be associated with an increased risk for various fungal infections (Carvalho et al. 2009). SNPs in IL-4 show an impact on the response to *C. albicans*, and SNPs in IL-10 have both favorable (ACC haplotype) and unfavorable (ATA haplotype) effects on susceptibility to develop IA (Carvalho et al. 2009). Recently, SNPs in chemokines have been identified that alter susceptibility to fungal infections. In patients with mycetoma, caused by *Madurella mycetomatis*, there was an association between susceptibility to infection and the CXCL8 251A allele and the CXCR2 +785C allele (van de Sande et al. 2007). These SNPs were correlated with an altered expression of CXCL8 and inefficient wound healing (van de Sande et al. 2007). CXCL8 is one of the major mediators of the inflammatory response, functioning as a chemoattractant, and a potent angiogenic factor (Table 1).

SNPs in CXCL10 were associated with an increased risk of IA in post-alloSCT patients. CXCL10 is secreted by several cell types in response to interferon-gamma and plays major roles in chemoattraction of macrophages, dendritic cells, and NK cells and in adhesion of T-cells to endothelial cells. The "CCAG" haplotype caused reduced CXCL10 expression in iDC exposed to *A. fumigatus* germ-tubes compared to wild type, which was significant since increased CXCL10 production was associated with surviving IA (Mezger et al. 2008b).

A further, clinically relevant complication following alloSCT is aGvHD and cGvHD. The severity of this pathophysiological condition is at least partly determined by point mutations in chemokine and cytokine genes.

Thus, paragraph 4 focuses on the relevance of SNPs on the development and intensity of GvHD.

Table 1 Polymorphisms in chemokine genes associated with fungal disease

Chemokine	Polymorphism	Key observation	References
CXCL10	C+11101T C+1642G A−1101G	Increased susceptibility to aspergillosis post HSCT	Mezger et al. (2008b)
CXCL8	CXCL8−251A	Slower wound healing and susceptibility to mycetoma	van de Sande et al. (2007)
CXCR2	CXCR2+785C	Associated with susceptibility to mycetoma	van de Sande et al. (2007)

4 Graft vs. Host Disease

aGvHD is a severe complication of hematopoietic stem cell allograft and the major cause of early transplant-related mortality and long-term complications. The pathophysiology of an aGvHD can be considered as a "cytokine storm" (Ferrara and Deeg 1991). Despite improved immunosuppressive prophylaxis, aGvHD occurs even in patients receiving a graft from a matched sibling donor, and the risk increases in unrelated or histo-incompatible donor transplantations (Anasetti and Hansen 1994). Patients who fail to respond to the first-line therapy with immunosuppressive agents have a poor prognosis, with high transplant-related mortality (Van Lint et al. 1998) due to GvHD itself and its treatment complications, such as opportunistic infections. Several second-line therapies have been proposed for the management of unresponsive GvHD, without obtaining a beneficial effect on patient's outcome or overall long-term survival (Martin et al. 1990, 1991).

4.1 SNPs in Cytokine and Chemokine Genes Associated with GvHD

There are numerous reports that describe associations between genetic polymorphisms in immune-relevant genes and the occurrence of aGvHD and cGvHD.

During aGvHD, a massive up-regulation of pro-inflammatory cytokines such as tumor necrosis factor (TNF) and IL-6 stimulate allo-reactive donor lymphocytes to attack recipient tissues. In consequence, increased circulating levels of IL-6 predict the occurrence and severity of GvHD (Imamura et al. 1994).

Mullighan et al. analyzed 22 polymorphisms in 11 immunoregulatory genes by PCR using sequence-specific primers in 160 related myeloablative transplants (Mullighan et al. 2004). They were able to demonstrate that an intronic polymorphism in the *TNFA* gene (TNF 488A) was associated with the risk of aGvHD (odds ratio [OR] 16.9), grades II–IV aGvHD (OR 3.3), cGvHD (OR 12.5), and early death posttransplant (OR 3.4).

Recently, the group of Visentainer (Viel et al. 2007) published a follow-up study on their work from 2005 (Visentainer et al. 2005) on the relationship between cytokine gene polymorphisms and GvHD in allogeneic stem cell transplant recipients. Now, this group studied 122 donor/recipient pairs who received HLA-identical transplants from siblings between June 1996 and June 2006. Donor/recipient alleles for TNFA −238 and IL2-330/+166 SNP were investigated by PCR-SSP. Interestingly, no association was observed between the risk of GvHD and these SNP. However, they describe that the polymorphism TNFA −238GA is associated with the occurrence and severity of cGvHD. The probability of cGvHD in patients with GA genotype at position −238 of *TNFA* gene was 91.7% in contrast to 59.4% in patients with the GG genotype ($p = 0.038$), and the probability of

extensive cGvHD in patients with TNFA −238GA was 91.7% compared with 46.3% in patients with TNFA −238GG ($p = 0.0046$).

Furthermore, various groups were able to show an association between SNPs in the IL-6 gene and GvHD. IL-6 is a subsidiary of TNF having analogical pro-inflammatory properties. Karabon et al. revealed an association between SNPs within the promoters of IL-6 (−174G/C) and IL-10 (−1082G/A, −819C/T, −592C/A) genes and the outcome of allogeneic sibling HSCT (Karabon et al. 2005). Ninety-three recipients and 74 donors were typed for IL-6 and IL-10 alleles. Recipient IL-6 G genotype was associated with increased IL-6 activity and C-reactive protein production. In univariate analyzes, recipient IL-6 G and donor IL-6 GG were associated with an increased risk for aGvHD. In contrast, recipient IL-10 GCC/GCC and donor IL-10 ACC decreased the risk of aGvHD. Multivariate analyzes confirmed the independent contribution of recipient IL-10 GCC/GCC (OR = 0.085, $p = 0.046$) and donor IL-6 GG (OR = 3.934, $p = 0.034$) genotypes to the risk of aGvHD (Table 2).

Ambruzova et al. performed a preliminary study in which they could show that again the polymorphism −174 G/C in IL-6 is associated with the risk of aGvHD and survival after alloSCT (Ambruzova et al. 2008). They screened a Czech population of 56 patients and their HLA-matched sibling donors. Recently, the same group (Ambruzova et al. 2009) published results from a much larger patient cohort of 166 HLA-identical alloSCT pairs. The group genotyped the chemokine CCL2 −2518A/G and CCL2 −2076A/T SNPs using sequence-specific primers. CCL2, also known as monocyte chemotactic protein-1, recruits monocytes, T cells, and dendritic cells to sites of tissue injury and infection. The presence of the CCL2 −2076TT genotype

Table 2 Polymorphisms in chemokine genes associated with graft vs. host disease

Chemokine	Polymorphism	Key observation	References
TNFA	TNFA 488A	Risk of acute and chronic GvHD and early death postTx	Mullighan et al. (2004)
TNFA	TNFA −238GA	Risk of chronic GvHD increased	Viel et al. (2007)
IL-6	IL-6 −174GC	Risk of acute GvHD increased	Karabon et al. (2005)
IL-6	IL-6 −174GC	Risk of acute GvHD increased	Ambruzova et al. (2008)
IL-10	IL-10 −1082GA, −819CT, −592CA	Risk of acute GvHD decreased	Karabon et al. (2005)
CCL5	CCL5 −28CG	Higher incidence of chronic GvHD, extensive GvHD, severe GvHD	Kim et al. (2007)
CCR9	CCR9 −926AG	Increased incidence of acute and chronic skin GvHD	Inamoto et al. (2009)
IL-23R	IL-23R 1142GA	Reduced incidence of acute GvHD	Gruhn et al. (2009)
IL-23R	IL-23R 1142GA	Reduced incidence of acute GvHD	Elmaagacli et al. (2008)
IL-7Ralpha	IL-7Ra +1237AG	Survival after alloSCT	Shamim et al. (2006)

was associated with decrease of OS ($p = 0.04$) and increase of TRM ($p = 0.02$) in patients who have undergone transplantation by related donors.

Serial expression levels of CC Chemokine ligand 5 (CCL5, previously known as RANTES, a recruiter of granulocytes and T-cells into inflammatory sites) exhibited (after alloSCT) similar expression changes in patients than those exhibited by IL-6 and TNFa (Nomura et al. 2006). Genetic markers of the CCL5 promoter at positions −28 (rs1800825) and −403 (rs2107538) were analyzed in 72 recipients and donors using PCR and restriction fragment length polymorphism methods (Kim et al. 2007). This group found that the CG genotype of the CCL5 gene at position −28 in recipients was significantly associated with a higher incidence of chronic [cGvHD] ($p = 0.004$), extensive cGvHD ($p = 0.038$), and severe grade of cGvHD at presentation ($p = 0.017$) compared to the CC genotype. In terms of haplotype analysis, the recipients with AG haplotype of CCL5 gene also showed a higher incidence of cGvHD ($p = 0.003$), extensive cGvHD ($p = 0.023$), and more severe grade of cGvHD ($p = 0.020$).

4.2 Genetic Markers in Cytokine Receptor and Chemokine Receptor Genes Associated with GvHD

Recently, a few reports on the association of SNPs in cytokine and chemokine receptor genes and the occurrence of GvHD were published. Inamoto et al. (2009) reported that donor SNP in CCR9 affects the incidence of skin GvHD. CCR9 is unique because it is exclusively expressed in epithelial cells and in Peyer's patches of the small intestine; the specific ligand of this receptor is CCL25 (also known as thymus-expressed chemokine). They analyzed the SNP of donors in 167 consecutive patients who received allo-HSCT from an HLA-identical sibling donor. Genotypes were tested for associations with aGvHD and cGvHD in each organ and transplant outcome. Multivariate analyzes showed that the genotype 926AG was significantly associated with the incidence of acute stage ≥ 2 skin GvHD (hazard ratio [HR], 3.2; 95% confidence interval [CI], 1.1–9.1; $p = 0.032$) and chronic skin GvHD (HR, 4.1; 95% CI, 1.1–15; $p = 0.036$), but not with GvHD in other organs or with relapse or nonrelapse mortality. The authors conclude that more active homing of CCR9-926AG T-cells to Peyer's patches may produce changes in antigen presentation and result in increased incidence of skin GvHD.

Cullup et al. examined polymorphisms in the interleukin-1 receptor (IL-1Ra) antagonist gene in HLA-matched allogeneic bone marrow transplant patients and donors. IL-1Ra VNTR (allele 2) in the donor genotype was more frequent with milder aGvHD grades 0–II (29 out of 59 transplants) than severe GvHD grades III–IV (2 out of 18 transplants, $p = 0.0032$) (Cullup et al. 2001). This association was confirmed in a subgroup with cyclosporine monotherapy prophylaxis: donor possession of allele 2 was again associated with milder aGvHD, grades 0–II (19 out of 38 transplants), than grades III–IV (1 out of 14, $p = 0.0042$) transplants. They

concluded that the donor genotype for the IL-1Ra polymorphism has an apparent protective role against aGvHD following transplantation. Furthermore, the same group was able to show that patients homozygous for the IFN-γ Intron1 allele 3 had more severe (grade III–IV) aGvHD (Cavet et al. 2001).

Very recently, there was a study describing an analysis of the 1142G>A SNP in the interleukin-23 receptor gene (IL23R, which pairs with the receptor molecule IL12Rbeta1, both are required for IL23 signaling) in a cohort of 231 children who underwent alloSCT and their respective donors (Gruhn et al. 2009). The authors found a significantly reduced incidence of aGvHD grade II–IV in patients who were transplanted from a donor with the IL23R polymorphism (5.0% vs. 33.3%; $p = 0.009$). There was no case of aGvHD grade III–IV if this polymorphism occurred in the donor. These findings could be particularly relevant for children with inborn metabolic or immunologic disorders who do not benefit from a graft vs. tumor effect, and thus, selection of a donor with the IL23R polymorphism might be beneficial.

In parallel, a group from Essen, Germany, described a study in which they have analyzed the identical SNP in a cohort of 221 adult transplant recipients and their HLA-identical sibling donors and in a second cohort of 186 adult transplant recipients and their HLA-identical unrelated donors. Genotypes were tested for an association with GvHD by multivariate analysis. The donor's IL-23R genotype was significantly associated with a reduced risk of aGvHD in both cohorts for patients after transplantation. Analysis of all 407 transplant recipients showed that IL-23R (1142G>A, Arg381Gln) genotype of the donor was associated with a decreased risk of grades II–IV aGvHD (31.6% compared to 51.0%, $p = 0.02$) and grades III–IV severe aGvHD (3.9% compared to 23.4%, $p = 0.003$). Death in remission was significantly lower in patients who underwent transplantation from donors with variant IL23-R (11.7% vs. 27.7%, $p = 0.028$), whereas overall survival or relapse rates were not influenced significantly by the IL-23R genotype. The authors concluded that among recipients of hematopoietic cells from HLA-identical donors, the IL-23R (Arg381Gln) gene variant on the donor side has a protective effect on the occurrence of aGvHD in recipients after transplantation.

IL-7 is essential for T-cell development in the thymus and for the maintenance of peripheral T-cells. IL-7 signals through IL-7 receptor (IL-7R), which consists of the gamma-c-chain and an alpha-chain. Sequencing of IL-7R alpha has revealed the existence of four SNPs (+510C/T, +1237A/G, +2087T/C, and +3110A/G), which all give rise to amino acid substitutions. IL-7R alpha polymorphisms were determined in 195 recipient and donor pairs from either matched sibling donors or matched unrelated donors (MUD). Genotyping of 173 normal controls was performed in parallel (Shamim et al. 2006). In MUD transplants, the +1237A/G genotype of the donor was associated with survival after SCT, the mortality being highest and intermediate for the GG and AG genotypes, respectively ($p = 0.023$). This pattern was more pronounced with respect to treatment-related mortality ($p = 0.003$), while IL-7R alpha genotypes were unrelated to the risk of relapse of leukemia. The IL-7R alpha +1237A/G genotype of the recipient and the genotypes of the other three polymorphisms were not significantly associated with the

outcome of SCT. These findings suggest that the IL-7R alpha polymorphisms may be of importance for treatment-related mortality after SCT.

Finally, in a recent study, analyzing 936 patients receiving a HCT from unrelated donors, genotypes of SNPs in the IL-10 gene and the IL-10RB gene were evaluated as correlates with outcomes after transplantation (Tseng et al. 2009). This group found no statistically significant associations of polymorphisms at positions −3575, −2763, −1082, and −592 of the IL10 gene or codon 238 of the IL10RB gene with severe aGvHD, extensive cGvHD, or non-relapse mortality after hematopoietic cell transplantation. The authors concluded that, although genetic variation in the IL-10 pathway affects nonrelapse mortality in HLA-identical sibling transplants, their results indicate that genetic variation in the IL-10 pathway does not significant affect these outcomes in unrelated donor transplants, suggesting that the strength of the allo-immune response in the latter exceeds the anti-inflammatory activity of IL-10.

In parallel, another study by Azarpira et al. came to the same conclusion; these identical SNPs in the IL-10 promoter were found not to be associated with aGvHD in patients after alloSCT from matched sibling donors (Azarpira et al. 2008).

5 SNPs in Adaptive Immunity

T-lymphocytes play a major role in cell-mediated immunity; CTLA-4 is an inhibitory molecule that down-regulates T-cell activation. In 2007, there was the first report showing an association between polymorphisms at CTLA-4 and clinical outcome after allo-HSCT (Pérez-García et al. 2007). The CT60 genotype influences relapse and aGvHD, probably due to its action on CTLA-4 alternative splicing. Patients receiving stem cells ($n = 536$ HLA-identical sibling donors of allo-HSC transplants were genotyped) from a donor with at least 1 G allele in position CT60 had worse overall survival (56.2% vs. 69.8% at 5 years; $p = 0.001$; HR, 3.80; 95% CI, 1.75–8.22) due to a higher risk of relapse ($p = 0.049$; HR, 1.71; 95% CI, 1.00–2.93). aGvHD was more frequent in patients receiving CT60 AA stem cells ($p = 0.033$; HR, 1.54; 95% CI, 1.03–2.29) (Table 3).

Recently, the same group investigated the association between the CTLA4 CT60 A/G genotype and the incidence of leukemic relapse in 143 adult patients with AML in first complete remission (Pérez-García et al. 2009). Interestingly, the CT60 AA genotype was again associated with a higher rate of leukemic relapse (56.4% vs. 35.6%; $p = 0.004$; HR = 2.64; 95% CI = 1.36–5.14) and lower overall survival at 3 years (39.4% vs. 68.4%; $p = 0.004$; HR = 2.80; 95% CI = 1.39–5.64).

Table 3 Polymorphisms in chemokine genes associated with T-cell activation

Chemokine	Polymorphism	Key observation	References
CTLA-4	CTLA4 CT60AA	Higher risk of relapse and more frequent acute GvHD	Pérez-García et al. (2007)

6 Ethnicity and SNP Frequencies

The analysis of genetic variation in humans is identifying the genetic basis for population differences in susceptibility to various diseases. Endemic diseases apply a selective pressure to local populations, which drives ethnic differences in SNP frequencies. Adaptation to malaria is the most widely known example of genetic variation and disease susceptibility. Resistance to malaria is associated with a SNP in the β-globin gene that occurs at a frequency of approximately 25% in populations in sub-Saharan Africa (Kwiatkowski 2005). Ethnic differences in SNP frequencies have been observed in several genes that are important in the innate immune system. Toll-like receptors (TLRs) recognize structural motifs on microbial pathogens; TLR 4 shows two SNPs (Asp299Gly and Thr399Ile) that occur in Caucasians but not in Africans and are linked to a variety of conditions including hyperinflammatory response and susceptibility to sepsis (Misch and Hawn 2008). Variation in TLR9 occurs at a frequency of 15% in North American populations (Greene et al. 2009), and SNP, −1237T/C, has been linked to asthma in Caucasians (Lazarus et al. 2003). DC-SIGN is a receptor on dendritic cells that exhibits SNPs in the promoter region that have been correlated to human T-lymphotropic virus type 1 (HTLV-1). In a study of these SNPs in four Brazilian ethnic groups, it was found that the −336A and −139A SNPs were more common in Asians and the −201T was present only in Africans (Kashima et al. 2009). Ethnic related differences in IL10 (Rady et al. 2004) and TLRs (Lazarus et al. 2003; Misch and Hawn 2008) may have a bearing on susceptibility to infectious diseases. Such genetic variation could present a risk factor for allogeneic stem cell transplantation since the recipient may acquire some of the immune characteristics of the donor (Bochud et al. 2008).

Finally, it has to be pointed out that several of the studies mentioned in this review describe associations in unique genetic backgrounds, such as the studies of the group of Visentainer (Visentainer et al. 2005; Viel et al. 2007), which were performed in a limited Brazilian cohort of patients, or the association studies of Ambruzova et al., which analyzed small cohorts of the Czech population (Ambruzova et al. 2008).

7 Conclusion

In conclusion, the studies described impressively show that defined genetic makers in genes encoding for cytokines and chemokines are associated with the individual risk to develop severe complications after alloSCT. However, further studies are necessary with higher number of patients and genetic markers; genome-wide association analyses using arrays covering up to 1 million SNPs might be powerful options for further studies. These studies must consider appropriate population stratification, power, and consistency of association and should ideally include functional data to support the observed associations. In the future, stratification of

high risk patients, based on their individual genetic profiles, might lead to a reduction of morbidity and mortality in this patient cohort.

References

Ambruzova Z, Mrazek F, Raida L et al (2008) Association of IL-6 gene polymorphism with the outcome of allogeneic haematopoietic stem cell transplantation in Czech patients. Int J Immunogenet 35:401–403

Ambruzova Z, Mrazek F, Raida L et al (2009) Association of IL6 and CCL2 gene polymorphisms with the outcome of allogeneic haematopoietic stem cell transplantation. Bone Marrow Transplant 44:227–235

Anasetti C, Hansen JA (1994) Effect of HLA incompatibility in marrow transplantation from unrelated and HLA-mismatched related donors. Transfus Sci 15:221–230

Azarpira N, Ramzi M, Aghdaie MH et al (2008) Interleukin-10 gene polymorphism in bone marrow transplant recipients. Exp Clin Transplant 6:74–79

Baggiolini M, Loetscher P (2000) Chemokines in inflammation and immunity. Immunol Today 21:418–420

Bochud PY, Chien JW, Marr KA et al (2008) Toll-like receptor 4 polymorphisms and aspergillosis in stem-cell transplantation. N Engl J Med 359:1766–1777

Carvalho A, Cunha C, Pasqualotto AC et al (2009) Genetic variability of innate immunity impacts human susceptibility to fungal diseases. Int J Infect Dis [PubMed: 19828347]. DOI: 10.1016/j.ijid.2009.06.028

Cavet J, Dickinson AM, Norden J et al (2001) Interferon-gamma and interleukin-6 gene polymorphisms associate with graft-versus-host disease in HLA-matched sibling bone marrow transplantation. Blood 98:1594–1600

Chorley BN, Wang X, Campbell MR et al (2008) Discovery and verification of functional single nucleotide polymorphisms in regulatory genomic regions: current and developing technologies. Mutat Res 659:147–157

Cortez KJ, Lyman CA, Kottilil S et al (2006) Functional genomics of innate host defense molecules in normal human monocytes in response to *Aspergillus fumigatus*. Infect Immun 74:2353–2365

Cullup H, Dickinson AM, Jackson GH et al (2001) Donor interleukin 1 receptor antagonist genotype associated with acute graft-versus-host disease in human leucocyte antigen-matched sibling allogeneic transplants. Br J Haematol 113:807–813

Einsele H, Hebart H (2002) Cellular immunity to viral and fungal antigens after stem cell transplantation. Curr Opin Hematol 9:485–489

Elmaagacli AH, Koldehoff M, Landt O et al (2008) Relation of an interleukin-23 gene polymorphism to graft-versus-host disease after hematopoietic cell transplantation. Bone Marrow Transplant 41:821–826

Esche C, Stellato C, Beck LA (2005) Chemokines: key players in innate and adaptive immunity. J Invest Dermatol 125:615–628

Ferrara JLM, Deeg HL (1991) Graft-versus-host-disease. N Engl J Med 324:667–674

Gafa V, Remoli ME, Giacomini E et al (2007) *In vitro* infection of human dendritic cells by *Aspergillus fumigatus* conidia triggers the secretion of chemokines for neutrophil and Th1 lymphocyte recruitment. Microbes Infect 9:971–980

Greene JA, Moormann AM, Vulule J et al (2009) Toll-like receptor polymorphisms in malaria-endemic populations. Malar J 8:50

Gruhn B, Intek J, Pfaffendorf N et al (2009) Polymorphism of interleukin-23 receptor gene but not of NOD2/CARD15 is associated with graft-versus-host disease after hematopoietic stem cell transplantation in children. Biol Blood Marrow Transplant 15:1571–1577

Hollegaard MV, Bidwell JL (2006) Cytokine gene polymorphism in human disease: on-line databases, supplement 3. Genes Immun 7:269–276

Imamura M, Hashino S, Kobayashi H et al (1994) Serum cytokine levels in bone marrow transplantation: synergistic interaction of interleukin-6, interferon-gamma, and tumor necrosis factor-alpha in graft-versus-host disease. Bone Marrow Transplant 13:745–751

Inamoto Y, Murata M, Katsumi A et al (2009) Donor single nucleotide polymorphism in the CCR9 gene affects the incidence of skin GVHD. Bone Marrow Transplant 2010 Feb; 45(2):363–369

Karabon L, Wysoczanska B, Bogunia-Kubik K et al (2005) IL-6 and IL-10 promoter gene polymorphisms of patients and donors of allogeneic sibling hematopoietic stem cell transplants associate with the risk of acute graft-versus-host disease. Hum Immunol 66:700–710

Kashima S, Rodrigues ES, Azevedo R et al (2009) DC-SIGN (CD209) gene promoter polymorphisms in a Brazilian population and their association with human T-cell lymphotropic virus type 1 infection. J Gen Virol 90:927–934

Kim HS, Choi EH, Khan J et al (2005) Expression of genes encoding innate host defense molecules in normal human monocytes in response to Candida albicans. Infect Immun 73:3714–3724

Kim DH, Jung HD, Lee NY et al (2007) Single nucleotide polymorphism of CC chemokine ligand 5 promoter gene in recipients may predict the risk of chronic graft-versus-host disease and its severity after allogeneic transplantation. Transplantation 84:917–925

Kwiatkowski DP (2005) How malaria has affected the human genome and what human genetics can teach us about malaria. Am J Hum Genet 77:171–192

Lazarus R, Klimecki WT, Raby BA et al (2003) Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (TLR9): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. Genomics 81:85–91

Loeffler J, Haddad Z, Bonin M et al (2009) Interaction analyses of human monocytes co-cultured with different forms of *Aspergillus fumigatus*. J Med Microbiol 58:49–58

Luster AD (1998) Chemokines – chemotactic cytokines that mediate inflammation. N Engl J Med 338:436–445

Marr KA, Carter RA, Boeckh M et al (2002) Invasive aspergillosis in aloogeneic stem cell transplant recipients: changes in epidemiology and risk factors. Blood 100:4358–4366

Martin PJ, Schoch G, Fisher L et al (1990) A retrospective analysis of therapy for acute graft-versus-host disease: initial treatment. Blood 76:1464–1472

Martin PJ, Schoch G, Fisher L et al (1991) A retrospective analysis of therapy for acute graft-versus-host disease: secondary treatment. Blood 62:626–631

Mezger M, Kneitz S, Wozniok I et al (2008a) Proinflammatory response of immature human dendritic cells is mediated by dectin-1 after exposure to *Aspergillus fumigatus* germ tubes. J Infect Dis 197:924–931

Mezger M, Steffens M, Beyer M et al (2008b) Polymorphisms in the chemokine (C-X-C motif) ligand 10 are associated with invasive aspergillosis after allogeneic stem-cell transplantation and influence CXCL10 expression in monocyte-derived dendritic cells. Blood 111:534–536

Misch EA, Hawn TR (2008) Toll-like receptor polymorphisms and susceptibility to human disease. Clin Sci (Lond) 114:347–360

Morgan J, Wannemuehler KA, Marr KA et al (2005) Incidence of invasive aspergillosis following hematopoietic stem cell and solid organ transplantation: interim results of a prospective multicenter surveillance program. Med Mycol 43:49–58

Mullighan C, Heatley S, Doherty K et al (2004) Non-HLA immunogenetic polymorphisms and the risk of complications after allogeneic hemopoietic stem-cell transplantation. Transplantation 77:587–596

Naruse K, Ueno M, Satoh T et al (1996) A YAC contig of the human CC chemokine genes clustered on chromosome 17q11.2. Genomics 34:236–240

Nomura S, Ishii K, Kanazawa S et al (2006) Role of platelet-derived chemokines (RANTES and ENA-78) after stem cell transplantation. Transpl Immunol 15:247–253

Ok M, Latge JP, Baeuerlein C et al (2009) Immune responses of human immature dendritic cells can be modulated by the recombinant *Aspergillus fumigatus* antigen Aspf1. Clin Vaccine Immunol 16:1485–1492

Pérez-García A, De la Cámara R, Román-Gómez J et al (2007) CTLA-4 polymorphisms and clinical outcome after allogeneic stem cell transplantation from HLA-identical sibling donors. Blood 110:461–467

Pérez-García A, Brunet S, Berlanga JJ et al (2009) CTLA-4 genotype and relapse incidence in patients with acute myeloid leukemia in first complete remission after induction chemotherapy. Leukemia 23:486–491

Rady PL, Matalon R, Grady J et al (2004) Comprehensive analysis of genetic polymorphisms in the interleukin-10 promoter: implications for immune regulation in specific ethnic populations. Genet Test 8:194–203

Ruffini PA, Morandi P, Cabioglu N et al (2007) Manipulating the chemokine–chemokine receptor network to treat cancer. Cancer 109:2392–2404

Shamim Z, Ryder LP, Heilmann C et al (2006) Genetic polymorphisms in the genes encoding human interleukin-7 receptor-alpha: prognostic significance in allogeneic stem cell transplantation. Bone Marrow Transplant 37:485–491

Stevens D (2000) Aspergillosis. In: *Cecil textbook of medicine*, L. Goldman, Editor. p. 1875–1877, Elsevier, Maryland Heights

Szymczak WA, Deepe GS Jr (2009) The CCL7-CCL2-CCR2 axis regulates IL-4 production in lungs and fungal immunity. J Immunol 183:1964–1974

Traynor TR, Huffnagle GB (2001) Role of chemokines in fungal infections. Med Mycol 39:41–50

Tseng LH, Storer B, Petersdorf E et al (2009) IL10 and IL10 receptor gene variation and outcomes after unrelated and related hematopoietic cell transplantation. Transplantation 87:704–710

van de Sande WW, Fahal A, Verbrugh H et al (2007) Polymorphisms in genes involved in innate immunity predispose toward mycetoma susceptibility. J Immunol 179:3065–3074

Van Lint MT, Uderzo C, Locasciulli A et al (1998) Early treatment of acute graft-versus-host disease with high- or low-dose 6-methylprednisolone: a multicenter randomized trial from the Italian Group for Bone Marrow Transplantation. Blood 92:2288–2293

Viel DO, Tsuneto LT, Sossai CR et al (2007) IL2 and TNFA gene polymorphisms and the risk of graft-versus-host disease after allogeneic haematopoietic stem cell transplantation. Scand J Immunol 66:703–710

Visentainer JE, Lieber SR, Persoli LB et al (2005) Relationship between cytokine gene polymorphisms and graft-versus-host disease after allogeneic stem cell transplantation in a Brazilian population. Cytokine 32:171–177

Wagner W, Roderburg C, Wein F et al (2007) Molecular and secretory profiles of human mesenchymal stromal cells and their abilities to maintain primitive hematopoietic progenitors. Stem Cells 25:2638–2647

Wu VY, Walz DA, McCoy LE (1977) Purification and characterization of human and bovine platelet factor 4. Prep Biochem 7:479–493

Zeiser R, Beilhack A, Negrin RS (2006) Acute graft-versus-host disease-challenge for a broader application of allogeneic hematopoietic cell transplantation. Curr Stem Cell Res Ther 1:203–212

The Chemokine System: A Possible Therapeutic Target in Acute Graft Versus Host Disease

Nicolai A. Kittan and Gerhard C. Hildebrandt

Contents

Abstract Allogeneic hematopoetic stem cell transplantation often presents the only chance for cure in a number of malignant and nonmalignant hematologic diseases. However, its beneficial effects are counterweighed by the development of potentially lethal complications, most importantly the development of acute and chronic graft-vs.-host disease (GVHD). Alloantigen-reactive immune responses mediate injury and destruction of GVHD target organs, including the gastrointestinal tract, the liver, the skin, and the lung. Donor leukocyte infiltration into the respective tissues is orchestrated by interactions between chemokines and chemokine receptors, which will be reviewed using a basic science – clinical comparative approach.

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1 Introduction

For numerous malignant and nonmalignant diseases, allogeneic hematopoetic stem cell transplantation (HSCT) is the only curative treatment available today. However, its use and benefits are limited by the development of serious and life-threatening complications, most importantly, acute and chronic graft-vs.-host disease (GVHD), both of which are major causes for morbidity and treatment-related mortality (TRM). The pathophysiology of acute GVHD (aGVHD) has been a strong focus of both clinical and basic science over the last decades. The current concept involves three phases as follows: (1) conditioning toxicity to host tissue with subsequent expression of inflammatory cytokines and chemokines, resulting in the activation of host antigen presenting cells (APCs), (2) donor T cell activation, expansion, and differentiation, and (3) host tissue injury by infiltrating donor immune cells through direct cell to cell related mechanisms of cytotoxicity and the production of soluble cytotoxic mediators (Goker et al. 2001; Ferrara et al. 2009).

Distinct from aGVHD, chronic GVHD (cGVHD) pathophysiology is rather less understood, as both autoimmune-like processes, alloreactive T cells (Sullivan and Parkman 1983; Teshima et al. 2003; Cutler et al. 2001; Champlin et al. 2000), a shift from a Th1 to a Th2 immune response (Kataoka et al. 2001), immunodominant epitope-dependent organ involvement (Kaplan et al. 2004), and B-cell (auto-) antibody-production (Okamoto et al. 2000) seem to play a role.

Chemokines and their receptors comprise a complex system involved in leukocyte migration to target tissues and to inflammatory sites, in leukocyte activation, in the organization and structure of secondary lymphoid tissues, in hematopoiesis, and in angiogenesis (Pease and Williams 2006; Moser et al. 2004; Rollins 1997; Choi et al. 2007; Addison et al. 2000; Belperio et al. 2000; Strieter et al. 2005; Broxmeyer 2008; Ohl et al. 2003; Czermak et al. 1999; Muller et al. 2003). Following allogeneic HSCT, they are increasingly expressed in various GVHD target organs and contribute to organ injury and TRM (Mapara et al. 2006; New et al. 2002; Sugerman et al. 2004; Jaksch et al. 2005; Duffner et al. 2003; Hancock et al. 2000; Hildebrandt et al. 2004a, b, c, 2005; Piper et al. 2007; Terwey et al. 2005; Varona et al. 2005; Wysocki et al. 2004, 2005a, b; Bouazzaoui et al. 2009).

This article reviews the role of specific chemokines and their receptors in aGVHD and cGVHD and elucidates their potential as a target for preventive therapy or actual treatment of these deleterious complications following allogeneic HSCT.

2 Allogeneic Hematopoetic Stem Cell Transplantation and Graft Versus Host Disease

For many children and adults with hematologic malignancies (e.g., leukemia, lymphoma, and multiple myeloma) or nonmalignant diseases, including hemoglobinopathies and metabolic storage diseases, allogeneic HSCT provides the only

therapeutic option, with a potential for long-term remission and even cure. Until a few years ago, conceptual understanding of allogeneic HSCT in the treatment of cancer was based on the idea that lethal total body irradiation and high dose chemotherapy are critical means to fight underlying malignancy to the optimal extent, but at the same time will result in irreparable damage to the patient's hematopoesis. The purpose of transfusing donor stem cells that time was to rescue the patient from the emerging state of hematopoetic failure and fatal immunoinsufficiency. Nowadays, in the treatment of hematologic malignancies, the idea has shifted towards allogeneic HSCT as being the platform for long-lasting graft-vs.-leukemia (GVL) or graft-vs.-tumor (GVT) responses and adoptive immunotherapy, both of which imply alloantigen-specific and immunologically mediated disease eradication and surveillance (Truitt and Atasoylu 1991a, b; Oettel et al. 1994; Slavin et al. 1993; Fowler et al. 1996, 1997; Nash and Storb 1996; Barrett 1997; Datta et al. 1994; Fowler and Gress 2000; Riddell et al. 2002; Kolb et al. 2003; Mapara et al. 2003).

The clinical use of HSCT is nevertheless limited by the potential development of severe and life-threatening complications. Besides the increased risk for infections due to extensive immunosuppression, the most common and well described risk is GVHD.

Differences in the human leukocyte antigen (HLA) system between the stem cell donor and the recipient result in severe T cell-mediated inflammatory reactions against host tissue(s), which are summarized under the term "GVHD." However, GVHD can also be seen in completely HLA-matched transplantation due to immunologically relevant minor histocompatibility antigens (HAs) (Ferrara et al. 2009; Goulmy et al. 1996). According to former concepts to classify GVHD, acute disease (aGVHD) was distinguished from chronic disease (cGVHD) according to the time point of onset (before or after day 100 after transplantation). Nowadays, this concept has been modified, as with the introduction of reduced-intensity conditioning (RIC) regimens, late onset forms of aGVHD (appearing >100 days after HSCT) as well as an overlap syndrome sharing features of both acute and chronic disease are more frequently seen (Ferrara et al. 2009; Filipovich et al. 2005).

Incidence and severity of GVHD are related to the degree of HLA mismatch between donor and recipient, the amount of transplanted donor T cells within the graft, patient's age, and the chosen conditioning regimen (myeloablative vs. RIC), with an incidence ranging from 10 to 80%. Target organs in aGVHD include the immune system, skin, liver, gastrointestinal (GI) tract, and lung, and mortality can be as high as 95% (Ferrara et al. 2009; Cooke et al. 1998; Miklos et al. 2008; Cahn et al. 2005).

The pathophysiology of aGVHD involves three consecutive phases. Phase 1: Tissue damage, caused by the toxicity of the preparative conditioning regimen, is associated with the release of pro-inflammatory cytokines (e.g., TNF, IFNγ) and chemokines and with the activation of host and (later on) donor APCs (Sun et al. 2007; Hill et al. 1997). Phase 2: Antigen-loaded APCs migrate to secondary lymphoid organs, where they encounter and present their antigens to donor

T cells. This results in priming, proliferation, and differentiation of alloreactive CD4+ and CD8+ effector T cells. (Sun et al. 2007; Shlomchik et al. 1999; Teshima et al. 2002). Phase 3: The third phase of aGVHD, also called effector phase, is characterized by alloreactive, cytotoxic T lymphocytes (CTLs) directly infiltrating different GVHD target organs promoting tissue damage via apoptosis. T cell-derived inflammatory cytokines such as TNF, IFNγ, and IL-17 further contribute to target organ injury both on direct cytotoxic levels and indirectly by maintaining a pro-inflammatory environment, responsible for subsequent effector cell recruitment (Sun et al. 2007).

Much less is known about the pathophysiology of cGVHD, which can emerge from acute disease or appear de novo, usually within the first 2 years after HSCT. Its incidence varies from 25 to 80% (Baird and Pavletic 2006), and a limited form is separated from extensive disease, depending on the extent and severity of organ involvement. Recently, the NIH consensus approach for diagnosis and staging of cGVHD provided a scoring system based on the specificity of clinical signs and histopathology (Filipovich et al. 2005). In contrast to aGVHD, every host organ system can be potentially affected, with skin, eyes, oral cavity, GI tract, liver, and lungs being most commonly involved (Baird and Pavletic 2006; Lee 2005). Characteristics of cGVHD include (sub)acute inflammation alongside chronic, fibrotic organ changes. Based on experimental data and clinical observations, a shift towards Th2 immune responses, altered expression of transforming growth factor (TGF)-β, the production of autoantibodies, thymic dysfunction with defective negative selection, and a low regulatory T cell population have been implicated in the development of cGVHD (Teshima et al. 2003; Lee 2005; Martin 2008; Chu and Gress 2008). The response to immunosuppressive treatment is unpredictable and nonuniform, often displaying mixed responses in different organs. Although associated with lower relapse rates due to improved GVT and GVL effects, cGVHD therefore remains the major cause of nonrelapse mortality during long-term follow up after allogeneic HSCT, with 5-year survival rates as low as 40% (Ferrara et al. 2009; Filipovich et al. 2005; Baird and Pavletic 2006; Shulman et al. 1978; Pavletic et al. 2006a; Higman and Vogelsang 2004).

During all phases of aGVHD, chemokines promote and orchestrate the recruitment of immune cells, for example, APCs and effector cells, to secondary lymphoid organs and peripheral tissues. Recent findings that various chemokines like CCL2-5, CXCL1, CXCL9-11, CCL17, and CCL27 are up-regulated during aGVHD underline their pivotal role during this process (Mapara et al. 2006; New et al. 2002; Sugerman et al. 2004; Jaksch et al. 2005; Duffner et al. 2003; Hancock et al. 2000; Hildebrandt et al. 2004a, b, c, 2005; Piper et al. 2007; Terwey et al. 2005; Varona et al. 2005; Wysocki et al. 2004, 2005a, b; Bouazzaoui et al. 2009). In addition, a limited number of reports have recently commented on the chemokine–chemokine-receptor system in cGVHD (Kim et al. 2007; Morita et al. 2007). Therefore, interactions between chemokines and their receptors are of specific interest as potential targets for GVHD therapy and have become a growing focus of intensive research.

3 The Chemokine–Chemokine-Receptor System

Chemokines consist of a group of 8–14 kDa proteins, which signal through a family of seven transmembrane domain-containing G protein-coupled receptors (GPCRs), activating downstream effector pathways (Zlotnik and Yoshie 2000; Rot and Von Andrian 2004). The chemokine–chemokine-receptor system is characterized by a huge degree of redundancy and pleiotropy. Up to date, approximately 50 chemokines and 20 chemokine receptors are known. Chemokines activate different receptors, and at the same time, receptors can interact with multiple ligands. In addition, chemokines as well as their receptors can form functional dimers and high order oligomers, either with partners from their own (homodimerization, -oligomerization) or from a different subfamily (heterodimerization, -oligomerization), thus adding a high degree of variability to an already complex system. Chemokine ligands are currently classified into four families, the CC (CCL1-28), the CXC (CXCL1-16), the CXC3 (CXC3L1), and the XC (XCL1-2) family, depending on the pattern of the first two of four cysteine residues (Allen et al. 2007; Murphy et al. 2000; Murphy 2002; Viola and Luster 2008). The chemokine–chemokine-receptor system plays an important role in organizing and orchestrating leukocyte trafficking both in homeostasis and in states of inflammation. Chemokine expression can be enhanced by inflammatory cytokines (Mackay 2001) and have been associated with different human diseases like infection, autoimmunity, or cancer, in which they serve as chemoattractants, leading immune cells to the sites of antigen priming in secondary lymhoid organs as well as to peripheral tissues in different target organs (Viola and Luster 2008). Secretion and binding of the chemokine ligand to glycosaminoglycans (GAGs), expressed on the surface of endothelial cells, creates a substrate gradient attracting and steering leukocytes equipped with the appropriate receptor to the site of the highest concentration (Allen et al. 2007; Campbell et al. 1998). Leukocytes have been demonstrated to roll along the endothelium of vessels, a process that is regulated by the expression of integrins and selectins on leukocytes and endothelial cells. Concentration and clustering of integrins, caused by signaling events initiated by chemokine–chemokine-receptor binding, leads to high affinity contact of the leukocyte with the endothelium or components of the extracellular matrix at the site of inflammation, and consecutively to an arrest and transmigration through the vascular wall (Campbell et al. 1998; Constantin et al. 2000). But also during homeostasis, leukocytes regularly traffic to secondary lymphoid organs and peripheral tissues to keep up a constant immune surveillance.

4 The Role of the Chemokine–Chemokine-Receptor System in aGVHD

The role of chemokines and chemokine receptors in aGVHD has to been seen in the context of the different organs and the time course of the leading events; early changes have to be differentiated from late(r) events. Also, the expression on

different T cell subsets might play a role. For example, while the administration of CCR2-deficient CD8+ T cells resulted in a significantly decreased extent of leukocyte infiltration in GVHD target organs, unselected CD4+ and CD8+ CCR2−/− T cells had no effect on the course of disease (Terwey et al. 2005). While some chemokine expression studies, in which either the analysis comparing lethal vs. nonmyeloablative conditioning was restricted to the first 10 days after allogeneic HSCT, or in which chemokine expression levels were related to inflammatory organ infiltrates of unconditioned SCID recipient mice (Mapara et al. 2006; New et al. 2002), were limited to a smaller number of chemokines, other studies, looking at higher number of chemokines, were limited to one GVHD target organ only (Sugerman et al. 2004; Ichiba et al. 2003; Zhou et al. 2007). A comprehensive analysis of chemokine and chemokine receptor expression in the four major target organs (GI tract, liver, skin, and lung) of aGVHD following murine HSCT, which has been based on both kinetics and tropism of expression over a time period of 6 weeks, has been recently published by Bouazzaoui et al. (2009). This study provides important information for the initiation and planning of further experimental studies in the search of chemokines and their receptors as potential future targets in the treatment of GVHD. However, certain limitations have to be kept in mind in this study when interpreting this or any other experimental study published in this field up to date, which restrict their validity when applied to the patient setting: no immunosuppressive treatment was given for either GVHD prophylaxis or treatment and no infectious challenges were concurrently performed, both of which will have significant impact on chemokine expression *in vivo*.

In addition, chemokines have currently gained attention as potential biomarkers in the prediction of GVHD, as, for example, shown for CCL8 both in mice and men (Ota et al. 2009; Hori et al. 2008).

A fundamental requirement for the development of aGVHD is the close interaction between host APCs and donor T cells in secondary lymphoid organs. Here, alloantigen gets presented to the T cell, leading to T cell activation, proliferation, and generation of cellular effector cells. The progeny of cellular effectors will leave the lymphoid compartment and infiltrate peripheral aGVHD target organs (Ferrara et al. 2009; Beilhack et al. 2005).

CCR7, which is expressed on dendritic cells, naïve, and central memory T cells, is responsible for the recirculation of these cells into lymphoid organs in response to their ligands CCL19 and CCL21, and therefore is critical to the initiation of GVHD (Forster et al. 2008; Weninger et al. 2001; Yakoub-Agha et al. 2006; Sasaki et al. 2003). Additional chemokines, which are increasingly expressed in secondary lymphoid tissue after allogeneic HSCT, include CCL2-5, CCL8, CCL12, CXCL9-11, and XCL1, and are potentially involved in T cell activation and homing (Choi et al. 2007; New et al. 2002; Wysocki et al. 2004; Bouazzaoui et al. 2009; Serody et al. 2000), although these processes are not yet fully understood.

In this review we will focus primarily on the role of chemokines and their related receptors in the development of aGVHD in peripheral target organs (GI tract, liver, skin, and lung) by using a basic science – clinical comparative approach.

4.1 Chemokines and aGVHD of the Gastrointestinal Tract

Both pathophysiologically and clinically, the GI tract is of particular significance as a target organ of aGVHD. Accordingly, to better understand the mechanisms, regulating intestinal T cell trafficking during homeostasis and inflammation has been of major interest. CCR9 and its ligand CCL25 have been shown to participate in the recruitment of gut-tropic effector cells during homeostasis and inflammation (Saruta et al. 2007; Papadakis et al. 2001; Nishimura et al. 2009; Koenecke and Forster 2009). Hadeiba et al. report on a CCR9+ subset of tolerogenic, plasmacytoid dendritic cells, which migrate to the gut in response to CCL25, display tissue protective properties via the induction of regulatory T cells, and suppress antigen-specific immune responses, including aGVHD (Hadeiba et al. 2008).

An association for CCR5 with the development of aGVHD has been described both clinically and in experimental studies. The presence of the loss of function 32-nucleotide deletion (CCR5Δ32) in patients undergoing allogeneic HSCT resulted in a decreased incidence of aGVHD. Even more important, although the presence of the mutation in only the donor did not seem to alter the development of aGVHD, the presence of CCR5Δ32 genotype in both recipient and donor displayed highest protection, with none of the 11 patients suffering from aGVHD (Bogunia-Kubik et al. 2006). These findings go along with the prior reports on a genetic predisposition of donor or patient being responsible for the development of aGVHD in patients receiving allogeneic HSCT (Holler et al. 2004, 2006; Gruhn et al. 2009; Tseng et al. 2009; Ambruzova et al. 2009a, b; Markey et al. 2008; MacMillan et al. 2003a, b; Takahashi et al. 2000; Middleton et al. 1998).

Protection from GVHD in the absence of functional CCR5 surface expression has also been demonstrated by Murai and colleagues using a murine HSCT model, as they describe an important role for the expression of CCR5 on allogeneic donor T cells for their homing to Peyer's patches. Peyer's patches are an integral part of secondary lymphoid tissue, essentially involved in T cell priming and activation, and therefore, critically contributing to the initiation of aGVHD (Murai et al. 2003). However, in this study, recipient mice were not conditioned, and conflicting data demonstrating an even higher GVHD severity using a model, in which lethally irradiated mice were transplanted with CCR5 defective donor cells, have been reported by Wysocki et al. (Wysocki et al. 2004). The observed contrary outcome was probably due to conditioning regimen-related tissue toxicity, leading to increased proinflammatory chemokine expression in GVHD target organs, and, when compared to CCR5 wild-type cells, due to enhanced migratory properties of murine CCR5−/− T cells towards the CXC chemokine CXCL10, presumably using CXCR3 (Wysocki et al. 2004). Furthermore, lacking CCR5 on donor regulatory T cells (Tregs) may loosen a brake, which normally hinders GVHD propelling, therefore leading to more severe established disease (Wysocki et al. 2005a). In the study by Bouazzaoui et al., expression levels of CCL4 and CCL5, which both share CCR5 as a receptor with CCL3, were not significantly elevated in the GI tract of allogeneic recipients (Bouazzaoui et al. 2009), whereas another study

demonstrated an increase in colonic CCL5 expression on day 6 after HSCT (Mapara et al. 2006). High intestinal expression of CCL3 can be seen early, and most likely sources from the intestinal mucosa itself (Serody et al. 2000). In contrast to CCR5, CCR1 expression was not strongly elevated in the gut early after allogeneic HSCT (Bouazzaoui et al. 2009), suggesting that CCL3 (CCL5): CCR5 interactions rather than CCL4 or CCR1 are involved in early recruitment of T cells to the GI tract, promoting the initiation of aGVHD. However, CCL5:CCR1 interactions seem to indirectly contribute to GVHD target organ injury, as the absence of CCR1 on donor T cells resulted in generally suppressed alloreactive T cell activation, resulting in decreased injury to gut and liver as well (Choi et al. 2007).

Another potential target in the treatment of acute intestinal GVHD is CXCR3. Mapara et al. demonstrated that using myeloablative conditioning regimen by itself is sufficient to induce a significant but partially short-lived increase of CXCR3 ligands, especially CXCL10, in the colon, which was not seen after non-myeloablative conditioning. Subsequent development of aGVHD further increased CXCR3 ligand expression over the first 10 days, thus underlying the importance of Th1 immune responses in this early phase of aGVHD with respect to inflammatory chemokine induction (Mapara et al. 2006). The expression of the CXCR3 ligand family (CXCL9-11) remains elevated throughout the cellular phase of aGVHD (phase 3) (Bouazzaoui et al. 2009). Causal proof for a role of CXCR3 being expressed on CD8+ T cells in the development of intestinal aGVHD has been provided by Duffner et al. In animals transplanted with CD8+ CXCR3−/− donor T cells, T cells expanded and accumulated in the spleen and infiltration of the GI tract was reduced, leading to diminished intestinal GVHD as well as prolonged survival (Duffner et al. 2003). A confirmative study, in which prolonged administration of an anti-CXCR3 neutralizing antibody was successfully used in a mouse model of human GVHD, has been published by He et al. (2008).

Ueha et al. demonstrated a specific role for donor cell expressed CX3CL1 in the recruitment of alloreactive CD8+ T cells into the GI tract after allogeneic HSCT, when administration of an CX3CL1 antibody resulted in decreased numbers of CD8+ T cells in the gut, but did not show any effect on hepatic infiltrates (Ueha et al. 2007).

CCR6 has been implemented into the recruitment of alloreactive CD4+ T cells to GVHD target organs, including the GI tract, liver, and skin, as allogeneic HSCT with CCR6−/− resulted in significantly reduced disease severity (Varona et al. 2005). CCR6 may play a role in both effector and regulatory T cell function (Varona et al. 2006).

The exact role of other chemokines in the development of GI tract GVHD still remains to be defined. CXCR6 expression on CD8+ T cells contributes to the early recruitment of these cells to the liver, but not to the gut, early after allogeneic HSCT (Ueha et al. 2007; Sato et al. 2005). Consistent with these findings, intestinal expression of the CXCR6 ligand, CXCL16, was not increased early after transplantation (Bouazzaoui et al. 2009), and therapeutically, early interventions such as the use of neutralizing antibodies against CXCL16 did not alter the course of intestinal

GVHD (Ueha et al. 2007). However, as CXCL16 expression eventually rises over time (Bouazzaoui et al. 2009), and CXCR6 expression associates with increased T cell numbers in the GI tract, it cannot currently be excluded that CXCR6: CXCL16 interactions potentially contribute to T cell infiltration of the intestine at later time points.

Conflicting data has also been reported on CCR2 and its ligands. While Terwey et al. describe a defect in the migratory capacity of CD8+ CCR2−/− T cells, resulting in reduced infiltration in the gut, this could not confirmed by Hildebrandt and colleagues (Hildebrandt et al. 2004b; Terwey et al. 2005).

4.2 Chemokines and aGVHD of the Liver

The liver presents the second classical target organ of aGVHD. Hepatic GVHD is characterized by endothelial dysfunction, lymphocyte infiltration of the portal areas, and pericholangitis, which ultimately leads to bile duct destruction (Ferrara et al. 2009). Several chemokines and chemokine receptors have been reported to be increasingly expressed: CCL1-5, CCL7, CCL8, CXCL1, CXCL2, CXCL9, CXCL10, CXCL11, CXCL16, XCL1, CCR1, CCR5, CXCR2, CXCR6, and XCR1 (Choi et al. 2007; Mapara et al. 2006; New et al. 2002; Bouazzaoui et al. 2009; Ichiba et al. 2003; Murai et al. 1999), suggesting a central role of these chemokines in attracting alloreactive donor T cells during the course of disease.

In 1999, Murai and colleagues reported as one of the first chemokine receptors involved in GVHD-related liver injury that CCR5 expression on CD8+ T cells plays a substantial role in the hepatic migration of these cells when both the vivo neutralization of the receptor and one of the ligands, CCL3, resulted in significantly decreased T cell infiltration into the liver (Murai et al. 1999). As seen for the GI tract, CCR1, a second receptor for the CCR5 ligands CCL3-5, contributes to hepatic GVHD as well (Choi et al. 2007). However, at this point it is unclear whether the reduction in liver injury in the absence of CCR1 on donor T cells is primarily due to the general suppression of T cell activation or is co-mediated by an impaired migratory capacity in response to CCR1 ligands. Increased CCL3 expression not only derives from hepatic tissue, for example, bile duct epithelial cells and endothelial cells, but also from macrophages and infiltrating donor T cells (Serody et al. 2000), suggesting a chemokine-mediated feedback mechanism on the recruitment of CCR1+ and CCR5+ donor T cells to the liver. In contrast, Wysocki et al. showed an accumulation of CCR5 deficient T cells in the liver following allogeneic HSCT, as well as a higher sensitivity of these cells to CXCR3 ligands was postulated. As at the same time liver histopathology was not increased, the authors speculated that the cells were rather being trapped by the sinusoidal epithelium than directly causing tissue damage (Wysocki et al. 2004).

Redundancy of function of the chemokine–chemokine receptor system provides evolutionary stability. Correspondingly, CD8+ T cells infiltrate the sites of inflammation in hepatic GVHD not only using CCR5 or CCR1 but also other chemokine

receptors, such as CXCR3 and CXCR6. Transplantation of allogeneic CXCR3−/− donor T cells or the use of anti-CXCR3 antibodies resulted in decreased hepatic injury (Duffner et al. 2003; He et al. 2008), and Sato et al. described a markedly reduced migratory capacity of CXCR6−/− CD8+ but not CD4+ T cells to the liver following allogeneic HSCT (Sato et al. 2005). Furthermore, the latter study was confirmed targeting CXCL16, the ligand of CXCR6, when administration of anti-CXCL16 antibodies led to a reduction in liver tissue damage (Ueha et al. 2007).

Terwey et al. reported the contribution of CCR2 on donor cells to CD8+ T cell-mediated hepatic aGVHD (Terwey et al. 2005), whereas in CD4+ T cell-mediated hepatic aGVHD, CCR2 deficiency of donor T cells rather led to increased T cell infiltrates (Rao et al. 2003), and when using a CD4+ and CD8+ T cell-mediated GVHD model, no significant effect of donor cell CCR2 deficiency on liver histopathology was found (Hildebrandt et al. 2004b).

4.3 Chemokines and aGVHD of the Skin

One of the most frequent sites of aGVHD is the skin (Goker et al. 2001; Ferrara et al. 2009; Breathnach and Katz 1987), usually preceding intestinal or hepatic involvement. Both experimental and clinical studies indicate an increased cutaneous expression of a number of chemokines and their receptors following allogeneic HSCT. Specifically, in murine studies, which are usually performed without immunosuppressive GVHD prophylaxis or treatment, elevated expression levels peaked rather early after transplantation within the first two weeks as shown for CXCL1, CXCL2, CXCL9-11, CCL2, CCL5-9, CCL11, CCL12, CCL19, and XCL1, correlating with some of their respective receptors, including CCR1, CCR5, CXCR3, and XCR1 (Mapara et al. 2006; Sugerman et al. 2004; Bouazzaoui et al. 2009). CCR2 expression was significantly induced by week 3, and CXCR3 demonstrated a second peak during the late cellular cytotoxic phase of aGVHD (phase 3) at week 6 after HSCT (Bouazzaoui et al. 2009). Clinically, the infiltration of CD4+ CCR10+ CCR7low CCR4+ CXCR3+ CCR6− T cells into GVHD skin correlated with increased epidermal expression of CCL27 (Faaij et al. 2006). Interestingly, this T cell population was not found in the GI tract of a (although limited) number of four patients with intestinal aGVHD, suggesting that CCR10–CCL27 interactions may be specifically relevant for tissue-specific migration of alloreactive T cells to the skin during aGVHD (Faaij et al. 2006; Reiss et al. 2001). CCR10 was not found on skin infiltrating CD8+ T cells in patients with cutaneous GVHD (Faaij et al. 2006), and other chemokine receptors such as CXCR3 may be predominantly responsible for CD8+ T cell recruitment to the skin (He et al. 2008; Flier et al. 2001). Piper et al. reported an association between CXCR3+ lymphocytes with one of its ligand, CXCL10, in biopsies of patients with skin GVHD, but not explicitly looked at CD4+ vs. CD8+ T cell subsets (Piper et al. 2007). Other chemokine receptors potentially involved in regulating the recruitment of T cells

into inflamed skin during GVHD are CCR5 (Morita et al. 2007; Palmer et al. 2009) or CCR9 (Inamoto et al. 2009).

A better understanding, why not every patient undergoing allogeneic HSCT develops cutaneous GVHD, may be deducted from the study by Inamoto et al., who demonstrated that the presence of the nonsynonymous single nucleotide polymorphism of CCR9 gene (926AG) in the donor substantially increases the risk to specifically develop GVHD target organ injury to the skin (Inamoto et al. 2009).

Langerhans cells are the major APC in the skin and substantially involved in the pathophysiology of cutaneous GVHD (Merad et al. 2002, 2004). Merad and co-workers elegantly showed that host Langerhans cells are critical to propel skin injury following allogeneic HSCT. Host Langerhans cells can persist for several months within the skin and can be responsible for the onset of skin GVHD at later time points. Alloreactive T cells infiltrating the skin deplete host Langerhans cells, induce the expression of CCL2 and CCL20, which are the ligands for Langerhans cell-expressed CCR2 and CCR6, respectively, and – predominantly using CCR6:CCL20 interactions, facilitated by CCR2:CCL2 – promote the recruitment of donor Langerhans cells into the skin (Merad et al. 2002, 2004). Consistently, in a clinical study on Langerhans cell chimerism in eight children undergoing allogeneic HSCT, Emile et al. reported that, in the two patients receiving a T cell-depleted transplant, no donor Langerhans cells were present in the skin, whereas in patients receiving a non-T cell-depleted transplant, donor Langerhans cells could be found (Emile et al. 1997).

4.4 Chemokines and aGVHD of the Lung

Acute noninfectious diffuse lung injury, classically defined as idiopathic pneumonia syndrome (IPS), has been associated with mortality rates of >70% (Clark et al. 1993; Kantrow et al. 1997) and has an incidence between 5 and 25% (Clark et al. 1993; Kantrow et al. 1997; Crawford and Hackman 1993; Crawford et al. 1993; Krowka et al. 1985; Weiner et al. 1986). IPS has not been traditionally considered as a form of aGVHD of the lung. However, over the last years, growing evidence indicates that alloreactive immune responses are involved (Cooke et al. 1998; Miklos et al. 2008; Kraetzel et al. 2008), leading to the concept of IPS as a form of pulmonary graft vs. host disease (pGVHD) (Miklos et al. 2008).

As infiltration of the lung by donor T cells, monocytes, and macrophages is a characteristic hallmark (Hildebrandt et al. 2004a; Cooke et al. 1998; Clark et al. 1998; Panoskaltsis-Mortari et al. 1997, 2001; Cooke et al. 2000), several studies investigated the role of chemokines both with respect to their expression levels and their mechanistic function in order to better understand the underlying mechanisms of leukocyte recruitment (Hildebrandt et al. 2004a, b, c, 2005; Wysocki et al. 2004; Serody et al. 2000; Panoskaltsis-Mortari et al. 2000, 2003). Several pro-inflammatory chemokines (CCL2, CCL3-5, CXCL9-11, XCL1, CCL2, CCL8, CCL12, and

CXCL1) and chemokine receptors (CXCR3, CCR1, CCR2, CCR5, CXCR6, and XCR1) are increasingly expressed during the development of acute pGVHD (Hildebrandt et al. 2004a, b, c, 2005; Serody et al. 2000; Panoskaltsis-Mortari et al. 2000; Hildebrandt et al. 2003, 2008). Functional studies have further revealed the relevance of a number of specific chemokine ligand–chemokine receptor interactions (Hildebrandt et al. 2004a, b, 2005; Serody et al. 2000; Panoskaltsis-Mortari et al. 2003) by either using genetically engineered ligand-/receptor-deficient animals or the *in vivo* administration of neutralizing antibodies.

Early after HSCT, increased expression of CXCL9 and CXCL10 was associated with the recruitment of CXCR3+ T cells into the lung (Hildebrandt et al. 2004a). In this study, both the use of CXCR3−/− donor cells as well as the use of anti-CXCL9 or anti-CXCL10 antibodies resulted in decreased T cell numbers in the lung, and subsequently in reduced lung injury. Interestingly, while isolated *in vivo* neutralization of either CXCL9 or CXCL10 partially reduced pulmonary infiltrates, an additive effect could be observed when both ligands were targeted simultaneously (Hildebrandt et al. 2004a). T cell-derived chemokines (CCL3, CCL5) and cytokines, for example, TNF, directly or indirectly through enhanced tissue injury and chemokine induction mediate the recruitment of CCR2+ monocytes and macrophages into the lung (Hildebrandt et al. 2003, 2004b, 2005; Cooke et al. 1998; Serody et al. 2000), which themselves contribute to pulmonary injury through the production of TNF (Hildebrandt et al. 2004c). Migration of donor monocytes/macrophages seems critically regulated through the increased pulmonary expression of the chemokine ligand CCL2, as both immunoneutralization of CCL2 and the absence of CCR2 on donor cells resulted in decreased inflammatory infiltrates and reduction in lung injury (Hildebrandt et al. 2004b), while CCR2 expression on host cells did not matter (Panoskaltsis-Mortari et al. 2004). A regulatory loop on leukocyte migration to the lung has additionally been characterized, as donor T cells through the production of inflammatory chemokines, such as CCL3 and CCL5, can promote subsequent T cell recruitment and propel the severity of disease (Hildebrandt et al. 2005; Serody et al. 2000), although for CCL3 this is not absolutely clear (Panoskaltsis-Mortari et al. 2003). These cascading events in the progression of acute pGVHD exemplify chemokines as critical mediators and potential therapeutic targets in this disease, and led Miklos et al. to the assumption that broad spectrum chemokine inhibition may prove efficient particularly in this disease (Miklos et al. 2009). Grainger et al. have developed a series of oligopeptides that act as functional chemokine inhibitors (Grainger and Reckless 2003). One of these oligopeptides is the broad spectrum chemokine inhibitor (BSCI) NR58-3.14.3, an anti-inflammatory agent, which suppresses the *in vitro* and *in vivo* migration of leucocytes in response to several chemokines, including CCL2, CXCL8 (IL-8), CCL3, and CCL5 (Reckless et al. 2001). Treating mice with this BSCI over the first 2 weeks following allogeneic HSCT resulted in improved pulmonary function and decreased pGVHD severity as well as in a minor reduction in hepatic GVHD, but did not affect intestinal GVHD (Miklos et al. 2009).

5 The Role of the Chemokine–Chemokine Receptor System in cGVHD

Studies on chemokines and chemokine receptors in cGVHD are limited. This may be due to various reasons, including the lack of a standardized classification system, which incorporates the variety of organs and symptoms involved, and the lack of a "one fits all" mouse model of cGVHD. While the former has been recently approached by the National Institute of Health Consensus Development Project on cGVHD (Filipovich et al. 2005; Pavletic et al. 2006a, b), the latter still remains a scientific challenge.

Clinically, a retrospective patient study carried out by Kim et al. described single nucleotide polymorphisms (SNPs) in the CCL5 promoter gene of HSCT recipients to be associated with a higher incidence and severity of cGVHD (Kim et al. 2007). Correspondingly, Morita et al. suggested a role for CCR5 expression on lymphocytes in the development of cGVHD of the skin (Morita et al. 2007).

Up to date, there are three major mouse models dealing with different aspects of cGVHD, involving autoantibody production, fibrosis, and thymic dysfunction. CD4+ T cell activation, resulting in B cell stimulation, and the production of autoantibodies are the main features of a so-called SLE-cGVHD model. The adoptive transfer of MHC mismatched, mature immune cells into a nonirradiated host leads to the generation of DNA-specific autoantibodies and immune-complex glomerulonephritis, both are characteristic findings in human systemic lupus erythematosus (SLE), but are rather infrequently seen in patients with cGVHD. Therefore, this model does not seem to be ideally suited for mimicking human cGVHD, and its validity maybe limited to specific pathophysiological aspects (Martin 2008; Chu and Gress 2008).

Unquestionable fibrosis of the skin and of different other organs is one of the key pathological mechanisms of chronic disease, which is reflected in the sclerodermatous (Scl)-cGVHD model. In this model, irradiated BALB/c (H-2^d) mice are transplanted with bone marrow and splenocytes from minor histocompatibility loci different B10.D2 (H-2^d) donors, resulting in fibrosis of skin, liver, lung, GI tract, and salivary glands. Zhou et al. analyzed mRNA expression levels of chemokines and chemokine receptors in the skin of Scl-cGVHD mice on d7, d30, d60, and d120 after HSCT (Zhou et al. 2007). Measurable skin thickening occurred after 3–5 weeks, and cytokine expression revealed a mixed Th1/Th2 profile with a shift towards a Th2 predominance during the course of skin fibrosis. Consistent with prior findings in mice and human scleroderma (Derk and Jimenez 2003; Zhang et al. 2002), upregulated chemokines included CCL2, CCL3, CCL5, and CCL7. Other chemokines increasingly expressed were the Th1-associated IFNγ-inducible chemokines CXCL9-11 and the Th2-associated chemokines CCL17 and CCL22. CCL17 is involved in the recruitment of CCR4+ lymphocytes to inflamed skin (Reiss et al. 2001). Elevated levels of CCL2, CCL17, and CCL22 were also found in the bronchoalveolar fluid of patients with idiopathic pulmonary fibrosis (IPF) and were predictive of poor outcome. Interestingly, relative and absolute numbers of

macrophages, lymphocytes, neutrophils, and eosinophils in that study did not differ between survivors and nonsurvivors. Likewise, the CD4+/CD8+ ratio was not different (Shinoda et al. 2009). CCL2 is able to induce collagen and TGF-β gene expression in fibroblasts, consistent with elevated TGF-β levels found at later stages in the murine Scl-cGVHD model (Zhang et al. 2002). CXCL9-11 were increased in accordance with the elevated expression of IFNγ noted during early fibrosis and the upregulation of both Th1- and Th2-associated chemokines points to a more complex pathophysiology involving both Th1 and Th2 mechanisms (Zhou et al. 2007).

The third experimental model focuses on the observed thymic dysfunction following allogeneic HSCT, and may help to explain the differences in cGVHD incidence between pediatric and adult patients. Sakoda et al. were able to show that impaired negative selection in the thymus results in the generation of autoreactive T cells, which can cause clinical cGVHD in mice resembling numerous features of human cGVHD (Sakoda et al. 2007). However, further studies employing this model are still pending.

Late onset noninfectious pulmonary complications can present both with restrictive lung function impairment [restrictive pulmonary function test (PFT) pattern, late interstitial pneumonitis (IP), and cryptogenic organizing pneumonia (COP)], air outflow obstruction [obstructive PFT pattern, obliterative bronchiolitis (BO), and bronchiolitis obliterans syndrome (BOS)], or a combination of both (Cooke and Hildebrandt 2006; Tichelli et al. 2008; Bolanos-Meade and Chien 2009). These forms of pulmonary disease are strongly associated with cGvHD and therefore, although not pathophysiologically fully understood, are being considered potential forms of pulmonary cGVHD (Cooke and Hildebrandt 2006; Tichelli et al. 2008; Bolanos-Meade and Chien 2009; Patriarca et al. 2004; Sakaida et al. 2003; Savani et al. 2006; Nishio et al. 2009; Freudenberger et al. 2003; Uderzo et al. 2007). A murine model of BO following allogeneic HSCT has been lacking until recently when Panoskaltsis-Mortari et al. demonstrated the development of obliterative changes in lungs of allogeneic recipients along with increasing levels of CXCL1 (human CXCL8) (Panoskaltsis-Mortari et al. 2007). While no functional study with respect to chemokines and their receptors in the development of BO after HSCT has yet been reported, other groups have shown the critical contribution of CCR2, CXCR2, and CXCR3 together with their respective ligands in the pathophysiology of BO after lung transplantation (Belperio et al. 2001, 2002, 2003, 2005), which may play a role following HSCT as well.

6 Current Possibilities and Perspectives for Targeting the Chemokine–Chemokine Receptor System in GVHD

Besides their described role in GVHD, chemokine and chemokine receptor expression and interactions are importantly involved in various diseases, for example, autoimmune diseases and HIV infection. For example, CCR5 is well known for

their pivotal role in HIV entry into the cell. Therefore, small molecules targeting these receptors have been a focus in the development of new treatment modalities, which resulted in the discovery of maraviroc, up-to-date the only CCR5 antagonist commercially available and FDA-approved for HIV therapy (Lieberman-Blum et al. 2008). As outlined in this review article, CCR5 and its ligands seem to play an integral role in the recruitment of effector T cells to GVHD organs, and targeting this receptor may present a promising alternative approach for disease modulation and therapy. However, it has to be kept in mind that, in one study, CCR5 deficiency of Tregs resulted in exaggerated GVHD severity (Wysocki et al. 2005a). Responses to treatment approaches targeting specific chemokines or chemokine receptors, therefore, may not be as uniform as expected, as functional relevance of the targeted structure may be distributed across different systems, and therefore, potentially contrary effects can occur.

Considering the importance of Th1-based inflammatory responses during early aGVHD and the relevance of increased CXCR3 ligand expression on the recruitment of alloreactive CXCR3+ donor T cells to GVHD target organs (Duffner et al. 2003; Hildebrandt et al. 2004a; He et al. 2008), CXCR3 presents another interesting target. Several patents for CXCR3 antagonists were already disclosed, but none has yet been approved for clinical use (Pease and Horuk 2009).

CCR9 has been recently identified as a critical homing-receptor for lymphocytes involved in GI inflammation like Crohn's disease (Saruta et al. 2007). With CCX282 an orally bioactive inhibitor has been designed, which is currently being tested in phase III clinical trials (Pease and Horuk 2009), and – depending on the results – may provide a promising approach in the treatment of intestinal GVHD as well.

As reviewed in this article, CCR5, CXCR3, and CCR9 present only a few of the potential targets among others (e.g., CCR2, CXCR6, CCL2, CCL3, CCL5, and CXCL16) within the chemokine–chemokine receptor system, for which selective neutralization or specific blockade through newly developed antibodies or small molecules seems to be indicated in future. However, the successful translation from bench to bedside may be hampered by the complexity, the redundancy, and the pleiotropy of the chemokine system. In addition, experimental data from models using chemokine receptor knock out animals have been somewhat controversial [e.g., CCR2 (Terwey et al. 2005) vs. (Hildebrandt et al. 2004b); CCR5 (Murai et al. 2003) vs. (Wysocki et al. 2004)]. The observed discrepancy may reflect mouse strain-dependent and conditioning regimen- or T cell dose-related characteristics, but it also suggests that, while blocking one single receptor or ligand, disease activity and progression are maintained, mediated through alternative chemokine–chemokine-receptor interactions (Wysocki et al. 2004), which either simply take over function or are even compensatory upregulated.

One way to overcome this problem could be the use of promiscuous antagonists, that is, agents, which inhibit the binding capacity or the intracellular signaling pathways of two or more receptors at the same time (Pease and Horuk 2009). Recently developed oligopeptides may fall under this category, as they act as functional chemokine inhibitors (Grainger and Reckless 2003; Fox et al. 2009),

and one member of this group of broad spectrum chemokine inhibitors, NR58-3.14.3, has been successfully tested in murine GVHD, reducing target organ injury to the lung and to the liver (Miklos et al. 2009).

Another novel and very interesting approach to treat aGVHD has been suggested by Hasegawa et al. (2008). They took advantage of the pathologic overexpression of CXCR3 ligands in GVHD target organs by using it as chemotactic signal for CXCR3-transfected Tregs, resulting in targeted delivery of these cells to GVHD target organs and in a reduction in GVHD severity (Hasegawa et al. 2008). Similar approaches being applied to other chemokine receptors may prove beneficial as well, as using this scenario, Treg-mediated immunosuppresion may rather be locally confined than systemically relevant.

Caveats of any modulation of immune responses, including the usage of specific chemokine or chemokine receptor blocking agents, the use of broad spectrum chemokine inhibitors, the application of Tregs or any other kind of immunosuppression, include the potential increase in susceptibility to infection and a potential loss of GVL. These issues have been insufficiently addressed in experimental studies so far and further clarifying studies are needed.

References

Addison CL, Daniel TO, Burdick MD, Liu H, Ehlert JE, Xue YY et al (2000) The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR + CXC chemokine-induced angiogenic activity. J Immunol 165(9):5269–5277

Allen SJ, Crown SE, Handel TM (2007) Chemokine: receptor structure, interactions, and antagonism. Annu Rev Immunol 25:787–820

Ambruzova Z, Mrazek F, Raida L, Jindra P, Vidan-Jeras B, Faber E et al (2009a) Association of IL6 and CCL2 gene polymorphisms with the outcome of allogeneic haematopoietic stem cell transplantation. Bone Marrow Transplant 44(4):227–235

Ambruzova Z, Mrazek F, Raida L, Stahelova A, Faber E, Indrak K et al (2009b) Possible impact of MADCAM1 gene single nucleotide polymorphisms to the outcome of allogeneic hematopoietic stem cell transplantation. Hum Immunol 70(6):457–460

Baird K, Pavletic SZ (2006) Chronic graft versus host disease. Curr Opin Hematol 13(6):426–435

Barrett AJ (1997) Mechanisms of the graft-versus-leukemia reaction. Stem Cells 15(4):248–258

Beilhack A, Schulz S, Baker J, Beilhack GF, Wieland CB, Herman EI et al (2005) *In vivo* analyses of early events in acute graft-versus-host disease reveal sequential infiltration of T-cell subsets. Blood 106(3):1113–1122

Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD et al (2000) CXC chemokines in angiogenesis. J Leukoc Biol 68(1):1–8

Belperio JA, Keane MP, Burdick MD, Lynch JP 3rd, Xue YY, Berlin A et al (2001) Critical role for the chemokine MCP-1/CCR2 in the pathogenesis of bronchiolitis obliterans syndrome. J Clin Invest 108(4):547–556

Belperio JA, Keane MP, Burdick MD, Lynch JP 3rd, Xue YY, Li K et al (2002) Critical role for CXCR3 chemokine biology in the pathogenesis of bronchiolitis obliterans syndrome. J Immunol 169(2):1037–1049

Belperio JA, Keane MP, Burdick MD, Lynch JP 3rd, Zisman DA, Xue YY et al (2003) Role of CXCL9/CXCR3 chemokine biology during pathogenesis of acute lung allograft rejection. J Immunol 171(9):4844–4852

Belperio JA, Keane MP, Burdick MD, Gomperts B, Xue YY, Hong K et al (2005) Role of CXCR2/CXCR2 ligands in vascular remodeling during bronchiolitis obliterans syndrome. J Clin Invest 115(5):1150–1162

Bogunia-Kubik K, Duda D, Suchnicki K, Lange A (2006) CCR5 deletion mutation and its association with the risk of developing acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. Haematologica 91(12):1628–1634

Bolanos-Meade J, Chien J (2009) Chronic graft-versus-host disease and the lung. In: Vogelsang GB, Pavletic SZ (eds) Chronic graft-versus-host disease. Cambridge University Press, UK, pp 229–237

Bouazzaoui A, Spacenko E, Mueller G, Miklos S, Huber E, Holler E et al (2009) Chemokine and chemokine receptor expression analysis in target organs of acute graft-versus-host disease. Genes Immun 10(8):687–701

Breathnach SM, Katz SI (1987) Immunopathology of cutaneous graft-versus-host disease. Am J Dermatopathol 9(4):343–348

Broxmeyer HE (2008) Chemokines in hematopoiesis. Curr Opin Hematol 15(1):49–58

Cahn JY, Klein JP, Lee SJ, Milpied N, Blaise D, Antin JH et al (2005) Prospective evaluation of 2 acute graft-versus-host (GVHD) grading systems: a joint Societe Francaise de Greffe de Moelle et Therapie Cellulaire (SFGM-TC), Dana Farber Cancer Institute (DFCI), and International Bone Marrow Transplant Registry (IBMTR) prospective study. Blood 106 (4):1495–1500

Campbell JJ, Hedrick J, Zlotnik A, Siani MA, Thompson DA, Butcher EC (1998) Chemokines and the arrest of lymphocytes rolling under flow conditions. Science 279(5349):381–384

Champlin RE, Schmitz N, Horowitz MM, Chapuis B, Chopra R, Cornelissen JJ et al (2000) Blood stem cells compared with bone marrow as a source of hematopoietic cells for allogeneic transplantation. IBMTR Histocompatibility and Stem Cell Sources Working Committee and the European Group for Blood and Marrow Transplantation (EBMT). Blood 95(12):3702–3709

Choi SW, Hildebrandt GC, Olkiewicz KM, Hanauer DA, Chaudhary MN, Silva IA et al (2007) CCR1/CCL5 (RANTES) receptor-ligand interactions modulate allogeneic T-cell responses and graft-versus-host disease following stem-cell transplantation. Blood 110(9):3447–3455

Chu YW, Gress RE (2008) Murine models of chronic graft-versus-host disease: insights and unresolved issues. Biol Blood Marrow Transplant 14(4):365–378

Clark J, Hansen J, Hertz M, Parkman R, Jensen L, Peavy H (1993) Idiopathic pneumonia syndrome after bone marrow transplantation. Am Rev Respir Dis 147:1601–1606

Clark JG, Madtes DK, Hackman RC, Chen W, Cheever MA, Martin PJ (1998) Lung injury induced by alloreactive Th1 cells is characterized by host-derived mononuclear cell inflammation and activation of alveolar macrophages. J Immunol 161:1913–1920

Constantin G, Majeed M, Giagulli C, Piccio L, Kim JY, Butcher EC et al (2000) Chemokines trigger immediate beta2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. Immunity 13(6):759–769

Cooke KR, Hildebrandt GC (2006) Pulmonary toxicity following hematopoietic cell transplantation: Is the lung a target organ of graft-versus-host disease? Curr Opin Organ Transplant 11 (1):69–77

Cooke KR, Krenger W, Hill G, Martin TR, Kobzik L, Brewer J et al (1998) Host reactive donor T cells are associated with lung injury after experimental allogeneic bone marrow transplantation. Blood 92(7):2571–2580

Cooke KR, Hill GR, Gerbitz A, Kobzik L, Martin TR, Crawford JM et al (2000) Hyporesponsiveness of donor cells to lipopolysaccharide stimulation reduces the severity of experimental idiopathic pneumonia syndrome: potential role for a gut-lung axis of inflammation. J Immunol 165(11):6612–6619

Crawford S, Hackman R (1993) Clinical course of idiopathic pneumonia after bone marrow transplantation. Am Rev Respir Dis 147:1393

Crawford S, Longton G, Storb R (1993) Acute graft-versus-host disease and the risks for idiopathic pneumonia after marrow transplantation for severe aplastic anemia. Bone Marrow Transplant 12:225

Cutler C, Giri S, Jeyapalan S, Paniagua D, Viswanathan A, Antin JH (2001) Acute and chronic graft-versus-host disease after allogeneic peripheral-blood stem-cell and bone marrow transplantation: a meta-analysis. J Clin Oncol 19(16):3685–3691

Czermak BJ, Sarma V, Bless NM, Schmal H, Friedl HP, Ward PA (1999) *In vitro* and *in vivo* dependency of chemokine generation on C5a and TNF-alpha. J Immunol 162(4):2321–2325

Datta AR, Barrett AJ, Jiang YZ, Guimaraes A, Mavroudis DA (1994) Distant T cell populations distinguish chronic myeloid leulaemia cells from lymphocytes in the same individual: a model for separating GVHD from GVL reactions. Bone Marrow Transplant 14:517–524

Derk CT, Jimenez SA (2003) Systemic sclerosis: current views of its pathogenesis. Autoimmun Rev 2(4):181–191

Duffner U, Lu B, Hildebrandt GC, Teshima T, Williams DL, Reddy P et al (2003) Role of CXCR3-induced donor T-cell migration in acute GVHD. Exp Hematol 31(10):897–902

Emile JF, Haddad E, Fraitag S, Canioni D, Fischer A, Brousse N (1997) Detection of donor-derived Langerhans cells in MHC class II immunodeficient patients after allogeneic bone marrow transplantation. Br J Haematol 98(2):480–484

Faaij CM, Lankester AC, Spierings E, Hoogeboom M, Bowman EP, Bierings M et al (2006) A possible role for CCL27/CTACK-CCR10 interaction in recruiting CD4 T cells to skin in human graft-versus-host disease. Br J Haematol 133(5):538–549

Ferrara JL, Levine JE, Reddy P, Holler E (2009) Graft-versus-host disease. Lancet 373 (9674):1550–1561

Filipovich AH, Weisdorf D, Pavletic S, Socie G, Wingard JR, Lee SJ et al (2005) National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. Biol Blood Marrow Transplant 11(12):945–956

Flier J, Boorsma DM, van Beek PJ, Nieboer C, Stoof TJ, Willemze R et al (2001) Differential expression of CXCR3 targeting chemokines CXCL10, CXCL9, and CXCL11 in different types of skin inflammation. J Pathol 194(4):398–405

Forster R, Davalos-Misslitz AC, Rot A (2008) CCR7 and its ligands: balancing immunity and tolerance. Nat Rev 8(5):362–371

Fowler DH, Gress RE (2000) Th2 and Tc2 cells in the regulation of GVHD, GVL, and graft rejection: considerations for the allogeneic transplantation therapy of leukemia and lymphoma. Leuk Lymphoma 38(3–4):221–234

Fowler DH, Breglio J, Nagel G, Eckhaus MA, Gress RE (1996) Allospecific CD8+ Tc1 and Tc2 populations in GVL and GVHD. J Immunol 157:4811

Fowler DH, Breglio J, Nagel G, Gress RE (1997) Allospecific CD4+ Th1/Th2 and CD8+ Tc1/Tc2 populations in murine GVL: type I cells generate GVL, type II cells abrogate GVL. Biol Blood Marrow Transplant 2:118

Fox DJ, Reckless J, Lingard H, Warren S, Grainger DJ (2009) Highly potent, orally available anti-inflammatory broad-spectrum chemokine inhibitors. J Med Chem 52(11):3591–3595

Freudenberger TD, Madtes DK, Curtis JR, Cummings P, Storer BE, Hackman RC (2003) Association between acute and chronic graft-versus-host disease and bronchiolitis obliterans organizing pneumonia in recipients of hematopoietic stem cell transplants. Blood 102 (10):3822–3828

Goker H, Haznedaroglu IC, Chao NJ (2001) Acute graft-vs-host disease: pathobiology and management. Exp Hematol 29(3):259–277

Goulmy E, Schipper R, Pool J, Blokland E, Falkenburg F (1996) Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. N Engl J Med 334(5): 281–285

Grainger DJ, Reckless J (2003) Broad-spectrum chemokine inhibitors (BSCIs) and their anti-inflammatory effects *in vivo*. Biochem Pharmacol 65(7):1027–1034

Gruhn B, Intek J, Pfaffendorf N, Zell R, Corbacioglu S, Zintl F et al (2009) Polymorphism of interleukin-23 receptor gene but not of NOD2/CARD15 is associated with graft-versus-host disease after hematopoietic stem cell transplantation in children. Biol Blood Marrow Transplant 15(12):1571–1577

Hadeiba H, Sato T, Habtezion A, Oderup C, Pan J, Butcher EC (2008) CCR9 expression defines tolerogenic plasmacytoid dendritic cells able to suppress acute graft-versus-host disease. Nat Immunol 9(11):1253–1260

Hancock WW, Lu B, Gao W, Csizmadia V, Faia K, King JA et al (2000) Requirement of the chemokine receptor CXCR3 for acute allograft rejection. J Exp Med 192(10):1515–1520

Hasegawa H, Inoue A, Kohno M, Lei J, Miyazaki T, Yoshie O et al (2008) Therapeutic effect of CXCR3-expressing regulatory T cells on liver, lung and intestinal damages in a murine acute GVHD model. Gene Ther 15(3):171–182

He S, Cao Q, Qiu Y, Mi J, Zhang JZ, Jin M et al (2008) A new approach to the blocking of alloreactive T cell-mediated graft-versus-host disease by *in vivo* administration of anti-CXCR3 neutralizing antibody. J Immunol 181(11):7581–7592

Higman MA, Vogelsang GB (2004) Chronic graft-versus-host disease. Br J Haematol 125(4): 435–454

Hildebrandt GC, Olkiewitz KO, Corrion LA, Chang Y, Liu C, Ferrara JLM et al (2003) Secretion of TNF-alpha by donor effector cells is critical in the development of Idiopathic Pneumonia Syndrome after allogeneic stem cell transplantation. Blood 102(11):3529

Hildebrandt GC, Corrion LA, Olkiewicz KM, Lu B, Lowler K, Duffner UA et al (2004a) Blockade of CXCR3 receptor:ligand interactions reduces leukocyte recruitment to the lung and the severity of experimental idiopathic pneumonia syndrome. J Immunol 173(3):2050–2059

Hildebrandt GC, Duffner UA, Olkiewicz KM, Corrion LA, Willmarth NE, Williams DL et al (2004b) A critical role for CCR2/MCP-1 interactions in the development of idiopathic pneumonia syndrome after allogeneic bone marrow transplantation. Blood 103(6):2417–2426

Hildebrandt GC, Olkiewicz KM, Corrion LA, Chang Y, Clouthier SG, Liu C et al (2004c) Donor-derived TNF-alpha regulates pulmonary chemokine expression and the development of idiopathic pneumonia syndrome after allogeneic bone marrow transplantation. Blood 104(2):586–593

Hildebrandt GC, Olkiewicz KM, Choi S, Corrion LA, Clouthier SG, Liu C et al (2005) Donor T-cell production of RANTES significantly contributes to the development of idiopathic pneumonia syndrome after allogeneic stem cell transplantation. Blood 105(6):2249–2257

Hildebrandt GC, Olkiewicz KM, Corrion L, Clouthier SG, Pierce EM, Liu C et al (2008) A role for TNF receptor type II in leukocyte infiltration into the lung during experimental idiopathic pneumonia syndrome. Biol Blood Marrow Transplant 14(4):385–396

Hill GR, Crawford JM, Cooke KR, Brinson YS, Pan L, Ferrara JL (1997) Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. Blood 90(8):3204–3213

Holler E, Rogler G, Herfarth H, Brenmoehl J, Wild PJ, Hahn J et al (2004) Both donor and recipient NOD2/CARD15 mutations associate with transplant-related mortality and GvHD following allogeneic stem cell transplantation. Blood 104(3):889–894

Holler E, Rogler G, Brenmoehl J, Hahn J, Herfarth H, Greinix H et al (2006) Prognostic significance of NOD2/CARD15 variants in HLA-identical sibling hematopoietic stem cell transplantation: effect on long-term outcome is confirmed in 2 independent cohorts and may be modulated by the type of gastrointestinal decontamination. Blood 107(10):4189–4193

Hori T, Naishiro Y, Sohma H, Suzuki N, Hatakeyama N, Yamamoto M et al (2008) CCL8 is a potential molecular candidate for the diagnosis of graft-versus-host disease. Blood 111 (8):4403–4412

Ichiba T, Teshima T, Kuick R, Misek DE, Liu C, Takada Y et al (2003) Early changes in gene expression profiles of hepatic GVHD uncovered by oligonucleotide microarrays. Blood 102(2):763–771

Inamoto Y, Murata M, Katsumi A, Kuwatsuka Y, Tsujimura A, Ishikawa Y et al (2009) Donor single nucleotide polymorphism in the CCR9 gene affects the incidence of skin GVHD. Bone Marrow Transplant 45(2):363–369

Jaksch M, Remberger M, Mattsson J (2005) Increased gene expression of chemokine receptors is correlated with acute graft-versus-host disease after allogeneic stem cell transplantation. Biol Blood Marrow Transplant 11(4):280–287

Kantrow SP, Hackman RC, Boeckh M, Myerson D, Crawford SW (1997) Idiopathic pneumonia syndrome: Changing spectrum of lung injury after marrow transplantation. Transplantation 63 (8):1079–1086

Kaplan DH, Anderson BE, McNiff JM, Jain D, Shlomchik MJ, Shlomchik WD (2004) Target antigens determine graft-versus-host disease phenotype. J Immunol 173(9):5467–5475

Kataoka Y, Iwasaki T, Kuroiwa T, Seto Y, Iwata N, Hashimoto N et al (2001) The role of donor T cells for target organ injuries in acute and chronic graft-versus-host disease. Immunology 103 (3):310–318

Kim DH, Jung HD, Lee NY, Sohn SK (2007) Single nucleotide polymorphism of CC chemokine ligand 5 promoter gene in recipients may predict the risk of chronic graft-versus-host disease and its severity after allogeneic transplantation. Transplantation 84(7):917–925

Koenecke C, Forster R (2009) CCR9 and inflammatory bowel disease. Expert Opin Ther Targets 13(3):297–306

Kolb HJ, Schmid C, Barrett AJ, Schendel DJ (2003) Graft-versus-leukemia reactions in allogeneic chimeras. Blood 103(3):767–776

Kraetzel K, Stoelcker B, Eissner G, Multhoff G, Pfeifer M, Holler E et al (2008) NKG2D-dependent effector function of bronchial epithelium-activated alloreactive T-cells. Eur Respir J 32(3):563–570

Krowka MJ, Rosenow EC 3rd, Hoagland HC (1985) Pulmonary complications of bone marrow transplantation. Chest 87(2):237–246

Lee SJ (2005) New approaches for preventing and treating chronic graft-versus-host disease. Blood 105(11):4200–4206

Lieberman-Blum SS, Fung HB, Bandres JC (2008) Maraviroc: a CCR5-receptor antagonist for the treatment of HIV-1 infection. Clin Ther 30(7):1228–1250

Mackay CR (2001) Chemokines: immunology's high impact factors. Nat Immunol 2(2):95–101

MacMillan ML, Radloff GA, DeFor TE, Weisdorf DJ, Davies SM (2003a) Interleukin-1 genotype and outcome of unrelated donor bone marrow transplantation. Br J Haematol 121(4):597–604

MacMillan ML, Radloff GA, Kiffmeyer WR, DeFor TE, Weisdorf DJ, Davies SM (2003b) High-producer interleukin-2 genotype increases risk for acute graft-versus-host disease after unrelated donor bone marrow transplantation. Transplantation 76(12):1758–1762

Mapara MY, Kim YM, Marx J, Sykes M (2003) Donor lymphocyte infusion-mediated graft-versus-leukemia effects in mixed chimeras established with a nonmyeloablative conditioning regimen: extinction of graft-versus-leukemia effects after conversion to full donor chimerism. Transplantation 76(2):297–305

Mapara MY, Leng C, Kim YM, Bronson R, Lokshin A, Luster A et al (2006) Expression of chemokines in GVHD target organs is influenced by conditioning and genetic factors and amplified by GVHR. Biol Blood Marrow Transplant 12(6):623–634

Markey KA, MacDonald KP, Hill GR (2008) Impact of cytokine gene polymorphisms on graft-vs-host disease. Tissue Antigens 72(6):507–516

Martin PJ (2008) Biology of chronic graft-versus-host disease: implications for a future therapeutic approach. Keio J Med 57(4):177–183

Merad M, Manz MG, Karsunky H, Wagers A, Peters W, Charo I et al (2002) Langerhans cells renew in the skin throughout life under steady-state conditions. Nat Immunol 3(12):1135–1141

Merad M, Hoffmann P, Ranheim E, Slaymaker S, Manz MG, Lira SA et al (2004) Depletion of host Langerhans cells before transplantation of donor alloreactive T cells prevents skin graft-versus-host disease. Nat Med 10(5):510–517

Middleton PG, Taylor PR, Jackson G, Proctor SJ, Dickinson AM (1998) Cytokine gene polymorphisms associating with severe acute graft-versus-host disease in HLA-identical sibling transplants. Blood 92(10):3943–3948

Miklos S, Mueller G, Chang Y, Schubert TE, Holler E, Hildebrandt GC (2008) Pulmonary function changes in experimental graft-versus-host disease of the lung. Biol Blood Marrow Transplant 14(9):1004–1016

Miklos S, Mueller G, Chang Y, Bouazzaoui A, Spacenko E, Schubert TE et al (2009) Preventive usage of broad spectrum chemokine inhibitor NR58-3.14.3 reduces the severity of pulmonary and hepatic graft-versus-host disease. Int J Hematol 89(3):383–397

Morita NI, Matsumura Y, Morita K, Miyachi Y (2007) Expression of CCR5 in graft-versus-host disease (GVHD) of the skin: immunohistochemical staining of 38 cases. J Dermatol 34(4):254–257

Moser B, Wolf M, Walz A, Loetscher P (2004) Chemokines: multiple levels of leukocyte migration control. Trends Immunol 25(2):75–84

Muller G, Hopken UE, Lipp M (2003) The impact of CCR7 and CXCR5 on lymphoid organ development and systemic immunity. Immunol Rev 195:117–135

Murai M, Yoneyama H, Harada A, Yi Z, Vestergaard C, Guo B et al (1999) Active participation of CCR5(+)CD8(+) T lymphocytes in the pathogenesis of liver injury in graft-versus-host disease. J Clin Invest 104(1):49–57

Murai M, Yoneyama H, Ezaki T, Suematsu M, Terashima Y, Harada A et al (2003) Peyer's patch is the essential site in initiating murine acute and lethal graft-versus-host reaction. Nat Immunol 4(2):154–160

Murphy PM (2002) International Union of Pharmacology. XXX. Update on chemokine receptor nomenclature. Pharmacol Rev 54(2):227–229

Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K et al (2000) International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Rev 52(1):145–176

Nash RA, Storb R (1996) Graft-versus-host effect after allogeneic hematopoietic stem cell transplantation: GVHD and GVL. Curr Opin Immunol 8:674–680

New JY, Li B, Koh WP, Ng HK, Tan SY, Yap EH et al (2002) T cell infiltration and chemokine expression: relevance to the disease localization in murine graft-versus-host disease. Bone Marrow Transplant 29(12):979–986

Nishimura M, Kuboi Y, Muramoto K, Kawano T, Imai T (2009) Chemokines as novel therapeutic targets for inflammatory bowel disease. Ann N Y Acad Sci 1173:350–356

Nishio N, Yagasaki H, Takahashi Y, Muramatsu H, Hama A, Tanaka M et al (2009) Late-onset non-infectious pulmonary complications following allogeneic hematopoietic stem cell transplantation in children. Bone Marrow Transplant 44(5):303–308

Oettel KR, Wesly OH, Albertini MR, Hank JA, Iliopolis O, Sosman JA et al (1994) Allogeneic T-cell clones able to selectively destroy Philadelphia chromosome-bearing (Ph1+) human leukemia lines can also recognize Ph1- cells from the same patient. Blood 83:3390–3402

Ohl L, Henning G, Krautwald S, Lipp M, Hardtke S, Bernhardt G et al (2003) Cooperating mechanisms of CXCR5 and CCR7 in development and organization of secondary lymphoid organs. J Exp Med 197(9):1199–1204

Okamoto I, Kohno K, Tanimoto T, Iwaki K, Ishihara T, Akamatsu S et al (2000) IL-18 prevents the development of chronic graft-versus-host disease in mice. J Immunol 164(11):6067–6074

Ota A, Yamamoto M, Hori T, Miyai S, Naishiro Y, Sohma H et al (2009) Upregulation of plasma CCL8 in mouse model of graft-vs-host disease. Exp Hematol 37(4):525–531

Palmer LA, Sale GE, Balogun JI, Li D, Jones D, Molldrem JJ et al (2009) Chemokine receptor CCR5 mediates allo-immune responses in Graft-vs-Host Disease. Biol Blood Marrow Transplant 16(3):311–319

Panoskaltsis-Mortari A, Taylor PA, Yaegar TM, Wangensteen OD, Bitterman PB, Ingbar DH et al (1997) The critical early proinflammatory events associated with idiopathic pneumonia

syndrome in irradiated murine allogenic recipients are due to donor T cell infusion and potentiated by cyclophoshamide. J Clin Invest 100(5):1015–1027

Panoskaltsis-Mortari A, Strieter RM, Hermanson JR, Fegeding KV, Murphy WJ, Farrell CL et al (2000) Induction of monocyte- and T-cell-attracting chemokines in the lung during the generation of idiopathic pneumonia syndrome following allogeneic murine bone marrow transplantation. Blood 96(3):834–839

Panoskaltsis-Mortari A, Hermanson JR, Haddad IY, Wangensteen OD, Blazar BR (2001) Intercellular adhesion molecule-I (ICAM-I, CD54) deficiency segregates the unique pathophysiological requirements for generating idiopathic pneumonia syndrome (IPS) versus graft-versus-host disease following allogeneic murine bone marrow transplantation. Biol Blood Marrow Transplant 7(7):368–377

Panoskaltsis-Mortari A, Hermanson JR, Taras E, Wangensteen OD, Serody JS, Blazar BR (2003) Acceleration of idiopathic pneumonia syndrome (IPS) in the absence of donor MIP-1 alpha (CCL3) after allogeneic BMT in mice. Blood 101(9):3714–3721

Panoskaltsis-Mortari A, Hermanson JR, Taras E, Wangensteen OD, Charo IF, Rollins BJ et al (2004) Post-BMT lung injury occurs independently of the expression of CCL2 or its receptor, CCR2, on host cells. Am J Physiol Lung Cell Mol Physiol 286(2):L284–L292

Panoskaltsis-Mortari A, Tram KV, Price AP, Wendt CH, Blazar BR (2007) A new murine model for bronchiolitis obliterans post-bone marrow transplant. Am J Respir Crit Care Med 176 (7):713–723

Papadakis KA, Prehn J, Moreno ST, Cheng L, Kouroumalis EA, Deem R et al (2001) CCR9-positive lymphocytes and thymus-expressed chemokine distinguish small bowel from colonic Crohn's disease. Gastroenterology 121(2):246–254

Patriarca F, Skert C, Sperotto A, Damiani D, Cerno M, Geromin A et al (2004) Incidence, outcome, and risk factors of late-onset noninfectious pulmonary complications after unrelated donor stem cell transplantation. Bone Marrow Transplant 33(7):751–758

Pavletic SZ, Lee SJ, Socie G, Vogelsang G (2006a) Chronic graft-versus-host disease: implications of the National Institutes of Health consensus development project on criteria for clinical trials. Bone Marrow Transplant 38(10):645–651

Pavletic SZ, Martin P, Lee SJ, Mitchell S, Jacobsohn D, Cowen EW et al (2006b) Measuring therapeutic response in chronic graft-versus-host disease: National Institute of Health Consensus Development Project on criteria for clinical trials in chronic graft-versus-host disease: IV. Response criteria working group report. Biol Blood Marrow Transpl 12:252–266

Pease JE, Horuk R (2009) Chemokine receptor antagonists: part 2. Expert Opin Ther Pat 19 (2):199–221

Pease JE, Williams TJ (2006) The attraction of chemokines as a target for specific anti-inflammatory therapy. Br J Pharmacol 147(Suppl 1):S212–S221

Piper KP, Horlock C, Curnow SJ, Arrazi J, Nicholls S, Mahendra P et al (2007) CXCL10-CXCR3 interactions play an important role in the pathogenesis of acute graft-versus-host disease in the skin following allogeneic stem-cell transplantation. Blood 110(12):3827–3832

Rao AR, Quinones MP, Garavito E, Kalkonde Y, Jimenez F, Gibbons C et al (2003) CC chemokine receptor 2 expression in donor cells serves an essential role in graft-versus-host-disease. J Immunol 171(9):4875–4885

Reckless J, Tatalick LM, Grainger DJ (2001) The pan-chemokine inhibitor NR58-3.14.3 abolishes tumour necrosis factor-alpha accumulation and leucocyte recruitment induced by lipopolysaccharide *in vivo*. Immunology 103(2):244–254

Reiss Y, Proudfoot AE, Power CA, Campbell JJ, Butcher EC (2001) CC chemokine receptor (CCR)4 and the CCR10 ligand cutaneous T cell-attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin. J Exp Med 194(10):1541–1547

Riddell SR, Murata M, Bryant S, Warren EH (2002) Minor histocompatibility antigens–targets of graft-versus-leukemia responses. Int J Hematol 76(Suppl 2):155–161

Rollins BJ (1997) Chemokines. Blood 90(3):909–928

Rot A, Von Andrian UH (2004) Chemokines in Innate and Adaptive Host Defense: Basic Chemokinese Grammar for Immune Cells. Annu Rev Immunol 22:891–928

Sakaida E, Nakaseko C, Harima A, Yokota A, Cho R, Saito Y et al (2003) Late-onset noninfectious pulmonary complications after allogeneic stem cell transplantation are significantly associated with chronic graft-versus-host disease and with the graft-versus-leukemia effect. Blood 102(12):4236–4242

Sakoda Y, Hashimoto D, Asakura S, Takeuchi K, Harada M, Tanimoto M et al (2007) Donor-derived thymic-dependent T cells cause chronic graft-versus-host disease. Blood 109 (4):1756–1764

Saruta M, Yu QT, Avanesyan A, Fleshner PR, Targan SR, Papadakis KA (2007) Phenotype and effector function of CC chemokine receptor 9-expressing lymphocytes in small intestinal Crohn's disease. J Immunol 178(5):3293–3300

Sasaki M, Hasegawa H, Kohno M, Inoue A, Ito MR, Fujita S (2003) Antagonist of secondary lymphoid-tissue chemokine (CCR ligand 21) prevents the development of chronic graft-versus-host disease in mice. J Immunol 170(1):588–596

Sato T, Thorlacius H, Johnston B, Staton TL, Xiang W, Littman DR et al (2005) Role for CXCR6 in recruitment of activated CD8+ lymphocytes to inflamed liver. J Immunol 174(1):277–283

Savani BN, Montero A, Srinivasan R, Singh A, Shenoy A, Mielke S et al (2006) Chronic GVHD and pretransplantation abnormalities in pulmonary function are the main determinants predicting worsening pulmonary function in long-term survivors after stem cell transplantation. Biol Blood Marrow Transplant 12(12):1261–1269

Serody JS, Burkett SE, Panoskaltsis-Mortari A, Ng-Cashin J, McMahon E, Matsushima GK et al (2000) T-lymphocyte production of macrophage inflammatory protein-1alpha is critical to the recruitment of CD8(+) T cells to the liver, lung, and spleen during graft-versus-host disease. Blood 96(9):2973–2980

Shinoda H, Tasaka S, Fujishima S, Yamasawa W, Miyamoto K, Nakano Y et al (2009) Elevated CC chemokine level in bronchoalveolar lavage fluid is predictive of a poor outcome of idiopathic pulmonary fibrosis. Respiration 78(3):285–292

Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J et al (1999) Prevention of graft-versus-host disease by inactivation of host antigen-presenting cells. Science 285(5426):412–415

Shulman HM, Sale GE, Lerner KG, Barker EA, Weiden PL, Sullivan K et al (1978) Chronic cutaneous graft-versus-host disease in man. Am J Pathol 91(3):545–570

Slavin S, Naparstek E, Nagler A et al (1993) Graft vs leukemia (GVL) effects with controlled GVHD by cell mediated immunotherapy (CMI) following allogeneic bone marrow transplantation (BMT). Blood 82:423a

Strieter RM, Burdick MD, Gomperts BN, Belperio JA, Keane MP (2005) CXC chemokines in angiogenesis. Cytokine Growth Factor Rev 16(6):593–609

Sugerman PB, Faber SB, Willis LM, Petrovic A, Murphy GF, Pappo J et al (2004) Kinetics of gene expression in murine cutaneous graft-versus-host disease. Am J Pathol 164(6):2189–2202

Sullivan KM, Parkman R (1983) The pathophysiology and treatment of graft-versus-host disease. Clin Haematol 12(3):775–789

Sun Y, Tawara I, Toubai T, Reddy P (2007) Pathophysiology of acute graft-versus-host disease: recent advances. Transl Res 150(4):197–214

Takahashi H, Furukawa T, Hashimoto S, Suzuki N, Kuroha T, Yamazaki F et al (2000) Contribution of TNF-alpha and IL-10 gene polymorphisms to graft-versus-host disease following allohematopoietic stem cell transplantation. Bone Marrow Transplant 26(12):1317–1323

Terwey TH, Kim TD, Kochman AA, Hubbard VM, Lu S, Zakrzewski JL et al (2005) CCR2 is required for CD8-induced graft-versus-host disease. Blood 106(9):3322–3330

Teshima T, Ordemann R, Reddy P, Gagin S, Liu C, Cooke KR et al (2002) Acute graft-versus-host disease does not require alloantigen expression on host epithelium. Nat Med 8(6):575–581

Teshima T, Reddy P, Liu C, Williams D, Cooke KR, Ferrara JL (2003) Impaired thymic negative selection causes autoimmune graft-versus-host disease. Blood 102(2):429–435

Tichelli A, Rovo A, Gratwohl A (2008) Late pulmonary, cardiovascular, and renal complications after hematopoietic stem cell transplantation and recommended screening practices. Hematology Am Soc Hematol Educ Program 1:125–133

Truitt RL, Atasoylu AA (1991a) Impact of pretransplant conditioning and donor T cells on chimerism, graft-versus-host disease, graft-versus-leukemia reactivity, and tolerance after bone marrow transplantation. Blood 77:2515–2523

Truitt RL, Atasoylu AA (1991b) Contribution of CD4+ and CD8+ T cells to graft-versus-host disease and graft-versus-leukemia reactivity after transplantation of MHC-compatible bone marrow. Bone Marrow Transplant 8:51–58

Tseng LH, Storer B, Petersdorf E, Lin MT, Chien JW, Grogan BM et al (2009) IL10 and IL10 receptor gene variation and outcomes after unrelated and related hematopoietic cell transplantation. Transplantation 87(5):704–710

Uderzo C, Pillon M, Corti P, Tridello G, Tana F, Zintl F et al (2007) Impact of cumulative anthracycline dose, preparative regimen and chronic graft-versus-host disease on pulmonary and cardiac function in children 5 years after allogeneic hematopoietic stem cell transplantation: a prospective evaluation on behalf of the EBMT Pediatric Diseases and Late Effects Working Parties. Bone Marrow Transplant 39(11):667–675

Ueha S, Murai M, Yoneyama H, Kitabatake M, Imai T, Shimaoka T et al (2007) Intervention of MAdCAM-1 or fractalkine alleviates graft-versus-host reaction associated intestinal injury while preserving graft-versus-tumor effects. J Leukoc Biol 81(1):176–185

Varona R, Cadenas V, Gomez L, Martinez AC, Marquez G (2005) CCR6 regulates CD4+ T-cell-mediated acute graft-versus-host disease responses. Blood 106(1):18–26

Varona R, Cadenas V, Lozano M, Moreno-Ortiz MC, Kremer L, Martinez AC et al (2006) CCR6 regulates the function of alloreactive and regulatory CD4+ T cells during acute graft-versus-host disease. Leuk Lymphoma 47(8):1469–1476

Viola A, Luster AD (2008) Chemokines and their receptors: drug targets in immunity and inflammation. Annu Rev Pharmacol Toxicol 48:171–197

Weiner RS, Mortimer MB, Gale RP, Gluckman E, Kay HEM, Kolb JJ et al (1986) Interstitial pneumonitis after bone marrow transplantation. Ann Intern Med 104:168–175

Weninger W, Crowley MA, Manjunath N, von Andrian UH (2001) Migratory properties of naive, effector, and memory CD8(+) T cells. J Exp Med 194(7):953–966

Wysocki CA, Burkett SB, Panoskaltsis-Mortari A, Kirby SL, Luster AD, McKinnon K et al (2004) Differential roles for CCR5 expression on donor T cells during graft-versus-host disease based on pretransplant conditioning. J Immunol 173(2):845–854

Wysocki CA, Jiang Q, Panoskaltsis-Mortari A, Taylor PA, McKinnon KP, Su L et al (2005a) Critical role for CCR5 in the function of donor CD4 + CD25+ regulatory T cells during acute graft-versus-host disease. Blood 106(9):3300–3307

Wysocki CA, Panoskaltsis-Mortari A, Blazar BR, Serody JS (2005b) Leukocyte migration and graft-versus-host disease. Blood 105(11):4191–4199

Yakoub-Agha I, Saule P, Depil S, Micol JB, Grutzmacher C, Boulanger-Villard F et al (2006) A high proportion of donor CD4+ T cells expressing the lymph node-homing chemokine receptor CCR7 increases incidence and severity of acute graft-versus-host disease in patients undergoing allogeneic stem cell transplantation for hematological malignancy. Leukemia 20(9):1557–1565

Zhang Y, McCormick LL, Desai SR, Wu C, Gilliam AC (2002) Murine sclerodermatous graft-versus-host disease, a model for human scleroderma: cutaneous cytokines, chemokines, and immune cell activation. J Immunol 168(6):3088–3098

Zhou L, Askew D, Wu C, Gilliam AC (2007) Cutaneous gene expression by DNA microarray in murine sclerodermatous graft-versus-host disease, a model for human scleroderma. J Invest Dermatol 127(2):281–292

Zlotnik A, Yoshie O (2000) Chemokines: a new classification system and their role in immunity. Immunity 12(2):121–127

Homing in on Acute Graft vs. Host Disease: Tissue-Specific T Regulatory and Th17 Cells

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Abstract Acute graft vs. host disease (aGVHD) is a major limitation of hematopoietic stem cell transplantation (HSCT), and it causes significant morbidity and mortality for this patient population. This immune-mediated injury occurs unpredictably and is caused by donor-derived T cells reacting to recipient alloantigens. Although donor Th1 cells play a critical role in aGVHD generation, numerous arms of both the innate and the adaptive immune systems along with determinants of lymphocyte trafficking are likely involved in the multifaceted cascade of immunological events that culminates in clinical aGVHD. T regulatory and Th17 cells are T cell subsets distinct from Th1 cells that are likely involved with aGVHD. Regulatory T cells (Tregs) have been implicated in the prevention of aGVHD in both mouse and man, while Th17 cells may modulate early inflammatory responses associated with aGVHD, especially those involving the skin and the lungs. Interestingly, these two lymphocyte subsets appear to be reciprocally regulated in part through retinoic acid, through cytokines such as IL-6, and via interactions with dendritic cells. Another area under tight regulation appears to be the homing of lymphocytes to lymph nodes, skin, and gut. Adhesion molecules including chemokine receptors, selectins, and integrins may identify specific T cell subsets with unique migratory functional properties during HSCT. Controlling the migration patterns of Th17 cells and Tregs represents a potential therapeutic target. A major goal of HSCT research will be to develop approaches to pharmacologically manipulate T cell subsets *in vivo* or to select, expand, and infuse T cell subsets that will maximize the targeted graft vs. tumor effect while minimizing the potentially fatal side effects of aGVHD. A better understanding of Tregs and their tissue specificity should lead to improvement in the success of HSCT.

Abbreviations

aGVHD	Acute graft vs. host disease
ATG	Antithymocyte globulin
ATRA	All-trans-retinoic acid
CCL	Chemokine ligand
CCRs	Chemokine receptors
cGVHD	Chronic graft vs. host disease
CLA	Cutaneous lymphocyte antigen
CTLA4	Cytotoxic T-lymphocyte associated antigen 4
E-selectin	Endothelial-cell selectin
Foxp3	Forkhead box protein P3
GITR	Glucocorticoid tumor necrosis factor receptor
GVT	Graft vs. tumor effect
HEVs	High endothelial venules
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
IDO	Indoleamine 2,3-dioxygenase

IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
MADCAM1	Mucosal vascular address in cell-adhesion molecule 1
MRD	Matched related donor
P-selectin	Platelet selectin
RIC	Reduced intensity chemotherapy
ROR	Retinoid-related orphan receptor
Tregs	Regulatory T cells
URD	Unrelated donor

1 Introduction

1.1 Acute Graft vs. Host Disease

Allogeneic HSCT is a curative therapy for fatal hematological disorders and hereditary immunodeficiency syndromes. In the context of treatment for hematological malignancies, HSCT is capable of eradicating residual malignant cells escaping chemotherapy and radiation via immune surveillance mechanisms known as graft vs. tumor effect (GVT). GVT is essential for the success of the transplant and for controlling disease relapse post-HSCT. Conversely, the donor immune system can also recognize recipient alloantigens as foreign, resulting in immune-mediated tissue injury known as graft vs. host disease (GVHD). GVHD is thought to be primarily a T cell mediated process. GVHD is the most important medical limitation of this procedure. GVHD is divided into two broad categories, acute and chronic, based on the phenotype of the disease (Filipovich et al. 2005). Classical acute GVHD (aGVHD) occurs in the first 100 days following HSCT and is characterized by the triad of dermatitis, gastroenteritis, and cholestatic hepatitis. aGVHD usually begins with a maculopapular rash involving the palms of the hands or soles of the feet. It can quickly spread to become a generalized erythroderma. The gastrointestinal tract also can be involved, leading to nausea, vomiting, anorexia, diarrhea, and even ileus or bloody diarrhea. When severe, aGVHD can be associated with bullous skin lesions and desquamation of cutaneous tissues and intestinal mucosa. Hepatic dysfunction usually is marked by a rise in serum bilirubin and occasionally the transaminases. aGVHD is a significant risk factor for the development of chronic GVHD (cGVHD), which in turn dictates long-term morbidity, quality of life, and nonrelapse mortality following HSCT (Arai and Vogelsang 2000; Przepiorka et al. 2001). Prophylaxis strategies with calcineurin inhibitors and either methotrexate or mycophenolate mofetil have reduced the incidence of aGVHD; however, aGVHD still affects 40–50% of patients undergoing a matched related donor (MRD) HSCT (Arai and Vogelsang 2000; Nash et al. 1992;

Weisdorf et al. 1991) and 50–80% of patients receiving human leukocyte antigen (HLA)-mismatched or unrelated donor (URD) transplants (Beatty et al. 1985, 1991). Although the triad of organ involvement is characteristic, aGVHD occurs unpredictably with regards to actual tissue involvement and severity following HSCT. When moderate to severe aGVHD occurs, it requires additional treatment with potent immunosuppressive agents, usually high-dose corticosteroids, which further increases the morbidity and infectious risk associated with transplantation. Unfortunately, only 50–60% of patients receiving treatment for aGVHD will have a durable response (Martin et al. 1990; Alousi et al. 2009). Most patients with severe aGVHD and many patients failing initial therapy die eventually from complications of aGVHD or its therapy (Martin et al. 1990, 1991). Thus, much effort has been placed on trying to understand the immunology of aGVHD as a means to improve patient outcomes.

1.2 T Cells and aGVHD

Recently, much interest has focused on T cell subsets and lymphocyte homing as a way to explain the clinical heterogeneity and the organ tropism of aGVHD. The chemokines, chemokine receptors (CCRs), and other adhesion molecules necessary for lymphocyte trafficking to lymph nodes, skin, gut, or to areas of inflammation have been established in the mouse and increasingly so in the human. These avenues are now being actively explored in HSCT recipients as a way to explain the organ involvement by aGVHD.

Differences in T cell differentiation and subtype could also be playing a role in the pathophysiology of aGVHD. Recently, a suppressive subset of CD4+ T cells has been identified. These regulatory T cells (Tregs) are characterized by high expression of the interleukin (IL)-2 receptor α chain (CD25) and intracellular expression of the transcription factor forkhead box protein P3 (Foxp3) (Sakaguchi et al. 1995; Shevach 2002; Fontenot et al. 2003; Hori et al. 2003). Physiologically, these cells have been implicated in the prevention of autoimmune diseases (Sakaguchi et al. 1995), host tolerance to chronic infections (Belkaid et al. 2002), and escape of immune surveillance by malignant cells (Curiel et al. 2004). There is also increasing evidence that high Treg frequencies post-HSCT are associated with reduced incidence or severity of aGVHD.

Another lineage of CD4+ T cells distinct from Tregs and Th1/Th2 cells, designated Th17 cells, has been identified. These Th17 cells are characterized by secretion of the pro-inflammatory cytokines, IL-17, IL-17F, IL-21, and IL-22. In contrast to the suppressive role of Tregs, Th17 cells appear to be associated with inflammation, the elimination of extracellular pathogens, autoimmunity, and solid organ allograft rejection. Their role in aGVHD is now being explored.

By combining increasing knowledge about specific T cell subsets and their patterns of homing, we can gain better insight into the immunology of aGVHD,

which may improve treatment options and outcomes for patients undergoing HSCT. In this review, we focus on recent progress in our understanding of naturally occurring Tregs with varying patterns of expression of chemokine receptors (CCRs) and other homing molecules and their relationship to the development of aGVHD. We also will describe briefly the emerging role of Th17 cells and their chemokine receptor expression in the pathophysiology of aGVHD.

2 Immunology of aGVHD

2.1 Model of Interactions

Based on data obtained from preclinical animal models of transplantation, a three-phase model for the development of aGVHD has been proposed. The first stage occurs prior to the infusion of the hematopoietic stem cell graft. During this initial stage, high doses of chemotherapy and radiation damage recipient tissues, causing the release of inflammatory cytokines IL-1 and TNF-α (Xun et al. 1994). In response to TNF-α secretion, dendritic cells increase expression of MHC antigens and co-stimulatory molecules, while lymphoid and peripheral tissues upregulate integrins and chemokines necessary for the migration of immune cells (Norton and Sloane 1991; Thornhill et al. 1991; Leeuwenberg et al. 1988). The graft, which contains hematopoietic stem cells along with donor lymphocytes, is then infused into the recipient, setting the stage for the second phase of aGVHD characterized by donor T cell activation. Recipient dendritic cells primed by inflammatory cytokines are thought to play a major role in the activation of donor CD4+ T cells via the presentation of disparate major and minor histocompatibility antigens (Shlomchik et al. 1999). The clonal expansion and differentiation of Th1 type CD4+ T cells are thought to drive the aGVHD reaction (Via and Finkelman 1993; Allen et al. 1993). These cells secrete Th1 cytokines including IL-2 and IFN-γ, leading to the third phase of aGVHD, the effector stage. Macrophages, NK cells, and CD8+ cytotoxic T cells stimulated by Th1 cytokines can exert end-organ damage via reactive oxygen species, TNF-α, perforin/granzyme, and Fas/Fas-ligand (CD95/CD95L), further perpetuating the above cycle (Shresta et al. 1998; Piguet et al. 1987; Graubert et al. 1997; Via et al. 1996). The culmination of these immunological events leads to the clinical syndrome that we recognize as aGVHD. Although the immunology of alloreactive T cells and the role of host dendritic cells in the inception of aGVHD has been reviewed extensively (Ferrara et al. 1999; Reddy 2003; Shlomchik 2007; Welniak et al. 2007; Socie and Blazar 2009), this model provides a useful framework in which to explore the relationship between antigen presentation, chemokine expression, and lymphocyte compartmentalization with the generation of organ-specific aGVHD.

2.2 Lymph Node Physiology

As implicated previously, secondary lymphoid organs play a critical role in the generation of aGVHD. In vivo bio-luminescence imaging of the mouse with transplanted luciferase-labeled allogeneic splenocytes demonstrated that naive but not memory donor T cells first localize to the lymph nodes and spleen within hours of infusion. During the next 2 days, activated T cells expand within these secondary lymphoid organs followed by migration over the next 3–6 days to the intestines, liver, and skin (Beilhack et al. 2005). Inhibiting lymphocyte entry into (or exit from) lymphoid tissues by either blocking antibodies/drugs, genetic manipulation, or surgical removal of organs greatly reduced the incidence and severity of aGVHD in murine models of transplantation (Kim et al. 2003; Murai et al. 2003; Beilhack et al. 2008). In human HSCT, preparative regimens containing total lymphoid irradiation followed by T cell depletion with anti-thymocyte globulin decreased the incidence of aGVHD to almost undetectable levels in patients with hematological malignancies (Lowsky et al. 2005).

The migration of donor T cells from the vascular compartment to the lymph node followed by lymph node egress and migration to the peripheral tissues requires a multi-step adhesion cascade involving CCRs, selectins, and integrins (von Andrian and Mempel 2003; Agace 2006; Sigmundsdottir and Butcher 2008). Naïve and central memory T cells express high levels of CD62L (L-selectin) and CCR7, which facilitate their migration to lymph nodes. CD62L and CCR7 can interact with peripheral node addressin (PNAD) and chemokine ligand (CCL)21, respectively, which are constituitively expressed on high endothelial venules (HEVs) and allow entry of the lymphocyte into the lymph node (von Andrian and Mempel 2003; Berg et al. 1991; Gunn et al. 1998). Interactions between the integrin $\alpha_4\beta_7$ with mucosal vascular addressin cell-adhesion molecule 1 (MADCAM1) also may play a role in mesenteric lymph node localization (Berlin et al. 1993; Arbones et al. 1994). The importance of lymph node compartmentalization in HSCT is illustrated further by the fact that naive ($CD44^{lo}CD62L^{hi}$) but not memory ($CD44^{hi}CD62^{lo}$) T cells are able to cause GVHD (Zhang et al. 2005). However, $CD44^{hi}CD62^{lo}$ T cells previously sensitized to recipient alloantigens can initiate GVHD, presumably by bypassing the initial activation step occurring in the lymph node.

2.3 Lymphocyte Compartmentalization

The lymph node environment and dendritic cells also play important roles in polarizing lymphocytes for homing phenotypes towards specific tissues (Agace 2006; Sigmundsdottir and Butcher 2008). During T cell activation, dendritic cells cause the upregulation of chemokine receptors and other adhesion molecules on

lymphoyctes. Remarkably, these homing receptors allow the lymphocytes to migrate back to the tissues where the antigen was first encountered by the dendritic cell. In Peyer's patches and mesenteric lymph nodes, antigen-experienced T cells up-regulate gut-homing markers including $\alpha_4\beta_7$ and CCR9 and reciprocally down-regulate skin-homing adhesion molecules via signaling by all-trans-retinoic acid (ATRA) produced by dendritic cells (Iwata et al. 2004; Mora et al. 2005; Dudda et al. 2005; Kim et al. 2008). Enzymes important for metabolizing retinol (vitamin A) to retinoic acid are not expressed by dendritic cells from peripheral tissues, which may contribute to the maintenance of lymphocyte tissue specificity (Sigmundsdottir and Butcher 2008; Iwata et al. 2004). When released back into the circulation by way of the efferent lymphatics and thoracic duct, these activated T cells migrate towards gastrointestinal tissues via $\alpha_4\beta_7$-MADCAM1 and CCR9-CCL25 interactions.

In an analogous situation, dendritic cells from the skin migrate toward peripheral lymph nodes where they are able to induce a skin-homing phenotype in T cells with subsequent downregulation of $\alpha_4\beta_7$ and CCR9 (Iwata et al. 2004; Mora et al. 2005; Dudda et al. 2005; Kim et al. 2008). T cells activated in peripheral nodes upregulate cutaneous lymphocyte antigen (CLA) and CCR4. CLA binds to endothelial-cell selectin (E-selectin) and platelet selectin (P-selectin), which are constitutively expressed on cutaneous tissues, while CCR4 interacts with CCL17 expressed on skin venules (von Andrian and Mempel 2003; Agace 2006; Sigmundsdottir and Butcher 2008). During inflammation, both the selectins and the CCL17 are upregulated by skin, thus facilitating lymphocyte entry into peripheral tissues (Agace 2006). CCR10 and its ligand CCL27 also may function in directing lymphocytes from the dermis to the epidermal junction (Agace 2006). The mechanism by which lymphocytes are polarized towards a skin-homing phenotype has been elucidated less clearly, but is thought to be related to vitamin D metabolites and possibly IL-12 (Sigmundsdottir et al. 2007; Picker et al. 1993). Additionally, skin-homing may occur by way of a default mechanism during T cell–dendritic cell interactions in the absence of retinoic acid signaling (Agace 2006).

The importance of the lymph node and the stereotypical involvement of some organs but not others strongly suggests that lymphocyte homing might play a role in aGVHD generation. The expression of specific homing molecules and CCRs can be used to define unique populations of effector and suppressor T cells. As noted earlier, two nonoverlapping antigen-experienced T cell populations have been well characterized in mice and in humans. One subset is characterized by $\alpha_4\beta_7$/CCR9 expression (gut-homing) and the other by CLA/CCR4 expression (skin-homing). Interestingly, these homing patterns correspond to the two most commonly involved tissues during aGVHD, skin, and gut. These populations of cells may have unique functions and, when perturbed, may result in specific pathological outcomes in HSCT. Homing of particular functional subsets of T cells such as Tregs and Th17 cells may explain the organ-specific nature of clinical aGVHD. Here we will consider the evidence for homing of these cells in control or induction of aGVHD.

3 Tregs, aGVHD, and Adhesion Molecules

3.1 History of Suppressor Cells

Suppressor T cells were first described over 30 years ago by Kondo and Gershon (Gershon and Kondo 1970). These cells were thought to regulate the immune system by secretion of antigen-specific factors. The failure to clone these factors in the 1980s led to widespread skepticism about suppressor T cells. This field lay dormant for several decades until suppressor T cells were "rediscovered" as Tregs in the 1990s. In a seminal paper, Sakaguchi et al. demonstrated that the depletion of CD4+CD25+ cells in mice led to the development of autoimmune-induced diabetes mellitus, thyroiditis, gastritis, and other disorders (Sakaguchi et al. 1995). Autoantibodies and immune-mediated end organ damage were significantly increased in BALB/c nu/nu mice receiving T cell suspensions obtained from BALB/c nu/+ mice depleted of CD4+CD25+ Tregs. This paper was the first report demonstrating that CD25 could be used as a marker to define a population of immunoregulatory T cells. Since this initial report, much work has been done on Tregs pertaining to their identification, development, mechanisms of action, homing characteristics, and their relationship to various human diseases and conditions.

3.2 Natural Treg Phenotype and Characterization

Tregs are a naturally occurring subset of T lymphocytes that make up about 5–10% of normal circulating CD4+ cells (Sakaguchi et al. 1995; Shevach 2002). Tregs develop in the thymus ("natural Treg") or they can be generated in the periphery from naive T cells ("induced Treg") (Chen et al. 2003; Izcue et al. 2006). Tregs suppress the proliferation of activated T cells via a cell-contact-dependent mechanism and in an antigen nonspecific manner. They are further characterized by relative hyporesponsiveness to stimulation by pan T cell activators (Shevach 2002). In vivo, they are thought to function in maintaining immunological self-tolerance. Tregs were identified initially by the expression of high levels of the IL-2 receptor α chain, CD25. Numerous other markers have been attributed to Treg phenotype and function including cytotoxic T-lymphocyte-associated antigen 4 (Takahashi et al. 2000), glucocorticoid tumor necrosis factor receptor (Shimizu et al. 2002), CD62L (Ermann et al. 2005; Taylor et al. 2004), CCR4 (Oswald-Richter et al. 2004), folate receptor 4 (FR4) (Yamaguchi et al. 2007), and low expression of the IL-7 receptor α chain, CD127 (Liu et al. 2006; Seddiki et al. 2006). However, none of these molecules are uniquely expressed on Tregs, and many of them can be identified on T cells without suppressor activity.

3.3 *Foxp3 Expression*

Perhaps the best characterized and most reliable marker for Tregs is Foxp3, a member of the fork-head/winged-helix family of transcription factors (Fontenot et al. 2003; Hori et al. 2003). The identification of Foxp3 as a critical transcription factor necessary for Treg development and function was derived from a combination of basic science, mouse genetics, and clinical medicine studies. Foxp3 mutations were first identified in an inbred mouse line named *scurfy* and later in an analogous human genetic disorder called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). These syndromes are characterized by a wasting illness associated with immune dysfunction, lymphoproliferation, diarrhea, rash, and numerous autoimmune/endocrine abnormalities. The phenotype of these inherited disorders was very similar to that occurring in mice depleted of CD4+CD25+ Tregs. Similarities between IPEX and aGVHD were also noted. Through a series of elegant experiments it was shown that Foxp3 was upregulated in unmanipulated CD4+CD25+ Tregs as compared to CD4+CD25− T cells, CD8+ T cells, and CD19+ B cells. In addition, forced expression of Foxp3 by CD25− T cells via retroviral transduction led to the development of a T cell subset with both *in vivo* and *in vivo* suppressive properties (Fontenot et al. 2003; Hori et al. 2003).

Initially it was felt that Foxp3 was an unambiguous marker for Tregs; however, later it was shown that recently activated T cells without regulatory characteristics can up-regulate Foxp3 transiently (Morgan et al. 2005; Gavin et al. 2006; Wang et al. 2007). So it appears that an all-inclusive, highly specific single marker for Tregs remains elusive. Recently, some interest has focused on GARP, also known as LRRC32, a cell surface molecule important for both Foxp3 expression and the suppressive properties of Tregs (Wang et al. 2008). More research will need to be done to further elucidate the properties of GARP and other molecules that are used to identify Tregs. The identification of more specific and preferably extracellular molecules may help with the use of Tregs as a diagnostic and therapeutic tool in the treatment of human diseases. Because of the intracellular location of this transcription factor and the need for fixation and permeabilization of cells prior to detection with antibodies, viable Tregs cannot be isolated currently based on the expression of Foxp3.

3.4 *Treg Mechanisms of Action*

The mechanisms by which Tregs mediate suppression have already been reviewed extensively (Tang and Bluestone 2008; Vignali et al. 2008). Briefly, Tregs function by various manners, including secretion of the immunosuppressive cytokines IL-10, IL-35, and TGF-β (Tang and Bluestone 2008; Takahashi et al. 1998; Thornton and Shevach 1998), granzyme/perforin-induced cell lysis (Qin

et al. 2006), metabolic disruption via cytokine (IL-2) deprivation (Pandiyan et al. 2007) or CD39/CD73-associated generation of adenosine metabolites, which suppresses T cells by binding to their adenosine receptor 2A (Deaglio et al. 2007), and modulation of dendritic cell function (Curti et al. 2009; Sharma et al. 2009; Chung et al. 2009). With regards to this last mechanism, special mention should be made of the upregulation of indoleamine 2,3-dioxygenase (IDO) on dendritic cells by Tregs and IFN-γ. Increased expression of IDO leads to depletion of tryptophan, which is an essential amino acid required by proliferating T cells. In addition, a byproduct of the tryptophan metabolism called kynurinine has potent immunosuppressive properties (Curti et al. 2009; Xu et al. 2008). Recent data suggests that IDO expression by dendritic cells could be an important mechanism of Treg-induced immune suppression following HSCT by increasing kynurinine (Jasperson et al. 2009). The proposed mechanism is through reduction of IL-6, an important cytokine for Th17 cell generation, thereby preventing aGVHD and linking Tregs and Th17 cells (Sharma et al. 2009; Chen et al. 2009). In the end, the exact mechanism by which Tregs induce immune regulation is not fully defined but likely involves numerous functions that vary depending on the clinical situation. The mechanisms of action may differ depending on whether the Treg is involved with the prevention of autoimmunity, resolution of ongoing inflammation, maintenance of immune homeostasis, or regulating alloimmune responses.

3.5 Tregs and Murine aGVHD

Much of the early work in this area focused on natural Treg suppression of autoimmune phenomena. However, mixed lymphocyte reactions showed that natural Tregs also could efficiently control the proliferation of alloreactive T cells (Taylor et al. 2001). This finding indicated a potential role for natural Tregs in the prevention of allograft rejection during solid organ transplantation and during the inhibition of aGVHD following HSCT. In preclinical animal models, several groups demonstrated that aGVHD severity and lethality could be attenuated by the co-administration of freshly isolated Tregs with T cell effectors when compared to mice receiving only T effectors, in whom aGVHD was rapidly fatal (Taylor et al. 2001; Cohen et al. 2002; Hoffmann et al. 2002). In a similar experiment, when allogeneic bone marrow and T cell grafts were depleted of CD4+CD25+ Tregs, the mice quickly succumbed to the effects of aGVHD (Taylor et al. 2001; Cohen et al. 2002). Interestingly, in the experiments involving the co-transfer of Tregs and T effector cells, supraphysiologic ratios of Tregs to T effectors (i.e., 1:2 or 1:1) were needed to induce this suppression, as physiologic ratios of 1:10 did not show a protective effect.

Further studies demonstrated that only the $CD62L^{hi}$ Treg subset could decrease the incidence and severity of aGVHD in murine models of transplantation (Ermann et al. 2005; Taylor et al. 2004). Similarly, in an autoimmune diabetes model, only

the CD62L^{hi} Tregs that also expressed high levels of CCR7 were able to prevent the induction of diabetes (Szanya et al. 2002). These data were important as they suggested that Treg subsets could be defined by the expression of adhesion molecules and CCRs, with each subset possessing unique *in vivo* functional properties. The importance of lymph node homing in aGVHD generation again was illustrated, and the experiments also suggested that the lymph node was a possible *in vivo* site for Treg-induced immune suppression. Indeed the CD62L^{hi} Tregs homed more efficiently to secondary lymphoid organs including the spleen, mesenteric, and peripheral LN, and were better able to prevent the proliferation of alloreactive T cells at these sites when compared to animals receiving the CD62^{lo} Treg infusions (Ermann et al. 2005; Taylor et al. 2004). This hypothesis was further supported by bio-luminescence imaging studies that showed early co-localization of T effector cells with Tregs in secondary lymphoid organs after transplant, followed by egress of Tregs from lymph nodes and migration to peripheral tissues (Nguyen et al. 2007). Although this model would explain how Tregs prevent the induction of aGVHD, it does not necessarily elucidate how Tregs can suppress established aGVHD (Jones et al. 2003). Alternatively, lymph node localization may function to polarize Tregs towards homing patterns for specific tissues, so that, when released back into circulation, they are able to mediate organ-specific immune suppression. Only preliminary data is available to support this hypothesis in HSCT (Engelhardt et al. 2008).

As noted earlier, the prevention of aGVHD by adoptive transfer requires the infusion of large numbers of Tregs, which may not be feasible in clinical practice due to the low frequency of natural Tregs in circulation (i.e., 5–10% of CD4+ T cells). To deal with this problem, various groups developed protocols to expand Tregs ex vivo through stimulation with anti-CD3 antibodies or allogeneic APCs and exogenous high-dose IL-2. In addition, activated Tregs appear to suppress immune responses more efficiently than resting cells, suggesting additional clinical benefit from the expansion process (Cohen et al. 2002; Taylor et al. 2002; Hoffmann et al. 2004). Along these lines, two groups independently showed that the infusion of ex vivo expanded Tregs could improve survival in murine models of aGVHD (Cohen et al. 2002; Taylor et al. 2002). Although both freshly isolated and expanded Tregs could prevent the induction of aGVHD when co-transferred with effector T cells, their activity in treating established aGVHD is less clear. Jones et al. showed that the infusion of Tregs up to 10 days after the initial infusion of CD8+ T cells could prevent aGVHD lethality in a MHC-matched model. Delayed administration of Tregs (>2 days after CD4+ infusion); however, could not prevent aGVHD mortality in a haploidentical model of transplant (Jones et al. 2003). These data suggest that Tregs can treat evolving aGVHD; however, optimal suppression seems to occur early in the disease process and prior to profound immune activation as in the setting of MHC mismatch. Taken as a whole, these observations imply that human Tregs could be expanded ex *vivo* and infused with the stem cell graft to prevent aGVHD or possibly to treat early aGVHD in HSCT.

3.6 Graft vs. Tumor Concerns

The immune mechanisms associated with GVT appear to be closely related to GVHD. This apparent association raised concern that the therapeutic use of Tregs to prevent aGVHD could lead to an increased risk of cancer relapse following HSCT. In murine models where animals were challenged with conventional T cells, Tregs, and either leukemia or lymphoma cell lines, Tregs could control aGVHD without disrupting GVT following MHC-matched or -mismatched transplants (Jones et al. 2003; Edinger et al. 2003; Trenado et al. 2003). This finding suggested that the immunological mechanism of GVT could be separated from GVHD, which would further support the therapeutic use of Tregs in human HSCT. However, some conflicting results have been obtained depending on the malignant cell line used (Trenado et al. 2003), and one human study has shown that higher Treg frequencies post-transplant were associated with an increased risk of relapse of chronic myelogenous leukemia (Nadal et al. 2007). Further research is needed to decipher the specific role of Tregs in aGVHD and GVT.

Prevention of stem cell graft rejection and immune reconstitution following transplant are key characteristics necessary for the long-term survival of patients undergoing HSCT. Interestingly, both donor and recipient Treg infusions facilitate donor hematopoietic progenitor cell engraftment, perhaps by suppressing recipient anti-donor immune responses (Taylor et al. 2004; Hanash and Levy 2005). Immune reconstitution also is improved with higher lymphocyte counts and increased frequencies of CD4+ and CD8 T+ cells post-transplant, indicating that normal immune development and function requires appropriate regulation by Tregs (Trenado et al. 2003). In summary, there is substantial evidence that naturally occurring Tregs are associated with a decrease in the incidence and severity of aGVHD in animal models of transplantation, improvement of immune reconstitution, and preservation of the beneficial effects of GVT.

3.7 Tregs and Human aGVHD

In spite of the overwhelming data supporting Treg prevention of aGVHD in murine models of transplantation, the role of Tregs in human aGVHD is less clear. Human HSCT is complex. Differing chemotherapy and immunosuppression regimens are used based on patient age, overall health, and disease status. Donors can be related, unrelated, HLA-identical, or HLA-mismatched. Stem cell grafts can be derived from bone marrow, peripheral blood, or cord blood. In addition, the graft can be manipulated with the removal or addition of specific lymphocyte subsets to facilitate engraftment, to prevent aGVHD, or to decrease relapse rates. All of these variations could potentially confound analysis of the role of Tregs in GVHD in humans. Numerous observational and retrospective studies have been performed

using different patient populations and transplant techniques. Not surprisingly, heterogeneous results have been obtained.

In one of the first human studies examining CD4+CD25+ Tregs in patients undergoing HLA-identical sibling transplantation, the frequency of CD4+ cells co-expressing CD25+ in the peripheral blood stem cell graft was significantly higher in those individuals who developed aGVHD (Stanzani et al. 2004). The *in-vitro* suppressive properties of these isolated Tregs were not analyzed in this study. The authors suggested that CD25 alone maybe insufficient to adequately identify human Tregs in the transplant setting. In a similar study, CD4+CD25+ Tregs were enumerated during the first 100 days following transplant in a series of patients primarily undergoing matched related sibling transplants. Here, there was no significant difference in the relative or absolute number of CD4+CD25+ Tregs in patients with or without aGVHD (Sanchez et al. 2004). Tregs were identified only by the expression of CD25 in both of these studies. It is difficult to reliably quantify Tregs in peripheral blood using only CD25, especially in HSCT patients who may have increased numbers of activated CD25-expressing T cells.

The later identification of Foxp3 as a more specific marker for Tregs has greatly facilitated Treg analysis in human transplantation. Initially, Foxp3 expression by peripheral blood mononuclear cells was analyzed by real-time quantitative polymerase chain reaction (PCR). The patient population studied was heterogeneous, consisting of patients with both HLA-matched and -mismatched, related or unrelated donors. Bone marrow was the stem cell source for all patients. Blood samples were obtained from the recipient at the time of aGVHD occurrence. Foxp3 mRNA expression was decreased significantly in patients with any aGVHD compared to patients without aGVHD or healthy controls. In addition, Foxp3 expression was inversely related to the severity of aGVHD, confirming the importance of Foxp3 for Treg analysis/identification and supporting the data previously obtained in murine studies (Miura et al. 2004).

In addition to PCR analysis, Tregs can also be enumerated using specific antibodies to Foxp3. Much of the early work analyzing the frequency and absolute numbers of human Foxp3+ Tregs was performed at the National Institutes of Health (NIH) using patients undergoing T cell depleted, HLA-identical sibling transplants. The frequency and absolute numbers of Foxp3+ Tregs were analyzed in these donors, stem cell grafts, and recipients both pre- and post-transplant. In patients undergoing myeloablative conditioning at the NIH, high absolute numbers of CD4+Foxp3+ cells in the stem cell graft or in the recipient at day +30–45 was associated with a reduced risk of grade II–IV (moderate to severe) aGVHD. The proportion of CD4+CD25+ T cells expressing Foxp3 at day +30 was also lower in patients developing aGVHD (Rezvani et al. 2006). In another study from the NIH, this time examining patients undergoing reduced intensity chemotherapy (RIC) transplantation, moderate-to-severe aGVHD was more likely to occur in patients whose donors had fewer Tregs. The absolute and relative frequencies of Tregs were increased in the donors of patients who did not develop aGVHD (Mielke et al. 2007). Tregs in the stem cell graft or in the patient before and early after transplant were not associated with the development of aGVHD in this study.

The above data were obtained from patients receiving T cell-depleted transplants. In patients receiving more conventional HSCT with cells from either a related or an unrelated donor, the infusion of stem cell grafts containing higher absolute numbers of Foxp3+ cells was associated with a significantly lower cumulative incidence of aGVHD (Pabst et al. 2007; Wolf et al. 2007). The association of Tregs with aGVHD incidence appeared to be strongest in patients undergoing myeloablative MRD, as the significance was lost by RIC patients when the cohort was stratified based on the intensity of the conditioning regimen (i.e., myeloablative vs. RIC) (Wolf et al. 2007). Additionally, improved survival was found in patients receiving both myeloablative conditioning and grafts from sibling donors with high Treg numbers (Wolf et al. 2007). The risk of relapse was not affected by the Treg content of the graft. These data suggest that Treg infusions or naturally occurring high Treg numbers could improve survival by decreasing treatment-related mortality associated with aGVHD. Alternatively, Tregs could improve post-transplant immune reconstitution, thereby leading to less frequent or severe infectious complications.

Initially it appeared that data from Foxp3 studies might explain why earlier studies were unable to demonstrate a relationship between human Tregs and the prevention of aGVHD. However, not all studies have shown a clear association between the number of Foxp3+ cells and incidence of aGVHD. In a series of pediatric patients undergoing either matched-related or unrelated transplants, Foxp3 expression by CD4+CD25+ cells was determined by real time PCR analysis. Post-transplant Foxp3 expression in patients was similar to that in healthy controls, irrespective of the presence or the absence of aGVHD (Seidel et al. 2006). Furthermore, these investigators demonstrated that Foxp3 expression was closely linked to the CD4+CD25+ T cell population/phenotype regardless of their suppressor potential. These data imply that recently activated naïve T cells can upregulate CD25 and express abundant amounts of Foxp3 mRNA, independent of the immunoregulatory function of the cells. Once again, these studies cast doubt on the use of any single marker to exclusively identify Tregs in the setting of human HSCT.

To date, conflicting results have been obtained in human studies examining the relationship between the suppressor activity of Tregs and the incidence of aGVHD. These discrepancies likely are due to numerous factors, including the clinical heterogeneity of human transplantation, timing of Treg analysis, and the origin of the sample in which Tregs were enumerated (i.e., recipient, donor, or stem cell graft). In addition, the differences in human studies could result from the difficulties with identifying and isolating pure Treg subsets. Although imperfect, the identification of Tregs by Foxp3 expression has given great insight into the role of Tregs in human transplantation. Currently, there is growing evidence that supports a role for human Tregs in the prevention of aGVHD. These data suggests that donor-derived Tregs influence transplant outcomes and Treg frequencies post-transplant. With growing acceptance of this concept, a logical next step is to explore the unique features and functions of Treg subsets as they relate to HSCT.

3.8 Adhesion Molecules and Tissue-Specific Tregs

aGVHD primarily involves the skin, gut, liver, secondary lymphoid organs, and possibly the lungs. The stereotypical involvement of specific organs by aGVHD strongly suggests that dysregulation of lymphocyte trafficking is important for the pathogenesis of aGVHD. The role of lymphocyte homing and expression of important adhesion molecules including selectins, CCRs, and integrins in aGVHD has been reviewed (Sackstein 2006; Wysocki et al. 2005a). Similar to conventional T cells, there is significant evidence that Treg localization after HSCT is of importance. The expression of adhesion molecules including selectins, CCRs, and integrins may serve to define Treg subsets with specific migratory patterns and suppressor properties (Huehn and Hamann 2005; Kim 2006; Wei et al. 2006).

Based on homing patterns, Tregs can be divided into two general populations: (1) lymphoid-homing (i.e., naïve-like), which express CCR7, CXCR4, CD62L and (2) nonlymphoid-homing (i.e., effector/memory-like), which variably express CCR2, CCR4, CCR5, CCR6, CCR8, CXCR3, CXCR6, CLA, and CD103 (Kim 2006; Huehn et al. 2004; Lee et al. 2007; Lim et al. 2006). As previously outlined, only the $CD62L^{hi}$ Treg population could decrease the lethality of aGVHD in animal models (Ermann et al. 2005; Taylor et al. 2004). Presumably this principle also will hold true for the CCR7+ subset of Tregs, since this molecule is often co-expressed with CD62L (Szanya et al. 2002). However, Tregs express diverse homing molecules and are present in both lymphoid and nonlymphoid tissues, suggesting that Tregs maintain immunologic tolerance at various sites. In addition, Tregs can suppress the initiation of aGVHD or established aGHVD, further supporting the idea of dual sites of immune regulation (i.e., suppression of allo-responses in secondary lymphoid organs during the priming phase and in target tissues during the effector phase of aGVHD. This latter function likely is facilitated by the expression of integrins, CCRs, and selectins.

In murine models of transplantation, Treg expression of CCR5 and CCR6 has been shown to be of critical importance in preventing the development of aGVHD (Varona et al. 2006; Wysocki et al. 2005b). Normally, CCR5 and CCR6 are present on various types of leukocytes, including subsets of T cells and dendritic cells, and serve to mediate chemoattraction of these cells to areas of inflammation. The ligands for CCR5 (CCL3, CCL4, and CCL5) and CCR6 (CCL20) are present in aGVHD target tissues and are increased during inflammation (Wysocki et al. 2005a; Varona et al. 2006). In these Treg experiments, the severity and mortality of aGVHD induced by wild-type T cells was increased when CCR5- or CCR6-deficient Tregs were infused into either an unconditioned GVHD animal model (Varona et al. 2006) or an irradiated murine model of transplantation (Wysocki et al. 2005b), respectively. The *in-vitro* suppressive properties of both of these Treg subsets (i.e., CCR5−/− and CCR6−/−) were maintained, suggesting that lack of CCRs did not result in loss of suppressor phenotype. In addition, Varona et al. demonstrated that unmanipulated CCR6+ Tregs exhibit decreased expression of CD62L but upregulate other homing molecules including CCR4, CCR8, CD29,

CD11a, and CLA (P-selectin ligand) (Varona et al. 2006). Similarly, the absence of CCR5 on Tregs resulted in normal *in-vivo* localization of Tregs in secondary lymphoid organs during the first week of transplantation; however, later homing of Tregs to specific target organs of aGVHD was inhibited (Wysocki et al. 2005b). Thus, it appears that Treg expression of CCR5 or CCR6 is not specific for cells pertinent to a single aGVHD target tissue, but instead is necessary for Treg migration to areas of inflammation following HSCT. In these models, the inhibition of aGVHD severity and mortality appears to be related to Treg-mediated suppression at peripheral sites as opposed to the lymph node.

To date, Treg expression of adhesion molecules and CCRs have been incompletely explored in human HSCT. Currently, there is direct evidence that tissue localization is important for Treg-mediated prevention of aGVHD in human HSCT, which in turn indirectly suggests that Treg expression of homing molecules is necessary and critical for *in vivo* function following transplant. In patients undergoing allogeneic HSCT, the frequency of mucosal Foxp3+ Tregs in intestinal biopsies as determined by double immunoenzymatic labeling was significantly higher in those individuals without gastrointestinal aGVHD when compared to either healthy controls or patients with symptomatic gut aGVHD (Rieger et al. 2006). In a similar study, the frequency of Foxp3+ Tregs in skin biopsies was related inversely to the severity of skin aGVHD and correlated with a positive response to treatment (Fondi et al. 2009). These data support the importance of Treg compartmentalization; however, Treg expression of homing receptors was not analyzed in either study. Therefore, the direct association between circulating tissue-specific Tregs with eventual tissue infiltration or the prevention of organ-specific aGVHD could not be assessed from this work.

3.9 Recent Work on Tissue-Specific Tregs and aGVHD

Our work has focused on identifying unique subsets of tissue-specific Tregs as they relate to the pathogenesis of organ-specific aGVHD in human HSCT.

As previously noted, Tregs can be generally divided into lymphoid- and non-lymphoid-homing subsets. Similar to other T cells, the nonlymphoid-homing Tregs can be further subdivided into mutually exclusive groups characterized by expression of either $\alpha_4\beta_7$/CCR9 (gut-homing) or CLA/CCR4 (skin-homing). The same principles and mechanisms that govern the regulation of selectins, integrins, and CCRs on other T cell subsets also seem to operate in Tregs (Kang et al. 2007; Siewert et al. 2007). Consistent with the known reciprocal regulation of these homing receptors, we have found an inverse relationship between $\alpha_4\beta_7$ and CLA expression by human Tregs early after HSCT. Furthermore, we found that increased frequencies of circulating CLA+ Tregs early after transplant was associated with the prevention of initial skin aGVHD, and that higher percentages of CLA+ Tregs and $\alpha_4\beta_7$+ Tregs were related inversely to the severity of skin or gut aGVHD, respectively

(Engelhardt et al. 2008). These studies suggest that circulating tissue-homing subsets of Tregs may regulate organ-specific risk and severity of aGVHD in human HSCT.

3.10 Summary of Tregs and aGVHD

In summary, Treg-mediated prevention of aGVHD morbidity and mortality may occur by several mechanisms of action that occur in diverse anatomical sites. Evidence seems to support immune regulation in both the lymph node and more peripherally in the target tissues of aGVHD. Furthermore, lymph node localization appears to be critical for appropriate tissue compartmentalization of lymphocytes. We suggest that early after HSCT Tregs may function to suppress initial activation of alloreactive T cells in secondary lymphoid organs. Then antigen-activated Tregs may upregulate adhesion molecules and leave the lymph node to exert their suppressor functions at distal sites. The induction of certain CCRs, such as CCR5 and CCR6, may direct Tregs to areas of ongoing epidermal and mucosal inflammation or to other activated lymph nodes. In addition, the lymph node may function to polarize Tregs for homing to specific tissues via induced expression of $\alpha_4\beta_7$/CCR9 or CLA/CCR4, thereby allowing Tregs to migrate to and concentrate in the tissues where the alloantigen was originally encountered. Many of the ligands for the above adhesion molecules are expressed constitutively by aGVHD target tissues and expression is increased during periods of inflammation induced by chemotherapy or established aGVHD. This inflammatory environment therefore supports Treg migration to these tissues to help suppress alloreactive responses and to reestablish immune homeostasis. Thus, a well orchestrated suppression of immune responses in both the lymph node and the peripheral tissues likely allows Tregs to prevent aGVHD.

4 Th17 Cells and aGVHD

4.1 Biology of Th17 Cells

Th17 cells are a newly identified lineage of T cells with distinct characteristics that separate them from the previously described Th1/2 subsets and Tregs [reviewed in Bettelli et al. (2007), Miossec et al. (2009) and Ouyang et al. (2008)]. Th17 cells are characterized by the production of proinflammatory cytokines including IL-17 (also called interleukin-17A), IL-17F, IL-21, and IL-22. Normally, these cells help to induce peripheral inflammation and function to coordinate host defenses against extracellular pathogens (Aujla et al. 2008). Th17 cells also have been implicated in several pathological states, including induction of autoimmunity and the rejection

of solid organ allografts (Bettelli et al. 2007; Miossec et al. 2009; Ouyang et al. 2008).

Normally, IL-6 in the presence of TGF-β promotes the differentiation of naïve T cells into Th17 cells via a STAT3 pathway (Miossec et al. 2009; Bettelli et al. 2006; Mangan et al. 2006). IL-21 and IL-23 further support Th17 expansion and survival (Miossec et al. 2009; Mangan et al. 2006), while the Th1 cytokine, IFN-γ, acts as a negative regulator. Murine retinoid-related orphan receptor (ROR)t (or its human counterpart RORc) is the key transcription factor necessary for Th17 differentiation (Miossec et al. 2009; Bettelli et al. 2006; Ivanov et al. 2007). IL-6, IL-21, and IL-23 in the appropriate setting help to induce expression of RORt (Bettelli et al. 2006; Ivanov et al. 2007). Interestingly, IL-6, a critical cytokine for Th17 commitment, has been shown to interfere with peripheral (i.e., induced) Treg cell generation through up-regulation of the TGF-β pathway inhibitor SMAD7 (Dominitzki et al. 2007). Thus, these two T cell subsets, one with an inflammatory phenotype and the other with a suppressor phenotype, appear to be related and reciprocally regulated in part by IL-6. Furthermore, retinoic acid, a mediator of induction of gut homing phenotype that has been shown to increase Foxp3 expression and decrease Th17 lineage commitment by enhancing TGF-β-induced SMAD3 signaling, simultaneously inhibits IL-6 and IL-23 pathways by decreasing RORt expression (Schambach et al. 2007; Elias et al. 2008; Kim 2008; Xiao et al. 2008; Mucida et al. 2007). Physiologically, this process may function to protect the gut from unwanted inflammation resulting from constant antigen exposure by skewing this mucosal milieu towards tolerance. These studies also suggest that the expression of gut-homing markers will be under-represented on Th17 cells; however, this concept needs to be explored further.

4.2 Th17 Cells and CCRs

It has been reported that circulating Th17 cells are characterized by the CCR profile of either CCR6+CCR4+ (Acosta-Rodriguez et al. 2007) or CCR2+CCR5– (Sato et al. 2007). Specifically, CCR6 expression appears to be upregulated on CD4+ T cells capable of producing IL-17 (Singh et al. 2008). However, more extensive analysis of Th17 cells isolated from adult peripheral blood, umbilical cord blood, or tonsillar lymphoid tissue has demonstrated that these cells are capable of expressing a diverse range of CCRs. Similar to Tregs, Th17 cells also seem to express either secondary lymphoid tissue homing receptors (CCR7, CXCR4, CD62L) or nonlymphoid homing molecules (CCR2, CCR4, CCR5, CCR6, CXCR3, and CXCR6) (Lim et al. 2008; Kim 2009). Although CCR6 is highly expressed by Th17 cells, this CCR is also found on approximately 50% of all circulating CD4+ memory T cells (Singh et al. 2008). In the end, there is likely no single CCR expression pattern that will universally and specifically identify all Th17 cells.

4.3 Th17 Cells and the aGVHD Disease Process

Because of their association with inflammation and autoimmunity, the relationship between Th17 cells and aGVHD is now being explored. Th17 cells have been indentified in secondary lymphoid organs and in aGVHD target tissues of animals undergoing allogeneic bone marrow transplant (Carlson et al. 2009; Kappel et al. 2009). In one preclinical model of transplant, the transfer of murine IL-17−/− CD4+ T cells led to delayed aGVHD development when compared to animals receiving wild-type CD4+ T cells. aGVHD still occurred and the mortality rate was unchanged in this setting (Kappel et al. 2009). In a similar set of experiments using a MHC-mismatched HSCT model, transfer of IL-17−/− T cells paradoxically caused increased aGVHD mortality and was associated with a skewed Th1 differentiation pattern in the donor T cells with associated liver and gut injury (Yi et al. 2008). Another group showed that the infusion of naïve CD4+ T cells that were polarized towards a Th17 phenotype resulted in significant aGVHD primarily involving the skin and the lung (Carlson et al. 2009). Overall, the data suggest that Th17 cells may modulate Th1 donor T cell differentiation, which in turn may affect end organ damage. Further evidence supporting this model was derived from animal models where the infusion IFN-γ−/− CD4+ T cells led to preferential differentiation of cells with a Th2 and Th17 phenotype, with subsequent increase in skin and pulmonary aGVHD and decrease in T cell expression of gut-homing associated adhesion molecules (Yi et al. 2009). Many questions remain with regards to Th17 cells as they relate to aGVHD in both murine models of transplantation and in human HSCT. The preferential involvement of skin and lung over gut tissues by Th17 cells does suggest that Th17 cell compartmentalization and expression of adhesion molecules may play a role in aGVHD development. Of particular interest in future studies will be the role of CCR6. This CCR is highly expressed on Th17 cells (Singh et al. 2008) and when absent on Tregs was shown previously to be associated with accelerated aGVHD lethality (Varona et al. 2006).

5 Summary

GVHD is one of the principal complications following HSCT that limit success. There is a large amount of data now in both animal models and humans after transplant to suggest that complex dynamics in the T cell compartment regulate the clinical expression of disease. Most studies suggest that the suppressive function of Tregs is essential for maintaining successful outcomes after transplantation. Recent data suggests that the location of Tregs and their ability to migrate to organs such as skin and gut significantly affect the expression of disease in target tissues. These findings may allow early stratification of clinical risk of skin or gut aGVHD following transplantation based on numbers and

phenotype of circulating Tregs. These studies also suggest that induction of Tregs with an appropriate phenotype or adoptive transfer of such cells could be considered in the future as a prophylactic or therapeutic intervention in transplantation. However, the biology of these cells *in vivo* is complex and still incompletely understood. Many practical questions remain as to the number and phenotype of cells that would be needed in clinical intervention, and the exact mechanisms by which Tregs maintain immune homeostasis in the setting of human HSCT.

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References

Acosta-Rodriguez EV et al (2007) Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nat Immunol 8(6):639–646

Agace WW (2006) Tissue-tropic effector T cells: generation and targeting opportunities. Nat Rev Immunol 6(9):682–692

Allen RD, Staley TA, Sidman CL (1993) Differential cytokine expression in acute and chronic murine graft-versus-host-disease. Eur J Immunol 23(2):333–337

Alousi AM et al (2009) Etanercept, mycophenolate, denileukin, or pentostatin plus corticosteroids for acute graft-versus-host disease: a randomized phase 2 trial from the Blood and Marrow Transplant Clinical Trials Network. Blood 114(3):511–517

Arai S, Vogelsang GB (2000) Management of graft-versus-host disease. Blood Rev 14(4): 190–204

Arbones ML et al (1994) Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. Immunity 1(4):247–260

Aujla SJ et al (2008) IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. Nat Med 14(3):275–281

Beatty PG et al (1985) Marrow transplantation from related donors other than HLA-identical siblings. N Engl J Med 313(13):765–771

Beatty PG et al (1991) Marrow transplantation from HLA-matched unrelated donors for treatment of hematologic malignancies. Transplantation 51(2):443–447

Beilhack A et al (2005) In vivo analyses of early events in acute graft-versus-host disease reveal sequential infiltration of T-cell subsets. Blood 106(3):1113–1122

Beilhack A et al (2008) Prevention of acute graft-versus-host disease by blocking T-cell entry to secondary lymphoid organs. Blood 111(5):2919–2928

Belkaid Y et al (2002) CD4+ CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature 420(6915):502–507

Berg EL et al (1991) The human peripheral lymph node vascular addressin is a ligand for LECAM-1, the peripheral lymph node homing receptor. J Cell Biol 114(2):343–349

Berlin C et al (1993) Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. Cell 74(1):185–195

Bettelli E et al (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 441(7090):235–238

Bettelli E, Oukka M, Kuchroo VK (2007) T(H)-17 cells in the circle of immunity and autoimmunity. Nat Immunol 8(4):345–350

Carlson MJ et al (2009) In vitro-differentiated TH17 cells mediate lethal acute graft-versus-host disease with severe cutaneous and pulmonary pathologic manifestations. Blood 113 (6):1365–1374

Chen W et al (2003) Conversion of peripheral CD4+ CD25- naive T cells to CD4+ CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 198(12):1875–1886

Chen X et al (2009) Blockade of interleukin-6 signaling augments regulatory T-cell reconstitution and attenuates the severity of graft-versus-host disease. Blood 114(4):891–900

Chung DJ et al (2009) Indoleamine 2, 3-dioxygenase-expressing mature human monocyte-derived dendritic cells expand potent autologous regulatory T cells. Blood 114(3):555–563

Cohen JL et al (2002) CD4(+)CD25(+) immunoregulatory T Cells: new therapeutics for graft-versus-host disease. J Exp Med 196(3):401–406

Curiel TJ et al (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10(9):942–949

Curti A et al (2009) The role of indoleamine 2, 3-dioxygenase in the induction of immune tolerance: focus on hematology. Blood 113(11):2394–2401

Deaglio S et al (2007) Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J Exp Med 204(6):1257–1265

Dominitzki S et al (2007) Cutting edge: trans-signaling via the soluble IL-6R abrogates the induction of FoxP3 in naive CD4+ CD25 T cells. J Immunol 179(4):2041–2045

Dudda JC et al (2005) Dendritic cells govern induction and reprogramming of polarized tissue-selective homing receptor patterns of T cells: important roles for soluble factors and tissue microenvironments. Eur J Immunol 35(4):1056–1065

Edinger M et al (2003) CD4+ CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. Nat Med 9(9):1144–1150

Elias KM et al (2008) Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. Blood 111(3):1013–1020

Engelhardt B et al (2008) Circulating gut- or skin-homing regulatory T cells (tregs) predict whether acute graft-versus-host disease (aGVHD) occurs in gut or skin following allogeneic stem cell transplantation (ASCT). Blood 112(11):264a

Ermann J et al (2005) Only the CD62L+ subpopulation of CD4+ CD25+ regulatory T cells protects from lethal acute GVHD. Blood 105(5):2220–2226

Ferrara JL, Levy R, Chao NJ (1999) Pathophysiologic mechanisms of acute graft-vs.-host disease. Biol Blood Marrow Transplant 5(6):347–356

Filipovich AH et al (2005) National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. Biol Blood Marrow Transplant 11(12):945–956

Fondi C et al (2009) Increase in FOXP3+ regulatory T cells in GVHD skin biopsies is associated with lower disease severity and treatment response. Biol Blood Marrow Transplant 15(8): 938–947

Fontenot JD, Gavin MA, Rudensky AY (2003) Foxp3 programs the development and function of CD4+ CD25+ regulatory T cells. Nat Immunol 4(4):330–336

Gavin MA et al (2006) Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. Proc Natl Acad Sci USA 103(17):6659–6664

Gershon RK, Kondo K (1970) Cell interactions in the induction of tolerance: the role of thymic lymphocytes. Immunology 18(5):723–737

Graubert TA et al (1997) Perforin/granzyme-dependent and independent mechanisms are both important for the development of graft-versus-host disease after murine bone marrow transplantation. J Clin Invest 100(4):904–911

Gunn MD et al (1998) A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. Proc Natl Acad Sci USA 95(1):258–263

Hanash AM, Levy RB (2005) Donor CD4+ CD25+ T cells promote engraftment and tolerance following MHC-mismatched hematopoietic cell transplantation. Blood 105(4):1828–1836
Hoffmann P et al (2002) Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. J Exp Med 196(3):389–399
Hoffmann P et al (2004) Large-scale *in vivo* expansion of polyclonal human CD4(+)CD25high regulatory T cells. Blood 104(3):895–903
Hori S, Nomura T, Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. Science 299(5609):1057–1061
Huehn J, Hamann A (2005) Homing to suppress: address codes for Treg migration. Trends Immunol 26(12):632–636
Huehn J et al (2004) Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells. J Exp Med 199(3):303–313
Ivanov II, Zhou L, Littman DR (2007) Transcriptional regulation of Th17 cell differentiation. Semin Immunol 19(6):409–417
Iwata M et al (2004) Retinoic acid imprints gut-homing specificity on T cells. Immunity 21(4):527–538
Izcue A, Coombes JL, Powrie F (2006) Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. Immunol Rev 212:256–271
Jasperson LK et al (2009) Inducing the tryptophan catabolic pathway, indoleamine 2, 3-dioxygenase (IDO), for suppression of graft-versus-host disease (GVHD) lethality. Blood 114(24):5062–5070
Jones SC, Murphy GF, Korngold R (2003) Post-hematopoietic cell transplantation control of graft-versus-host disease by donor CD425 T cells to allow an effective graft-versus-leukemia response. Biol Blood Marrow Transplant 9(4):243–256
Kang SG et al (2007) Vitamin A metabolites induce gut-homing FoxP3+ regulatory T cells. J Immunol 179(6):3724–3733
Kappel LW et al (2009) IL-17 contributes to CD4-mediated graft-versus-host disease. Blood 113(4):945–952
Kim CH (2006) Migration and function of FoxP3+ regulatory T cells in the hematolymphoid system. Exp Hematol 34(8):1033–1040
Kim CH (2008) Regulation of FoxP3 regulatory T cells and Th17 cells by retinoids. Clin Dev Immunol 2008:416910
Kim CH (2009) Migration and function of Th17 cells. Inflamm Allergy Drug Targets 8(3): 221–228
Kim YM et al (2003) Graft-versus-host disease can be separated from graft-versus-lymphoma effects by control of lymphocyte trafficking with FTY720. J Clin Invest 111(5):659–669
Kim TD et al (2008) Organ-derived dendritic cells have differential effects on alloreactive T cells. Blood 111(5):2929–2940
Lee JH, Kang SG, Kim CH (2007) FoxP3+ T cells undergo conventional first switch to lymphoid tissue homing receptors in thymus but accelerated second switch to nonlymphoid tissue homing receptors in secondary lymphoid tissues. J Immunol 178(1):301–311
Leeuwenberg JF et al (1988) Effects of tumor necrosis factor on the interferon-gamma-induced major histocompatibility complex class II antigen expression by human endothelial cells. Eur J Immunol 18(9):1469–1472
Lim HW, Broxmeyer HE, Kim CH (2006) Regulation of trafficking receptor expression in human forkhead box P3+ regulatory T cells. J Immunol 177(2):840–851
Lim HW et al (2008) Human Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3+ regulatory T cells. J Immunol 180(1):122–129
Liu W et al (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 203(7):1701–1711
Lowsky R et al (2005) Protective conditioning for acute graft-versus-host disease. N Engl J Med 353(13):1321–1331

Mangan PR et al (2006) Transforming growth factor-beta induces development of the T(H)17 lineage. Nature 441(7090):231–234
Martin PJ et al (1990) A retrospective analysis of therapy for acute graft-versus-host disease: initial treatment. Blood 76(8):1464–1472
Martin PJ et al (1991) A retrospective analysis of therapy for acute graft-versus-host disease: secondary treatment. Blood 77(8):1821–1828
Mielke S et al (2007) Reconstitution of FOXP3+ regulatory T cells (Tregs) after CD25-depleted allotransplantation in elderly patients and association with acute graft-versus-host disease. Blood 110(5):1689–1697
Miossec P, Korn T, Kuchroo VK (2009) Interleukin-17 and type 17 helper T cells. N Engl J Med 361(9):888–898
Miura Y et al (2004) Association of Foxp3 regulatory gene expression with graft-versus-host disease. Blood 104(7):2187–2193
Mora JR et al (2005) Reciprocal and dynamic control of CD8 T cell homing by dendritic cells from skin- and gut-associated lymphoid tissues. J Exp Med 201(2):303–316
Morgan ME et al (2005) Expression of FOXP3 mRNA is not confined to CD4+ CD25+T regulatory cells in humans. Hum Immunol 66(1):13–20
Mucida D et al (2007) Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science 317(5835):256–260
Murai M et al (2003) Peyer's patch is the essential site in initiating murine acute and lethal graft-versus-host reaction. Nat Immunol 4(2):154–160
Nadal E et al (2007) Increased frequencies of CD4(+)CD25(high) T(regs) correlate with disease relapse after allogeneic stem cell transplantation for chronic myeloid leukemia. Leukemia 21(3):472–479
Nash RA et al (1992) Acute graft-versus-host disease: analysis of risk factors after allogeneic marrow transplantation and prophylaxis with cyclosporine and methotrexate. Blood 80(7):1838–1845
Nguyen VH et al (2007) In vivo dynamics of regulatory T-cell trafficking and survival predict effective strategies to control graft-versus-host disease following allogeneic transplantation. Blood 109(6):2649–2656
Norton J, Sloane JP (1991) ICAM-1 expression on epidermal keratinocytes in cutaneous graft-versus-host disease. Transplantation 51(6):1203–1206
Oswald-Richter K et al (2004) HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. PLoS Biol 2(7):E198
Ouyang W, Kolls JK, Zheng Y (2008) The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity 28(4):454–467
Pabst C et al (2007) The graft content of donor T cells expressing gamma delta TCR+ and CD4+ foxp3+ predicts the risk of acute graft versus host disease after transplantation of allogeneic peripheral blood stem cells from unrelated donors. Clin Cancer Res 13(10): 2916–2922
Pandiyan P et al (2007) CD4+ CD25+ Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. Nat Immunol 8(12):1353–1362
Picker LJ et al (1993) Control of lymphocyte recirculation in man. II. Differential regulation of the cutaneous lymphocyte-associated antigen, a tissue-selective homing receptor for skin-homing T cells. J Immunol 50(3):1122–1136
Piguet PF et al (1987) Tumor necrosis factor/cachectin is an effector of skin and gut lesions of the acute phase of graft-vs.-host disease. J Exp Med 166(5):1280–1289
Przepiorka D et al (2001) Chronic graft-versus-host disease after allogeneic blood stem cell transplantation. Blood 98(6):1695–1700
Qin HY et al (2006) A novel mechanism of regulatory T cell-mediated down-regulation of autoimmunity. Int Immunol 18(7):1001–1015
Reddy P (2003) Pathophysiology of acute graft-versus-host disease. Hematol Oncol 21(4): 149–161

Rezvani K et al (2006) High donor FOXP3-positive regulatory T-cell (Treg) content is associated with a low risk of GVHD following HLA-matched allogeneic SCT. Blood 108(4):1291–1297

Rieger K et al (2006) Mucosal FOXP3+ regulatory T cells are numerically deficient in acute and chronic GvHD. Blood 107(4):1717–1723

Sackstein R (2006) A revision of Billingham's tenets: the central role of lymphocyte migration in acute graft-versus-host disease. Biol Blood Marrow Transplant 12(1 Suppl 1):2–8

Sakaguchi S, Sakaguchi N, Asano M et al (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 155(3): 1151–1164

Sanchez J et al (2004) Kinetic of regulatory CD25high and activated CD134+ (OX40) T lymphocytes during acute and chronic graft-versus-host disease after allogeneic bone marrow transplantation. Br J Haematol 126(5):697–703

Sato W, Aranami T, Yamamura T (2007) Cutting edge: human Th17 cells are identified as bearing CCR2+CCR5- phenotype. J Immunol 178(12):7525–7529

Schambach F et al (2007) Activation of retinoic acid receptor-alpha favours regulatory T cell induction at the expense of IL-17-secreting T helper cell differentiation. Eur J Immunol 37(9):2396–2399

Seddiki N et al (2006) Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med 203(7):1693–1700

Seidel MG et al (2006) Expression of the putatively regulatory T-cell marker FOXP3 by CD4(+) CD25+ T cells after pediatric hematopoietic stem cell transplantation. Haematologica 91(4):566–569

Sharma MD et al (2009) Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes. Blood 113(24):6102–6111

Shevach EM (2002) CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2(6):389–400

Shimizu J et al (2002) Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. Nat Immunol 3(2):135–142

Shlomchik WD (2007) Graft-versus-host disease. Nat Rev Immunol 7(5):340–352

Shlomchik WD et al (1999) Prevention of graft versus host disease by inactivation of host antigen-presenting cells. Science 285(5426):412–415

Shresta S et al (1998) How do cytotoxic lymphocytes kill their targets? Curr Opin Immunol 10(5):581–587

Siewert C et al (2007) Induction of organ-selective CD4+ regulatory T cell homing. Eur J Immunol 37(4):978–989

Sigmundsdottir H, Butcher EC (2008) Environmental cues, dendritic cells and the programming of tissue-selective lymphocyte trafficking. Nat Immunol 9(9):981–987

Sigmundsdottir H et al (2007) DCs metabolize sunlight-induced vitamin D3 to 'program' T cell attraction to the epidermal chemokine CCL27. Nat Immunol 8(3):285–293

Singh SP et al (2008) Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. J Immunol 180(1):214–221

Socie G, Blazar BR (2009) Acute graft-versus-host disease: from the bench to the bedside. Blood 114(20):4327–4336

Stanzani M et al (2004) CD25 expression on donor CD4+ or CD8+ T cells is associated with an increased risk for graft-versus-host disease after HLA-identical stem cell transplantation in humans. Blood 103(3):1140–1146

Szanya V et al (2002) The subpopulation of CD4+ CD25+ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. J Immunol 169(5):2461–2465

Takahashi T et al (1998) Immunologic self-tolerance maintained by CD25+ CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int Immunol 10(12):1969–1980

Takahashi T et al (2000) Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med 192(2):303–310

Tang Q, Bluestone JA (2008) The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol 9(3):239–244

Taylor PA, Noelle RJ, Blazar BR (2001) CD4(+)CD25(+) immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. J Exp Med 193(11): 1311–1318

Taylor PA, Lees CJ, Blazar BR (2002) The infusion of ex vivo activated and expanded CD4(+) CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. Blood 99(10):3493–3499

Taylor PA et al (2004) L-Selectin(hi) but not the L-selectin(lo) CD4+25+ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. Blood 104(12):3804–3812

Thornhill MH et al (1991) Tumor necrosis factor combines with IL-4 or IFN-gamma to selectively enhance endothelial cell adhesiveness for T cells. The contribution of vascular cell adhesion molecule-1-dependent and – independent binding mechanisms. J Immunol 146(2):592–598

Thornton AM, Shevach EM (1998) CD4+ CD25+ immunoregulatory T cells suppress polyclonal T cell activation *in vivo* by inhibiting interleukin 2 production. J Exp Med 188(2):287–296

Trenado A et al (2003) Recipient-type specific CD4+ CD25+ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. J Clin Invest 112(11):1688–1696

Varona R et al (2006) CCR6 regulates the function of alloreactive and regulatory CD4+ T cells during acute graft-versus-host disease. Leuk Lymphoma 47(8):1469–1476

Via CS, Finkelman FD (1993) Critical role of interleukin-2 in the development of acute graft-versus-host disease. Int Immunol 5(6):565–572

Via CS et al (1996) A major role for the Fas pathway in acute graft-versus-host disease. J Immunol 157(12):5387–5393

Vignali DA, Collison LW, Workman CJ (2008) How regulatory T cells work. Nat Rev Immunol 8(7):523–532

von Andrian UH, Mempel TR (2003) Homing and cellular traffic in lymph nodes. Nat Rev Immunol 3(11):867–878

Wang J et al (2007) Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. Eur J Immunol 37(1):129–138

Wang R et al (2008) Identification of a regulatory T cell specific cell surface molecule that mediates suppressive signals and induces Foxp3 expression. PLoS One 3(7):e2705

Wei S, Kryczek I, Zou W (2006) Regulatory T-cell compartmentalization and trafficking. Blood 108(2):426–431

Weisdorf D et al (1991) Risk factors for acute graft-versus-host disease in histocompatible donor bone marrow transplantation. Transplantation 51(6):1197–1203

Welniak LA, Blazar BR, Murphy WJ (2007) Immunobiology of allogeneic hematopoietic stem cell transplantation. Annu Rev Immunol 25:139–170

Wolf D et al (2007) Regulatory T-cells in the graft and the risk of acute graft-versus-host disease after allogeneic stem cell transplantation. Transplantation 83(8):1107–1113

Wysocki CA et al (2005a) Leukocyte migration and graft-versus-host disease. Blood 105 (11):4191–4199

Wysocki CA et al (2005b) Critical role for CCR5 in the function of donor CD4+ CD25+ regulatory T cells during acute graft-versus-host disease. Blood 106(9):3300–3307

Xiao S et al (2008) Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. J Immunol 181(4):2277–2284

Xu H et al (2008) IDO: a double-edged sword for T(H)1/T(H)2 regulation. Immunol Lett 121(1):1–6

Xun CQ et al (1994) Effect of total body irradiation, busulfan-cyclophosphamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft-versus-host disease in H-2-incompatible transplanted SCID mice. Blood 83(8):2360–2367

Yamaguchi T et al (2007) Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. Immunity 27(1):145–159

Yi T et al (2008) Absence of donor Th17 leads to augmented Th1 differentiation and exacerbated acute graft-versus-host disease. Blood 112(5):2101–2110

Yi T et al (2009) Reciprocal differentiation and tissue-specific pathogenesis of Th1, Th2, and Th17 cells in graft-versus-host disease. Blood 114(14):3101–3112

Zhang Y et al (2005) Host-reactive CD8+ memory stem cells in graft-versus-host disease. Nat Med 11(12):1299–1305

Part IV
Clinical Hematology

The Chemokine Network in Acute Myelogenous Leukemia: Molecular Mechanisms Involved in Leukemogenesis and Therapeutic Implications

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Contents

Abstract Acute myelogenous leukemia (AML) is a bone marrow disease in which the leukemic cells show constitutive release of a wide range of CCL and CXCL chemokines and express several chemokine receptors. The AML cell release of various chemokines is often correlated and three release clusters have been

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identified: CCL2–4/CXCL1/8, CCL5/CXCL9–11, and CCL13/17/22/24/CXCL5. CXCL8 is the chemokine usually released at highest levels. Based on their overall constitutive release profile, patients can be classified into distinct subsets that differ in their T cell chemotaxis towards the leukemic cells. The release profile is modified by hypoxia, differentiation status, pharmacological interventions, and T cell cytokine responses. The best investigated single chemokine in AML is CXCL12 that binds to CXCR4. CXCL12/CXCR4 is important in leukemogenesis through regulation of AML cell migration, and CXCR4 expression is an adverse prognostic factor for patient survival after chemotherapy. Even though AML cells usually release high levels of several chemokines, there is no general increase of serum chemokine levels in these patients and the levels are also influenced by patient age, disease status, chemotherapy regimen, and complicating infections. However, serum CXCL8 levels seem to partly reflect the leukemic cell burden in AML. Specific chemokine inhibitors are currently being developed, although redundancy and pleiotropy of the chemokine system are obstacles in drug development.

Abbreviations

AML	Acute myelogenous leukemia
ATRA	All-trans retinoic acid
FAK	Focal adhesion kinase
HIF	Hypoxia inducible factor
IL	Interleukin
ITD	Internal tandem duplication
MMP	Matrix metalloproteases
MOZ	Monocyte zinc finger
NK	Natural killer
TNF	Tumor necrosis factor
VLA	Very late antigen

1 Introduction

Chemokines are involved in the regulation of cell survival, proliferation, and trafficking (Bendall 2005; Tanaka et al. 2005; Balkwill 2004; Rosenkilde and Schwartz 2004; Allavena et al. 2005). All these processes are important in the development of acute myelogenous leukemia (AML), an aggressive bone marrow malignancy, and the AML patients are often subclassified according to their prognosis, that is, risk of primary therapy resistance or later disease relapse (Estey and Döhner 2006; Harris et al. 1999). Primary human AML cells usually show constitutive release of a wide range of chemokines and have several chemokine receptors on the cell's surface. These chemokine/chemokine receptor expression patterns are probably important for both disease development (i.e., leukemogenesis) and chemosensitivity (i.e., response to therapy).

2 Primary Human AML Cells Often Show Constitutive Chemokine Release

The AML cells as well as their neighboring bone marrow stromal cells produce survival- and growth-regulatory cytokines, including chemokines belonging to both the CCL and CXCL subclasses (Bruserud et al. 2007; Balkwill 2004). The remaining normal hematopoietic cells and bone marrow infiltrating immunocompetent cells also release chemokines and express a wide range of chemokine receptors (Bruserud et al. 2007; Laurence 2006; Moser and Loetscher 2002; Christopherson and Hromas 2001; Mantovani et al. 2004; Broxmeyer 2008; Homey et al. 2002; Qin et al. 1998; Kim 2006; Honczarenko et al. 2006; Cignetti et al. 2003; Jin et al. 2007). For example, the stromal cells (1) release CCL2, CCL4, CCL5, CCL20, CXCL8, CXCL12, and CX3CL1 and (2) express CCR1, CCR7, CCR9, CXCR4, CXCR5, and CXCR6 (Honczarenko et al. 2006). The chemokines thereby constitute a bidirectional interacting network between leukemic and nonleukemic cells.

2.1 Constitutive Chemokine Release by Primary Human AML Cells

A broad constitutive chemokine release profile is often detected in AML, but the profile shows both qualitative and quantitative differences between individual patients (Bruserud et al. 2007). The release of different chemokines is often correlated so that distinct release clusters can be identified: (1) CCL2–4/CXCL1/8, (2) CCL5/CXCL9–11 (possibly also CCL23), and (3) CCL13/17/22/24/CXCL5 (possibly also CXCL6). This means that individual patients usually show either high or low release for all chemokines within the same cluster; the molecular mechanisms behind this coordinated release are not known, but common transcriptional regulation seems to be important for at least the CCL2–4/CXCL1/8 cluster. It should be emphasized that there is a wide variation in the release of each chemokine between individual patients, and this is illustrated by the summary of the overall results presented in Table 1 (Bruserud et al. 2007). For many patients, additional chemokines are also released. Individual AML patients can therefore be subclassified based on their overall chemokine release profile (Bruserud et al. 2007):

- A relatively large group (approximately 20–30% of patients) shows undetectable or low levels of most chemokines with decreased *in vitro* chemotaxis of immunocompetent cells towards the AML cells
- The majority of the other patients shows relatively high release for the CCL2–4/CXCL1/8 chemokine cluster eventually in combination with other single chemokines
- The remaining minority shows high CCL2–4/CXCL1/8 levels and in addition high levels of the CCL13/17/22/24/CXCL5 and CCL5/CXCL9–11 clusters

Table 1 The variation in constitutive chemokine release by primary human AML cells derived from different patients; a summary of the results for 68 consecutive patients (adapted from Bruserud et al. (2007))

Chemokine	Patients with detectable release			
	Detection limit (pg/ml)	Number	Median level (pg/ml)	Variation range
CCL1	4	43	617	6.1–>1,000
CCL2	**5**	**59**	**1,720**	**7.3–5,722**
CCL3	**75**	**53**	**5,209**	**102–13,836**
CCL4	**150**	**53**	**2,902**	**151–26,420**
CCL5	1.2	67	236	2.0–2,288
CCL7	27	40	751	42–935
CCL13	5	45	47.4	5.4–238
CCL17	11	31	114	11.7–3,704
CCL20	4.5	49	128	4.7–1,393
CCL22	**260**	**41**	**1,088**	**263– > 4,000**
CCL23	9	28	19.8	12.9–23.3
CCL24	13	39	394	15.2–5,080
CCL26	4.5	15	13.1	5.1–82.8
CCL28	7	14	7.8	7.1–41.7
CXCL1	**60**	**50**	**7,196**	**67–13,610**
CXCL4	**0.05**	**59**	**0.34**	**0.05–8.7**
CXCL5	**40**	**42**	**1,067**	**41–>20,000**
CXCL6	3.2	30	53.9	4.2–2,328
CXCL8	**30**	**64**	**22,720**	**42–33,720**
CXCL9	60	30	822	78–15,815
CXCL10	**60**	**48**	**1,782**	**64.2–24,906**
CXCL11	40	28	168	40.7–3,980
CXCL12	18	10	37.5	28.5–623
CXCL13	3.5	38	189	3.5–1,303

Primary human AML cells were cultured for 48 h before chemokine levels were determined in the supernatants. The results are presented as the concentration in pg/ml, the exception being CXCL4 that is presented as IU/ml. Chemokines marked in bold showed detectable levels for at least 40 of the 68 patients and a median level >1,000 pg/ml. CCL11, CCL21, CCL25, and CCL27 were also investigated but detectable release was not observed for any patient

No single chemokine or chemokine cluster showed any correlations with clinical or biological AML cell characteristics (i.e., morphology, membrane molecule expression, genetic abnormalities) in this study (Bruserud et al. 2007). Taken together, these observations therefore suggest that the chemokine release profile rather than single chemokines should be examined in biological studies of human AML.

2.2 Modulation of the Constitutive Chemokine Release

Even though the constitutive chemokine release by primary human AML cells seems to be carefully controlled and appears in clusters (see Sect. 2.1), several

factors can modulate the release profile. However, this modulation will often be similar for chemokines within the same cluster.

2.2.1 Differentiation Induction

Cytokines, chemotherapeutics, all-trans retinoic acid (ATRA), and vitamin D_3 can induce differentiation of AML blasts towards a dendritic cell phenotype (Bruserud and Gjertsen 2000). This phenotype includes altered chemokine levels with high release of CCL17 and CCL22 similar to normal dendritic cells but usually without effects on other chemokines in the CCL13/17/22/24/CXCL5 cluster (Olsnes et al. 2008).

2.2.2 Tissue Oxygenation

The oxygen pressure (pO_2) in human bone marrow is decreased and is estimated to be 50–55 mmHg (atmospheric pO_2 corresponding to 140–160 mmHg) (Harrison et al. 2002; Cummins and Taylor 2005). The most important hypoxia-responsive transcription factor is HIF-1, which is known to directly regulate CXCL12 and CXCR4 expression and increase the expression of proangiogenic CXCL8 (Wenger et al. 2005; Hirota and Semenza 2006; Lisy and Peet 2008). Exposure of primary human AML cells to hypoxia increases HIF-1 levels and the release of several other chemokines especially within the CCL2–4/CXCL1/8 cluster (Hatfield, unpublished data).

2.2.3 Pharmacological Interventions

NF-κB is important for transcriptional regulation of several chemokines and can be targeted by specific inhibitors and by the proteasomal inhibitor bortezomib. NF-κB expression by primary AML cells correlates with mRNA and protein levels of the CCL2–4/CXCL1/8 release cluster, an observation further supporting that common transcriptional regulation is important for this clustering (Bruserud et al. 2007). The specific inhibitor BMS345541 decreases the release of these chemokines, and bortezomib also decreases these chemokines, except for CXCL8, which is increased (Bruserud et al. 2007; Olsnes et al. 2009). The most likely explanation for the CXCL8 discrepancy between these two drugs is that bortezomib has additional effects and not only inhibits NF-κB.

The protein kinase C δ agonist PEP005 induces growth inhibition and apoptosis of primary human AML cells together with increased release of several T cell chemotactic chemokines, especially chemokines within the CCL2–4/CXCL1/8 and CCL5/CXCL9–11 clusters (Olsnes et al. 2009). Such a combination of direct antileukemic effects and immunostimulation through increased local T cell recruitment is uncommon and may result in synergistic antileukemic effects.

The drug JTE-607 inhibits the release of several cytokines. In a murine AML model, it had an antileukemic effect comparable to the maximum tolerable dose of cytarabine and was associated with decreased CXCL8 levels (Uesato et al. 2006). These decreased CXCL8 levels may be caused by decreased constitutive AML cell release, but it is not known whether the antileukemic activity depends on this effect. Furthermore, all-trans retinoic acid (ATRA) is mandatory in the treatment of acute promyelocytic leukemia (APL) (Bruserud and Gjertsen 2000), and it is also tried in the treatment of other AML variants (Bruserud et al. 2006). In vitro studies have shown that ATRA or vitamin D_3 derivatives can increase CXCR1 expression (Zahn et al. 1997) as well as decrease CXCL8 release by myeloid leukemia cells (Dubois et al. 1994; Srivastava and Ambrus 2004). The overall chemokine release profiles were not characterized in these pharmacological studies, and it is not known whether other chemokines within the CCL2–4/CXCL1/8 cluster also are affected. It is not known whether such effects contribute to the disease-stabilization observed for a subset of AML patients receiving ATRA-based palliative therapy (Bruserud et al. 2006).

2.2.4 Nonleukemic Stromal Cells

The bidirectional crosstalk between primary human AML cells and their neighboring nonleukemic stromal cells alters AML cell release of both CCL and CXCL chemokines. This has been observed both for fibroblasts, osteoblasts, and endothelial cells, but the wide variation in chemokine release between individual patients is maintained even in the presence of stromal cells (Bruserud et al. 2004; Olsnes et al. 2008; Glenjen et al. 2003, 2004; Hatfield et al. 2006, 2009).

Leukemic cells from most AML patients show a high constitutive release of CXCL8 (Bruserud et al. 2007). The cytokine crosstalk between AML cells and microvascular endothelial cells, fibroblasts, or osteoblasts increases the local CXCL8 levels and the proliferation of these nonleukemic stromal cells (Bruserud et al. 2004; Hatfield et al. 2006, 2008, 2009; Ryningen et al. 2005). Furthermore, CXCL8-binding receptors are expressed both by AML cells and endothelial cells (Bruserud et al. 2007; Tobler et al. 1993; Strieter et al. 1995; Xie 2001); AML-derived CXCL8 may therefore be involved both in autocrine and paracrine circuits in the bone marrow microenvironment.

The high levels of proangiogenic CXCL8 may contribute to the increased microvessel density in AML bone marrow (de Bont et al. 2001; Hatfield et al. 2005). Primary AML cells show constitutive release of several additional proangiogenic mediators, although there are both qualitative and quantitative differences between individual patients (Bruserud et al. 2007; Lee et al. 2007a). Among these proangiogenic nonchemokine mediators are angiopoietin-1 (Ang-1), Ang-2, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), interleukin-6 (IL-6), matrix metalloproteases (MMPs), and IL-1. The AML cells also show constitutive release of antiangiogenic molecules, including CXCL9–11, IL-12, and thrombospondin (Bruserud et al. 2004;

Hatfield et al. 2005). However, the constitutive release of antiangiogenic CXCL9–11 is lower than the CXCL8 release (Bruserud et al. 2007), showing that at least for the angioregulatory chemokines the balance is in favor of angiogenesis.

2.2.5 Cellular Immune Responses

Leukemia-directed T cell reactivity is important for the antileukemic effect of allogeneic stem cell transplantation (Ersvaer et al. 2007a, b; Paczesny et al. 2010; Engelhardt and Crowe 2010; Kittan and Hildebrandt 2010; Löffler et al. 2010), and antileukemic immune effects may also be important in patients receiving conventional chemotherapy (Ersvær et al. 2007b). IFN-γ is released at high levels by activated T cells derived from healthy individuals (Bruserud et al. 1993), patients receiving allogeneic (Bruserud et al. 1993) and autologous (Wendelbo et al. 2004a) stem cell transplantation, and patients with severe chemotherapy-induced cytopenia (Wendelbo et al. 2004b); IFN-γ reduces the constitutive release of proangiogenic CXCL8 and increases antiangiogenic CXCL9–11 by primary human AML cells (Ersvaer et al. 2007a). Antiangiogenic effects may thereby become a part of antileukemic T cell reactivity.

3 Chemokine Receptors on Primary Human AML Cells

We previously examined CCR1–5 and CXCR1–4 expression at the protein level by primary human AML cells (Bruserud et al. 2007). These nine receptors can bind 18 CCL (CCL2–5, 7, 8, 11–17, 22–24, 26, 28) and 11 CXCL chemokines (CXCL1–3,5–12) (Bendall 2005; Tanaka et al. 2005; Balkwill 2004; Rosenkilde and Schwartz 2004; Allavena et al. 2005). When comparing the expression for the total AML cell populations, the chemokine receptor expression varied considerably: (1) CCR3 and CXCR1 showed low levels for all patients; (2) CCR5, CXCR2, and CXCR3A generally showed intermediate expression; and (3) CCR1, CCR2, CCR4, and CXCR4 showed relatively high expression (Bruserud et al. 2007).

We have now analyzed the associations among the expression of these nine chemokine receptors, genetic abnormalities, and differentiation status for the patients included in our previous study (Bruserud et al. 2007). No clustering of receptor expression was observed similar to the chemokine release. Surprisingly, these additional studies demonstrated that Flt3-internal tandem duplication (ITD) was associated with decreased CCR1 and CXCR4 expression in these relatively old patients with severe leukemization (Fig. 1). Furthermore, high CCR1 and CCR2 expression among total AML cells was also associated with morphological signs of monocytic differentiation (Fig. 1), and the expression of these two receptors was also inversely correlated with expression of the CD34 stem cell marker (data not shown).

The chemokine receptor expression varies within the AML cell population in each patient (Bruserud et al. 2007). We observed increased expression of several

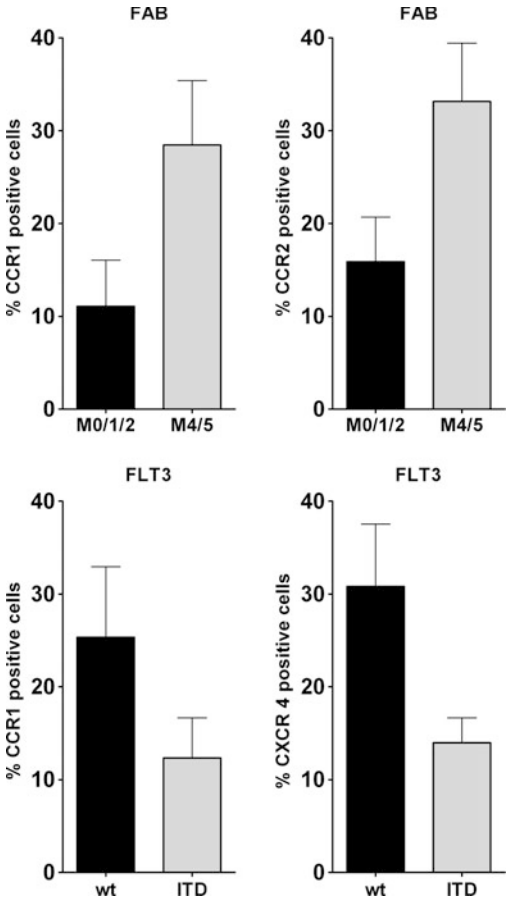

Fig. 1 Chemokine receptor expression by primary human AML cells (Bruserud et al. 2007). Receptor expression was analyzed by flow cytometry and the results are presented as the percentage of positive cells. Morphological signs of differentiation was classified according to the French–American–British classification, and monocytic differentiation is then classified as M4/M5. The figure shows that primary AML cells with morphological signs of monocytic differentiation have an increased expression of CCR1 (Mann-Whitney, U-test, $p = 0.03$) and CCR2 ($p = 0.023$) (*upper part*). Furthermore, Flt3-ITD, which is a genetic abnormality associated with adverse prognosis, is associated with decreased expression of CCR1 ($p = 0.048$) and CXCR4 ($p = 0.047$) (*lower part*)

receptors by the leukemic $CD34^+$ subset (often only a minority) compared with the $CD34^-$ cell subset in the same patient (Bruserud et al. 2007). This was most clearly seen for CCR5 and CXCR3A and also for CCR1, CCR2, and CCR4, and this difference was not altered by *in vitro* exposure to hematopoietic growth factors.

Our previous studies have demonstrated that primary human AML cells show constitutive release of several chemokines, and as described earlier, the leukemic cells also express the receptors for these chemokines. Even though autocrine circuits are formed thereby, this receptor/ligand expression is not associated with autocrine proliferation. Furthermore, for most patients exogenous chemokines do not affect spontaneous or cytokine-dependent AML cell proliferation either, although altered proliferation is observed for a minority of patients, with growth enhancement being most common. For these exceptional patients, altered proliferation was observed also for the more immature clonogenic cells. Thus, most chemokines have only minor direct effects on growth regulation in the AML cells.

4 The CXCL12/CXCR4 System in Human AML

4.1 CXCR4 and CXCL12 Expression in AML Bone Marrow

CXCR4 expression is detectable at the mRNA level for the large majority of patients (Cignetti et al. 2003), and studies at the protein level have confirmed this (Bruserud et al. 2007; Möhle et al. 1998) with an average percentage of $CXCR4^+$ cells comparable to normal $CD34^+$ hematopoietic cells (Möhle et al. 1998). However, the variation between patients is much wider than the variation between normal $CD34^+$ cells from healthy individuals (Möhle et al. 1998). Some studies suggest that CXCR4 expression is strongest for AML cells with a monocytic phenotype and in APL (Löffler et al. 2010; Cignetti et al. 2003; Möhle et al. 2000); in case of monocytic differentiation, the increased CXCR4 expression seems to be a part of a more complex phenotype with increased expression of other chemokine receptors (CCR1, CCR2), costimulatory molecules (CD40, CD86), death receptors (TNFR1, TNFR2, Fas), and several adhesion molecules (Burger and Kipps 2006; Brouwer et al. 2001).

Detectable release of CXCL12, the only CXCR4 ligand, by primary human AML cells is seen only for a minority of patients (Bruserud et al. 2007; Cignetti et al. 2003). Less than half of the patients show detectable mRNA expression (Cignetti et al. 2003), and when investigating CXCL12 release by *in vitro* cultured AML cells, low but detectable levels were seen only for ten out of 68 patients (Table 1) (Bruserud et al. 2007). Thus, autocrine CXCR4/CXCL12 loops are probably uncommon in human AML. The major source of CXCL12 in AML bone marrow seems to be the constitutive release by various stromal cells (Brouwer et al. 2001), including osteoblasts in endosteal stem cell niches and endothelial cells in vascular niches. At these sites, CXCL12 may facilitate survival, self-renewal, and localization of normal stem cells and possibly also leukemic cells (Broxmeyer 2008).

4.2 Biological Effects of CXCR4-Initiated Signaling in AML

CXCR4 cooperates with the very late antigen (VLA)-4 and other integrins, the hyaluronan receptor CD44, and possibly also the surface sialomucin podocalyxin in the regulation of AML cell adhesion and migration (Burger 2009; Burger and Bürkle 2007; Burger et al. 2003; Riccioni et al. 2006; Voermans et al. 2002; Jin et al. 2006; Tavernier-Tardy et al. 2009). Both CXCR4 and VLA-4 seem to mediate resistance to cytarabine-induced apoptosis through these interactions (Burger et al. 2003). CXCR4 is thereby a part of a larger functional entity that seems important for anchoring AML cells to the bone marrow and for possibly facilitating their migration to stem cell niches, with the maintenance of their immature phenotype (Rombouts et al. 2004). Finally, the hypoxic bone marrow microenvironment

causes upregulation of CXCR4 expression, failure to internalize CXCR4 in response to CXCL12 ligation, and altered shedding of soluble CXCR4 (Fiegl et al. 2009). Thus, CXCR4 is not only important for migration and differentiation but also for the adaption to the hypoxic microenvironment.

Cellular microparticles are submicron vesicles that are shed from the plasma membrane, and $CXCR4^+$ microparticles are detected both in the peripheral blood and bone marrow plasma of healthy individuals as well as AML patients (Kalinkovich et al. 2006). $CXCR4^+$ microparticles are increased in AML and express CD45, whereas most microparticles in healthy individuals express CD41. In vitro studies have demonstrated that these microparticles can transfer biologically active CXCL12 to AML cells.

Whether CXCR4 is important for AML cell migration outside the human bone marrow remains controversial. One study described an association between the *CXCR4 G801A* gene polymorphism and extramedullary disease (Dommange et al. 2006), but this association was not observed in another study (Ponziani et al. 2008). Other chemokines may also influence extramedullary AML cell trafficking since another study described a correlation between extramedullary AML and coexpression of CCL2/CCR2 (Cignetti et al. 2003). Finally, two relatively small studies including only 11 and 21 patients, respectively, showed conflicting results with regard to whether CXCR4 is important for engraftment of human AML cells in NOD/SCID mice (Tavor et al. 2004; Monaco et al. 2004a, b).

4.3 CXCR4/CXCL12 Has a Prognostic Impact in Human AML

CXCR4 expression is significantly increased in AML cells derived from patients with Flt3-ITD (Rombouts et al. 2004) (Fig. 1). Therefore, to investigate the prognostic impact of CXCR4 expression independent of the Flt3-ITD effect, Konoplev et al. (Konoplev et al. 2007) analysed survival after chemotherapy for patients with normal karyotype and no Flt3-ITD. CXCR4 was expressed by the AML cells for 70% of the patients. The initial complete remission rate did not differ, but patients with $CXCR4^+$ leukemic cells had decreased event-free and overall long-term survival. This was later confirmed by others (Spoo et al. 2007). Taken together, these results suggest that high CXCR4 expression has an adverse prognostic impact independent of Flt3-ITD.

A small study investigated the prognostic impact of CXCR4, VLA-4, and focal adhesion kinase (FAK) in AML (Tavernier-Tardy et al. 2009). CXCR4 cooperates with VLA-4 in AML cell migration (Burger et al. 2003), and FAK is also important in cell adhesion by regulating multiple signal-transduction pathways (Sieg et al. 2000). The expression of each single molecule was associated with decreased overall survival, but the strongest impact was observed for patients showing combined expression of at least two or all three markers. These observations suggest that the adverse prognosis associated with CXCR4 reflects the impact of a more complex phenotype.

4.4 CXCR4 as a Possible Therapeutic Target in Human AML

Several CXCR4 inhibitors have been developed (Zeng et al. 2006, 2009; Tavor et al. 2008; Liesveld et al. 2007; Nervi et al. 2009; Li et al. 2008), and clinical studies have demonstrated that CXCR4 inhibition can be used for mobilization of peripheral blood stem cells (Calandra et al. 2010). However, the use of CXCR4 inhibitors in AML therapy is also supported by several experimental observations:

- CXCR4 inhibitors decrease chemotaxis of human AML cell lines against CXCL12 or bone marrow stromal cells (Zeng et al. 2009; Tavor et al. 2008; Li et al. 2008) and inhibit transmigration of AML cells through stromal and endothelial cell monolayers (Liesveld et al. 2007)
- Bone marrow stromal cells have a protective effect against chemotherapy-induced apoptosis in primary human AML cells, and CXCR4-antagonists decrease this protection and enhance the proapoptotic effects of the cytotoxic drug cytarabine (Zeng et al. 2009; Tavor et al. 2008). This effect is possibly mediated through inhibition of CXCL12-mediated activation of ERK and AKT (Zeng et al. 2009; Tavor et al. 2008). The chemosensitizing effect has also been detected *in vivo* in murine AML models (Nervi et al. 2009)
- Flt3-ITD activates CXCR4 signaling, and CXCR4 inhibition then increases the sensitivity of Flt3-ITD$^+$ leukemic cells to proapoptotic Flt3 inhibitors (Zeng et al. 2009)
- CXCR4 inhibition induces differentiation and proliferation arrest in U937 AML cells, possibly through inhibition of CXCL12-dependent elastase that is constitutively expressed (Tavor et al. 2008)
- Studies in murine models have shown that CXCR4 inhibitors decrease bone marrow homing and thereby mobilize both normal and leukemic cells from the bone marrow to the blood (Zeng et al. 2009; Nervi et al. 2009)

These effects were observed with the inhibitory polypeptide RCP168 or the second-generation small molecule reversible CXCR4 inhibitors AMD3465 or AMD3100. Similar effects can also be induced by berberine, an isoquinoline derivative that inhibits stromal cell release of CXCL12 (Li et al. 2008).

5 Leukemogenesis Through Transcriptional Regulation in the Chemokine System

MEIS1 is a HOX cofactor that contributes to leukemogenesis in AML (Bruserud et al. 2006). Results from an animal AML model demonstrated that MEIS1 upregulated Flt3 and occupied regulatory sequences of the Flt3 as well as the *CCL3, CCL4,* and *CXCL4* genes (Argiropoulos et al. 2008). CCL3 was then important for the marrow-repopulating activity of AML cells, suggesting that altered chemokine expression is involved in leukemogenesis.

The NF-κB transcription factor is another regulator of chemokine expression in AML cells (Bruserud et al. 2007), and it is also regarded as important in leukemogenesis (Olsnes et al. 2009; Reikvam et al. 2009). Furthermore, the histone acetyltransferase Monocyte zinc finger (MOZ) increases CXCL8 release through a direct interaction with the p65 subunit of the NF-κB complex; MOZ can also be rearranged in human AML, and the fusion protein formed with the coactivator CREB binding protein (CBP) is then important in leukemogenesis (Bruserud et al. 2006). Thus, AML-associated genetic abnormalities that are regarded as important contributors in leukemogenesis may mediate their leukemogenic effects through the chemokine regulator NF-κB and thereby increase expression of CXCL8 and possibly also other NF-κB regulated chemokines (Bruserud et al. 2007). The same mechanism may be operative for translocations involving the RUNX1 or AML-1 transcription factor because MOZ also interacts with this transcription factor and thereby increase CCL3 expression (Mrózek et al. 2004; Bristow and Shore 2003), another member of the CCL2–4/CXCL1/8 release cluster (see Sect. 2.1).

Even though the molecular details behind transcriptional regulation of chemokine expression in primary human AML cells are largely unknown, the regulation seems to involve several transcription factors (NF-κB, MOZ, RUNX1) that can be involved in AML-associated genetic abnormalities. These results suggest that several chemokines and not only CXCL12/CXCR4 may contribute in leukemogenesis.

6 Chemokine Serum Levels in AML

Even though AML cells show constitutive release of several chemokines, there is no general increase in the serum levels of these mediators in untreated patients. However, increased CXCL8 serum levels are detected for patients with untreated disease and especially for patients with monocyte AML variants (Hsu et al. 2002; Liu et al. 1999; Negaard et al. 2009). These levels normalize when patients achieve complete hematological remission (Hsu et al. 2002), but increased levels can later be detected as a part of the acute phase reaction during febrile neutropenia and especially in patients with septicemia or septic shock (Ostermann et al. 1994; Bruserud et al. 1996; Schönbohn et al. 1995). Furthermore, increased levels of CCL2, CXCL10 (only younger patients), and CXCL12 have also been detected in patients with untreated disease (Kalinkovich et al. 2006; Mazur et al. 2007; Olsnes et al. 2006). The increased levels of total CXCL12 are then accompanied by decreased levels of the functional noncleaved form (Kalinkovich et al. 2006). Neither CCL2 nor CXCL10 levels are affected by chemotherapy (Mazur et al. 2007; Olsnes et al. 2006), and increased CXCL10 levels persist even after induction of hematological remission (Olsnes et al. 2006). Finally, CCL17 levels are decreased and CCL18 levels are not altered in patients with untreated disease, and CCL17 levels will decrease further following intensive chemotherapy and during febrile neutropenia (Olsnes et al. 2006; Struyf et al. 2003). We therefore

conclude that systemic chemokine levels in patients with untreated AML are determined by several factors and not only by the constitutive AML cell release.

7 Chemotaxis of Immunocompetent Cells in Human AML

7.1 T Cell Chemotaxis

Experimental studies have demonstrated that T cells are able to migrate towards primary AML cells, but the T cell chemotaxis varies between patients and is decreased for those patients who do not show constitutive chemokine release (see Sect. 2.1) (Bruserud et al. 2007). CCL5 and CXCL10 contribute to the chemotaxis but it is likely that other chemokines are also involved because AML cells often show constitutive release of several T cell chemotactic chemokines, including CCL1–5/7/11/13/17/20–22 and CXCL6/8–12 (Bruserud et al. 2007; Olsnes et al. 2006). Especially, CXCL8 is usually released at high levels for most patients and normal $CD4^+$ as well as $CD8^+$ T cells migrate after stimulation with CXCL8 (Ward et al. 1998). However, the T cell population in untreated AML patients is abnormal with increased numbers of circulating T cells, cytotoxic $CD3^+56^+$ T cells are frequently oligoclonal and in a higher state of activation with abnormal gene expression profiles, and these T cells are unable to form effective immune synapses with autologous AML cells (Le Dieu et al. 2009). The T cell population normalizes after remission induction, but it is not known whether chemotaxis towards AML cells is abnormal in untreated AML or after achievement of complete hematological remission.

7.2 Chemotaxis of Regulatory T Cells

T lymphocytes generally express several chemokine receptors (Ward et al. 1998; Campbell et al. 2003; Muller et al. 2002), and regulatory T (Treg) cells show a distinct expression profile and seem to be highly attracted by CCR4 ligation (CCL17, CCL22) and by ligation of CCR8 (CCL1) that seems to be more selectively expressed on Treg cells (Engelhardt and Crowe 2010; Iellem et al. 2001). Primary AML cells can be induced to differentiate towards AML-dendritic cells with high release of CCL17 and CCL22 (Olsnes et al. 2008; Köhler et al. 2000), and several normal immunocompetent T cell subsets ($CD4^+$ and $CD8^+$ T cells, Treg cells) show increased migration towards such cells (Olsnes et al. 2008). However, even in the presence of CCL17/CCL22-neutralizing antibodies, the number of migrating cells was higher than for primary AML cells, an observation clearly demonstrating that other chemokines are also involved.

Treg cells seem to have a stronger migration towards dendritic AML cells than other T cell subsets (Olsnes et al. 2008). Animal studies suggest that circulating Treg cells express CXCR4, CCR2, CCR5, CCR6, and CCR9 in addition to CCR 4 and CCR8 (see above) (Lee et al. 2007b; Yi et al. 2006). This expression profile shows that Treg chemotaxis will depend on the overall local chemokine network, although it is known that certain chemokines may have a predominant role in certain clinical situations (Haas et al. 2007; Olkhanud et al. 2009).

The frequency of circulating Treg cells is increased in patients with untreated AML (Szczepansky et al. 2009). The increased levels persist after remission induction, an observation suggesting that this is a disease-induced and chemoresistant immunomodulation with a biological impact even after achievement of disease control. The constitutive AML cell release of several Treg-recruiting chemokines may lead to colocalization of leukemic and Treg cells. This may explain the adverse prognostic impact of high pretherapy levels of circulating Treg cells (Szczepansky et al. 2009).

7.3 Chemotaxis of Monocytes

Previous *in vitro* studies have shown that the migration of normal monocytes towards primary human AML cells differs between patients (Legdeur et al. 1997, 2001). For a minority of patients this migration is low, but for most patients a high degree of migration is observed and CCL2 (a ligand of the CCR2 receptor) is the most important single chemotactic chemokine. These observations are also consistent with the studies of constitutive chemokine release by primary human AML cells (see Sect. 2.1); CCL2 is released at relatively high levels for most patients but often together with other CCR2 ligands or monocyte-chemotactic chemokines (Bruserud et al. 2007). The high monocyte migration towards AML cells is therefore expected. Furthermore, CD40 ligation of AML cells will increase the release of chemotactic chemokines, including CCL5 and CXCL8, and thereby increase monocyte as well as T and NK cell chemotaxis (Costello et al. 2000). The recruited monocytes may then have cytotoxic effects against AML cells, but the CCL2 effect is limited to monocyte migration without any effect on the antileukemic cytotoxicity of the recruited cells (Legdeur et al. 1997, 2001). Alternatively, the recruited monocytes may represent an AML-stimulating mechanism through their release of proangiogenic mediators (Dimberg 2010).

7.4 Chemotaxis of Natural Killer Cells

Natural killer (NK) cells can also mediate antileukemic activity, and they express several chemokine receptors (ligands given in parenthesis), including CCR1 (CCL2/3/5/7/14–16/23), CCR4 (CCL17/22), CCR6 (CCL20), CCR7 (CCL19/21)

CXCR1 (CXCL6/7/8), CXCR3 (CXCL9–11), CXCR4 (CXCL12), CXCR6 (CXCL16), and CX_3CR1 (CX3CL1) (Maghasachi 2010). The expression of the individual receptors may differ between various NK cell subsets and may also be dependent on the activation status of the cells, as described in detail by Maghasachi (Maghasachi 2010). However, it can be seen that many of these ligands are constitutively released by AML cells (see Table 1 and Sect. 2.1), including the chemokines within the CCL2–4/CXCL1/8 cluster that are released for most patients. One would therefore expect NK cells to migrate towards primary human AML cells.

8 Chemokine-Mediated Suppression of Normal Hematopoiesis

AML is a bone marrow disease, and leukemia-induced bone marrow failure is an important clinical characteristic (Estey and Döhner 2006). Previous experimental studies have shown that several chemokines have direct or indirect effects on normal hematopoiesis. First, several chemokines seem to directly inhibit normal hematopoiesis, including CCL3, CXCL4, CXCL5, and CXCL8 (Dimberg 2010; Lambert et al. 2007). For CCL chemokines, the suppression seems to be linked to a specific molecular motif that was identified in the inhibitory CCL3 but not in the noninhibitory CCL5 (Ottersbach et al. 2006). Residues within this region probably contribute to the binding of other inhibitory chemokines to their receptors (Bondue et al. 2002; Lecomte-Raclet et al. 2000), and based on the comparison of CCL3 and CCL5, the inhibition is probably mediated through the formation of a helical turn preceding the first β-strand in CCL3 (Ottersbach et al. 2006). Second, CCL18 and CCL2 seem to stimulate hematopoiesis (Broxmeyer 2008), but this is probably an indirect effect mediated through growth factor release from neighboring monocytes (Wimmer et al. 2006). Finally, injection of chemokines into mice has demonstrated that several of these mediators affect normal hematopoiesis, but it is not known whether direct or indirect effects are most important. Dose-dependent *in vivo* suppression has then been demonstrated for CCL2, CCL3, CCL19, CCL20, CXCL4, CXCL5, CXCL8, CXCL9, and XCL1 (Broxmeyer et al. 2006). Several chemokine combinations showed synergistic inhibitory effects, and suppression of hematopoiesis was associated with accelerated recovery in response to the toxic effects of cytarabine.

Taken together, these observations demonstrate that chemokine-induced suppression of hematopoiesis seems more common than stimulation, and Table 1 shows that several of the suppressing chemokines are constitutively released at high levels by primary human AML cells, especially the cluster I chemokines CCL2–4/CXCL5/8. Constitutive chemokine release may thereby contribute to the disease-associated bone marrow failure in AML. Chemokine effects on normal hematopoiesis thus differ from leukemic hematopoiesis where most chemokines either have no or weak enhancing effects on AML cell proliferation (see Sect. 3). Finally, enhancement of chemokine-mediated myelosuppression may represent a

possible therapeutic strategy for myeloprotection in patients receiving intensive anticancer therapy, and this may be achieved through pharmacological inhibition of the chemokine-degrading decoy receptors (Bonecchi et al. 2010).

9 Concluding Remarks

Hanahan et al. (Hanahan and Weinberg 2000) suggested that malignant diseases have six fundamental hallmarks, and Mantovani et al. (Mantovani 2009) later suggested that cancer-associated inflammation is a seventh hallmark. The local chemokine network can affect all these hallmarks in human AML (discussed in detail earlier):

- The three characteristics associated with cancer cell proliferation are limitless replicative potential, self-sufficiency in growth signals, and insensitivity to antigrowth signals. Chemokines can affect the growth of primary human AML cells directly, but in our available *in vitro* models, this is observed only for a minority of patients (Bruserud et al. 2007) and paracrine mechanisms are more likely to be involved. The importance of paracrine circuits is also supported by experimental studies describing expression of several chemokine receptors, by bone marrow stromal cells, including CCR1, CCR7, CCR9, CXCR4–6 (Honczarenko et al. 2006). Among these receptors, CCR1 binds at least three of the chemokines secreted by AML cells, namely CCL3, CCL5, and CCL13. When cultured in serum-free medium, the stromal cells release several chemokines that can bind to receptors expressed by the AML cells, including CCL2, CCL4, CCL5, CCL20, CXCL8, CXCL12, and CXC3L1 (Honczarenko et al. 2006).
- *Evading apoptosis*. Inhibition of chemokine signaling (i.e., CXCR4 antagonists) potentiates proapoptotic chemotherapy effects (Fig. 2).
- *Sustained angiogenesis*. Several proangiogenic chemokines are constitutively released by the AML cells at high levels for almost all patients, while antiangiogenic chemokines are released at lower levels (Bruserud et al. 2007; Dimberg 2010).
- *Tissue evasion and metastasis*. The CXCL12/CXCR4 system and CCL2/CCR2 are important for AML cell migration and thereby for bone marrow infiltration.
- *Inflammatory microenvironment*. The clinical importance of an inflammatory microenvironment in AML is generally accepted only for patients receiving allogeneic stem cell transplantation, and for these patients chemokine-targeting therapy is now considered as an immunomodulatory treatment. However, the balance between various immunocompetent cells may then be of particular importance (Szczepansky et al. 2009), especially in patients treated with allogeneic stem cell transplantation where this balance is essential for induction of antileukemic T cell reactivity vs. the risk of GVHD due to excess proinflammatory reactivity towards host antigens (Kittan and Hildebrandt 2010).

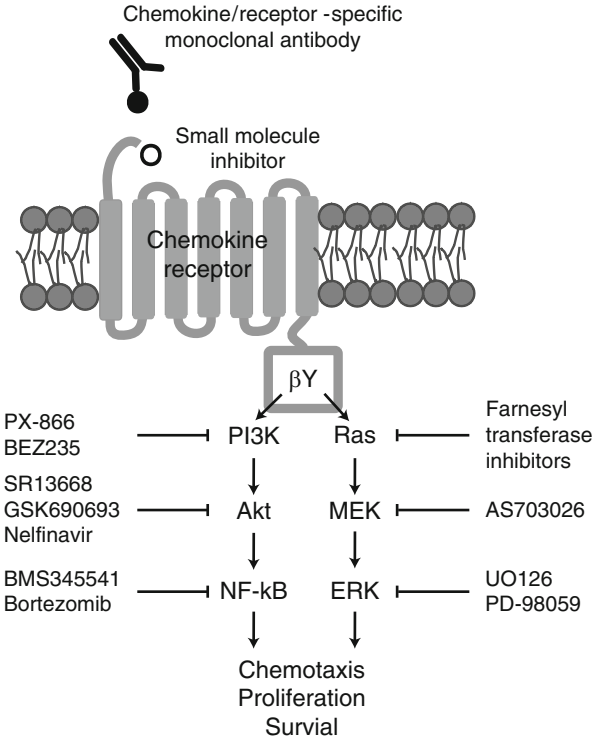

Fig. 2 Pharmacological targeting of the chemokine system, a general overview of possible strategies (for additional references see (Bruserud et al. 2007; Olsnes et al. 2009; Zebisch et al. 2007; Hatfield et al. 2005; Tavor et al. 2008; Calandra et al. 2010)). First, specific inhibition can be achieved through specific targeting of chemokines or chemokine receptors. Monoclonal antibodies can then be used either to neutralize chemokines or to inhibit chemokine receptors. Small molecule inhibitors can also effectively target specific chemokine receptors, and nonfunctional chemokines can inhibit chemokine oligomerization or interfere with the binding of chemokines to the extracellular matrix or cell surfaces. Second, chemokine-induced signaling downstream of the chemokine receptors can be altered by specific inhibitors; this last strategy will not be specific for chemokine-initiated signaling because other receptors may also affect the same pathways. Various pathways can then be involved in the intracellular signaling downstream of the receptor, and these are coupled to heterotrimeric G-proteins (subunits α, β, and γ, only the two last functional units being presented in the figure). Specific inhibitors have been developed against several of these mediators, as indicated in the figure, including protein kinase inhibitors, farnesyl transferase inhibitors, and proteasome inhibitors. Here we show only two of the possible signaling pathways that can be activated after receptor ligation and activation of the functional G-protein unit (the βγ-dimer). These inhibitors represent experimental tools, but several of them are also used in clinical therapy, for example, bortezomib and farnesyl transferase inhibitors. (βγ, the βγ subunit of the heterotrimeric G-protein; PI3K, phosphoinositide 3-kinase; Akt, serine/threonine-specific protein kinase and also known as protein kinase B; NF-κB, nuclear factor-κB; Ras, small GTPase; MEK, MAPK/ERK kinase and also known as MAPK kinase; ERK, extracellular-signal regulated kinase and also known as mitogen-activated protein kinase or MAPK)

Thus, therapeutic targeting of the chemokine system would interfere with fundamental cancer cell characteristics or important paracrine mechanisms. This therapeutic targeting of the chemokine system can include specific agents directed against the chemokines or their receptors (Fig. 2). However, downstream intracellular signaling involves several pathways, and specific inhibitors of intracellular mediators are now considered for cancer treatment and would then be expected to modulate chemokine effects on the malignant cells. Modulation of the chemokine network may also become useful in patients receiving allogeneic stem cell transplantation and possibly also when immunotherapy is tried in combination with conventional chemotherapy. Finally, analysis of AML-associated chemokine mRNA expression in bone marrow may become useful in monitoring of treatment responses and detection of minimal residual disease. A recent study described that mRNA expression of CCL23 together with six other disease markers could be used for early detection of AML relapse (Steinbach et al. 2006). Thus, a better understanding of the chemokine system in human AML will probably lead to the development of new diagnostic tools as well as new therapeutic strategies.

References

Allavena P, Marchesi F, Mantovani A (2005) The role of chemokines and their receptors in tumor progression and invasion: potential new targets of biological therapy. Curr Cancer Ther Rev 1:81–92

Argiropoulos B, Palmqvist L, Yung E, Kuchenbauer F, Heuser M, Sly LM, Wan A, Krystal G, Humphries RK (2008) Linkage of Meis1 leukemogenic activity to multiple downstream effectors including Trib2 and Ccl3. Exp Hematol 36:845–859

Balkwill F (2004) Cancer and the chemokine network. Nat Rev Cancer 4:540–550

Bendall L (2005) Chemokines and their receptors in disease. Histol Histopathol 20:907–926

Bondue A, Jao SC, Blanpain C, Parmentier M, LiWang PJ (2002) Characterization of the role of the N-loop of MIP-1 beta in CCR5 binding. Biochemistry 41:13548–13555

Bonecchi R, Savino B, Borroni EM, Mantovani A, Locati M (2010) Chemokine decoy receptors: structure-function and biological properties. Curr Top Microbiol Immunol, DOI 10.1007/82_2010_19

Bristow CA, Shore P (2003) Transcriptional regulation of the human MIP-1alpha promoter by RUNX1 and MOZ. Nucleic Acids Res 31:2735–2744

Brouwer RE, Hoefnagel J, Borger van Der Burg B, Jedema I, Zwinderman KH, Starrenburg IC, Kluin-Nelemans HC, Barge RM, Willemze R, Falkenburg JH (2001) Expression of co-stimulatory and adhesion molecules and chemokine or apoptosis receptors on acute myeloid leukaemia: high CD40 and CD11a expression correlates with poor prognosis. Br J Haematol 115:298–308

Broxmeyer HE (2008) Chemokines in hematopoiesis. Curr Opin Hematol 15:49–58

Broxmeyer HE, Pelus LM, Kim CH, Hangoc G, Cooper S, Hromas R (2006) Synergistic inhibition *in vivo* of bone marrow myeloid progenitors by myelosuppressive chemokines and chemokine-accelerated recovery of progenitors after treatment of mice with Ara-C. Exp Hematol 34:1069–1077

Bruserud Ø, Gjertsen BT (2000) New strategies for the treatment of acute myelogenous leukemia: differentiation induction – present use and future possibilities. Stem Cells 18:157–165

Bruserud Ø, Hamann W, Patel S, Ehninger G, Schmidt H, Pawelec G (1993) IFN-gamma and TNF-alpha secretion by $CD4^+$ and $CD8^+$ TCR $\alpha\beta^+$ T-cell clones derived early after allogeneic bone marrow transplantation. Eur J Haematol 51:73–79

Bruserud Ø, Halstensen A, Peen E, Solberg CO (1996) Serum levels of adhesion molecules and cytokines in patients with acute leukaemia. Leuk Lymphoma 23:423–430

Bruserud Ø, Ryningen A, Wergeland L, Glenjen NI, Gjertsen BT (2004) Osteoblasts increase proliferation and release of pro-angiogenic interleukin 8 by native human acute myelogenous leukemia blasts. Haematologica 89:391–402

Bruserud Ø, Stapnes C, Tronstad KJ, Ryningen A, Anensen N, Gjertsen BT (2006) Protein lysine acetylation in normal and leukaemic haematopoiesis: HDACs as possible therapeutic targets in adult AML. Expert Opin Ther Targets 10:51–68

Bruserud Ø, Ryningen A, Olsnes AM, Stordrange L, Øyan AM, Kalland KH, Gjertsen BT (2007) Subclassification of patients with acute myelogenous leukemia based on chemokine responsiveness and constitutive chemokine release by their leukemic cells. Haematologica 92:332–341

Burger JA (2009) CXCR4 in acute myelogenous leukemia (AML): when too much attraction is bad for you. Leuk Res 33:747–748

Burger JA, Bürkle A (2007) The CXCR4 chemokine receptor in acute and chronic leukaemia: a marrow homing receptor and potential therapeutic target. Br J Haematol 137:288–296

Burger JA, Kipps TJ (2006) CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. Blood 107:1761–1767

Burger JA, Spoo A, Dwenger A, Burger M, Behringer D (2003) CXCR4 chemokine receptors (CD184) and α4β1 integrins mediate spontaneous migration of human $CD34^+$ progenitors and acute myeloid leukaemia cells beneath marrow stromal cells (pseudoemperipolesis). Br J Haematol 122:579–589

Calandra G, Bridger G, Fricker S (2010) CXCR4 in clinical hematology. Curr Top Microbiol Immunol, DOI 10.1007/82_2010_26

Campbell DJ, Kim CH, Butcher EC (2003) Chemokines in the systemic organization of immunity. Immunol Rev 195:58–71

Christopherson K, Hromas R (2001) Chemokine regulation of normal and pathologic immune responses. Stem Cells 19:388–396

Cignetti A, Vallario A, Roato I, Circosta P, Strola G, Scielzo C, Allione B, Garetto L, Caligaris-Cappio F, Ghia P (2003) The characterization of chemokine production and chemokine receptor expression reveals possible functional cross-talks in AML blasts with monocytic differentiation. Exp Hematol 31:495–503

Costello RT, Mallet F, Chambost H, Sainty D, Arnoulet C, Gastaut JA, Olive D (2000) Acute myeloid leukaemia triggering via CD40 induces leukocyte chemoattraction and cytotoxicity against allogenic or autologous leukemic targets. Leukemia 14:123–128

Cummins EP, Taylor CT (2005) Hypoxia-responsive transcription factors. Pflugers Arch 450:363–371

de Bont ES, Vellenga E, Molema G, van Wering E, de Leij LF, Kamps WA (2001) A possible role for spontaneous interleukin-8 production by acute myeloid leukemic cells in angiogenesis related processes: work in progress. Med Pediatr Oncol 37:511–517

Dimberg A (2010) Chemokines in angiogenesis. Curr Top Microbiol Immunol, DOI 10.1007/82_2010_21

Dommange F, Cartron G, Espanel C, Gallay N, Domenech J, Benboubker L, Ohresser M, Colombat P, Binet C, Watier H, Herault O, GOELAMS Study Group (2006) CXCL12 polymorphism and malignant cell dissemination/tissue infiltration in acute myeloid leukemia. FASEB J 20:1913–1915

Dubois C, Schlageter MH, de Gentile A, Balitrand N, Toubert ME, Krawice I, Fenaux P, Castaigne S, Najean Y, Degos L (1994) Modulation of IL-8, IL-1 beta, and G-CSF secretion by all-trans retinoic acid in acute promyelocytic leukemia. Leukemia 8:1750–1757

Engelhardt BE, Crowe JE (2010) Homing in an acute graft-versus-host disease: Tissue-specific T regulatory and Th 17 cells. Curr Top Microbiol Immunol, DOI 10.1007/82_2010_24

Ersvaer E, Skavland J, Ulvestad E, Gjertsen BT, Bruserud Ø (2007a) Effects of interferon gamma on native human acute myelogenous leukaemia cells. Cancer Immunol Immunother 56:13–24

Ersvær E, Olsnes AM, Bruserud Ø (2007b) The immunological dilemma: cellular innate and adaptive immune response versus acute myelogenous leukemia. Open Hematol J 1:1–14

Estey E, Döhner H (2006) Acute myeloid leukaemia. Lancet 368:1894–1907

Fiegl M, Samudio I, Clise-Dwyer K, Burks JK, Mnjoyan Z, Andreeff M (2009) CXCR4 expression and biologic activity in acute myeloid leukemia are dependent on oxygen partial pressure. Blood 113:1504–1512

Glenjen N, Hovland R, Wergeland L, Wendelbo Ø, Ernst P, Bruserud Ø (2003) The angioregulatory phenotype of native human acute myelogenous leukemia cells: influence of karyotype, Flt3 abnormalities and differentiation status. Eur J Haematol 71:163–173

Glenjen N, Ersvaer E, Ryningen A, Bruserud Ø (2004) In vitro effects of native human acute myelogenous leukemia blasts on fibroblasts and osteoblasts. Int J Cancer 111:858–867

Haas J, Schopp L, Storch-Hagenlocher B, Fritzsching B, Jacobi C, Milkova L, Fritz B, Schwarz A, Suri-Payer E, Hensel M, Wildemann B (2007) Specific recruitment of regulatory T-cells into the CSF in lymphomatous and carcinomatous meningitis. Blood 111:761–766

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100:57–70

Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, Lister TA, Bloomfield CD (1999) World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. J Clin Oncol 17:3835–3849

Harrison JS, Rameshwar P, Chang V, Bandari P (2002) Oxygen saturation in the bone marrow of healthy volunteers. Blood 99:394

Hatfield KJ, Olsnes AM, Gjertsen BT, Bruserud Ø (2005) Antiangiogenic therapy in acute myelogenous leukemia: targeting of vascular endothelial growth factor and interleukin 8 as possible antileukemic strategies. Curr Cancer Drug Targets 5:229–248

Hatfield K, Ryningen A, Corbascio M, Bruserud Ø (2006) Microvascular endothelial cells increase proliferation and inhibit apoptosis of native human acute myelogenous leukemia blasts. Int J Cancer 119:2313–2321

Hatfield KJ, Hovland R, Øyan AM, Kalland KH, Ryningen A, Gjertsen BT, Bruserud Ø (2008) Release of angiopoietin-1 by primary human acute myelogenous leukemia cells is associated with mutations of nucleophosmin, increased by bone marrow stromal cells and possibly antagonized by high systemic angiopoietin-2 levels. Leukemia 22:287–293

Hatfield K, Øyan AM, Ersvaer E, Kalland KH, Lassalle P, Gjertsen BT, Bruserud Ø (2009) Primary human acute myeloid leukaemia cells increase the proliferation of microvascular endothelial cells through the release of soluble mediators. Br J Haematol 144:53–68

Hirota K, Semenza GL (2006) Regulation of angiogenesis by hypoxia-inducible factor 1. Crit Rev Oncol Hematol 59:15–26

Homey B, Muller A, Zlotnik A (2002) Chemokines: agents for the immunotherapy of cancer? Nat Rev Immunol 2:175–184

Honczarenko M, Le Y, Swierkowski M, Ghiran I, Glodek AM, Silberstein LE (2006) Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors. Stem Cells 24:1030–1041

Hsu HC, Lee YM, Tsai WH, Jiang ML, Ho CH, Ho CK, Wang SY (2002) Circulating levels of thrombopoietic and inflammatory cytokines in patients with acute myeloblastic leukemia and myelodysplastic syndrome. Oncology 63:64–69

Iellem A, Mariani M, Lang R, Recalde H, Panina-Bordignon P, Sinigaglia F, D'Ambrosio D (2001) Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by $CD4^{+}CD25^{+}$ regulatory T cells. J Exp Med 194:847–853

Jin L, Hope KJ, Zhai Q, Smadja-Joffe F, Dick JE (2006) Targeting of CD44 eradicates human acute myeloid leukemic stem cells. Nat Med 12:1167–1174

Jin JO, Park HY, Kim JW, Park JI, Hong YS, Min do S, Kwak JY (2007) Phosphatidic acid induces the differentiation of human acute promyelocytic leukemic cells into dendritic cell-like. J Cell Biochem 100:191–203

Kalinkovich A, Tavor S, Avigdor A, Kahn J, Brill A, Petit I, Goichberg P, Tesio M, Netzer N, Naparstek E, Hardan I, Nagler A, Resnick I, Tsimanis A, Lapidot T (2006) Functional CXCR4-expressing microparticles and SDF-1 correlate with circulating acute myelogenous leukemia cells. Cancer Res 66:11013–11020

Kim CH (2006) Migration and function of FoxP3+ regulatory T cells in the hematolymphoid system. Exp Hematol 34:1033–1040

Kittan NA, Hildebrandt GC (2010) The chemokine system – a possible therapeutic target in acute graft versus host disease. Curr Top Microbiol Immunol, DOI 10.1007/82_2010_23

Köhler T, Plettig R, Wetzstein W, Schmitz M, Ritter M, Mohr B, Schaekel U, Ehninger G, Bornhäuser M (2000) Cytokine-driven differentiation of blasts from patients with acute myelogenous and lymphoblastic leukemia into dendritic cells. Stem Cells 18:139–147

Konoplev S, Rassidakis GZ, Estey E, Kantarjian H, Liakou CI, Huang X, Xiao L, Andreeff M, Konopleva M, Medeiros LJ (2007) Overexpression of CXCR4 predicts adverse overall and event-free survival in patients with unmutated FLT3 acute myeloid leukemia with normal karyotype. Cancer 109:1152–1156

Lambert MP, Rauova L, Bailey M, Sola-Visner MC, Kowalska MA, Poncz M (2007) Platelet factor 4 is a negative autocrine *in vivo* regulator of megakaryopoiesis: clinical and therapeutic implications. Blood 110:1153–1160

Laurence AD (2006) Location, movement and survival: the role of chemokines in haematopoiesis and malignancy. Br J Haematol 132:255–267

Le Dieu R, Taussig DC, Ramsay AG, Mitter R, Miraki-Moud F, Fatah R, Lee AM, Lister TA, Gribben JG (2009) Peripheral blood T cells in AML patients at diagnosis have abnormal phenotype and genotype and form defective immune synapses with AML blasts. Blood 114:3909–3916

Lecomte-Raclet L, Rholam M, Alemany M, Lazar N, Simenel C, Delepierre M, Han ZC, Cohen P, Caen JP (2000) Dual structural requirements for multilineage hematopoietic-suppressive activity of chemokine-derived peptides. Biochemistry 39:9612–9622

Lee CY, Tien HF, Hu CY, Chou WC, Lin LI (2007a) Marrow angiogenesis-associated factors as prognostic biomarkers in patients with acute myelogenous leukaemia. Br J Cancer 97:877–882

Lee JH, Kang SG, Kim CH (2007b) FoxP3+ T cells undergo conventional first switch to lymphoid tissue homing receptors in thymus but accelerated second switch to nonlymphoid tissue homing receptors in secondary lymphoid tissues. J Immunol 178:301–311

Legdeur MC, Beelen RH, Schuurhuis GJ, Broekhoven MG, van de Loosdrecht AA, Tekstra J, Langenhuijsen MM, Ossenkoppele GJ (1997) A functional study on the migration of human monocytes to human leukemic cell lines and the role of monocyte chemoattractant protein-1. Leukemia 11:1904–1908

Legdeur MC, Broekhoven MG, Schuurhuis GJ, Beelen RH, Ossenkoppele GJ (2001) Monocyte-chemoattractant-protein-1-mediated migration of human monocytes towards blasts from patients with acute myeloid leukemia. Cancer Immunol Immunother 50:16–22

Li H, Guo L, Jie S, Liu W, Zhu J, Du W, Fan L, Wang X, Fu B, Huang S (2008) Berberine inhibits SDF-1-induced AML cells and leukemic stem cells migration via regulation of SDF-1 level in bone marrow stromal cells. Biomed Pharmacother 62:573–578

Liesveld JL, Bechelli J, Rosell K, Lu C, Bridger G, Phillips G 2nd, Abboud CN (2007) Effects of AMD3100 on transmigration and survival of acute myelogenous leukemia cells. Leuk Res 31:1553–1563

Lisy K, Peet DJ (2008) Turn me on: regulating HIF transcriptional activity. Cell Death Differ 15:642–649

Liu J, Zeng H, Zhang Y (1999) Study on the expression of interleukin-8 and its receptors in acute leukemia. Zhonghua Xue Ye Xue Za Zhi 20:24–26

Löffler J, Mezger M, Ok M, Oliver Morton C, Einsele H (2010) Genetic polymorphisms in the cytokine and chemokine system – their possible importance in allogeneic stem cell transplantation. Curr Top Microbiol Immunol, DOI 10.1007/82_2010_22

Maghasachi AA (2010) Role of chemokines in the biology of natural killer cells. Curr Top Microbiol Immunol, DOI 10.1007/82_2010_20

Mantovani A (2009) Cancer: inflaming metastasis. Nature 457:36–37

Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M (2004) The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 25:677–686

Mazur G, Wróbel T, Butrym A, Kapelko-Słowik K, Poreba R, Kuliczkowski K (2007) Increased monocyte chemoattractant protein 1 (MCP-1/CCL-2) serum level in acute myeloid leukemia. Neoplasma 54:285–289

Möhle R, Bautz F, Rafii S, Moore MA, Brugger W, Kanz L (1998) The chemokine receptor CXCR-4 is expressed on $CD34^+$ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. Blood 91:4523–4530

Möhle R, Schittenhelm M, Failenschmid C, Bautz F, Kratz-Albers K, Serve H, Brugger W, Kanz L (2000) Functional response of leukaemic blasts to stromal cell-derived factor-1 correlates with preferential expression of the chemokine receptor CXCR4 in acute myelomonocytic and lymphoblastic leukaemia. Br J Haematol 110:563–572

Monaco G, Belmont JW, Konopleva M, Andreeff M, Tavor S, Petit I, Kollet O, Lapidot T (2004a) Correlation between CXCR4 and homing or engraftment of acute myelogenous leukemia. Cancer Res 64:6832

Monaco G, Konopleva M, Munsell M, Leysath C, Wang RY, Jackson CE, Korbling M, Estey E, Belmont J, Andreeff M (2004b) Engraftment of acute myeloid leukemia in NOD/SCID mice is independent of CXCR4 and predicts poor patient survival. Stem Cells 22:188–201

Moser B, Loetscher P (2002) Lymphocyte traffic control by chemokines. Nat Immunol 2:123–128

Mrózek K, Heerema NA, Bloomfield CD (2004) Cytogenetics in acute leukemia. Blood Rev 18:115–136

Muller G, Hopken UE, Stein H, Lipp M (2002) Systemic immunoregulatory and pathogenic functions of homeostatic chemokine receptors. J Leukoc Biol 72:1–8

Negaard HF, Iversen N, Bowitz-Lothe IM, Sandset PM, Steinsvik B, Østenstad B, Iversen PO (2009) Increased bone marrow microvascular density in haematological malignancies is associated with differential regulation of angiogenic factors. Leukemia 23:162–169

Nervi B, Ramirez P, Rettig MP, Uy GL, Holt MS, Ritchey JK, Prior JL, Piwnica-Worms D, Bridger G, Ley TJ, DiPersio JF (2009) Chemosensitization of acute myeloid leukemia (AML) following mobilization by the CXCR4 antagonist AMD3100. Blood 113:6206–6214

Olkhanud PB, Baatar D, Bodogai M, Hakim F, Gress R, Anderson RL, Deng J, Xu M, Briest S, Biragyn A (2009) Breast cancer lung metastasis requires expression of chemokine receptor CCR4 and regulatory T cells. Cancer Res 69:5996–6004

Olsnes AM, Motorin D, Ryningen A, Zaritskey AY, Bruserud Ø (2006) T lymphocyte chemotactic chemokines in acute myelogenous leukemia (AML): local release by native human AML blasts and systemic levels of CXCL10 (IP-10), CCL5 (RANTES) and CCL17 (TARC). Cancer Immunol Immunother 55:830–840

Olsnes AM, Ryningen A, Ersvaer E, Bruserud Ø (2008) In vitro induction of a dendritic cell phenotype in primary human acute myelogenous leukemia (AML) blasts alters the chemokine release profile and increases the levels of T cell chemotactic CCL17 and CCL22. J Interferon Cytokine Res 28:297–310

Olsnes AM, Ersvær E, Ryningen A, Paulsen K, Hampson P, Lord JM, Gjertsen BT, Kristoffersen EK, Bruserud Ø (2009) The protein kinase C agonist PEP005 increases NF-kappaB expression, induces differentiation and increases constitutive chemokine release by primary acute myeloid leukaemia cells. Br J Haematol 145:761–774

Ostermann H, Rothenburger M, Mesters RM, van de Loo J, Kienast J (1994) Cytokine response to infection in patients with acute myelogenous leukaemia following intensive chemotherapy. Br J Haematol 88:332–337

Ottersbach K, McLean J, Isaacs NW, Graham GJ (2006) A310 helical turn is essential for the proliferation-inhibiting properties of macrophage inflammatory protein-1 alpha (CCL3). Blood 107:1284–1291

Paczesny S, Hanauer D, Sun Y, Reddy P (2010) New perspectives on the biology of acute GVHD. Bone Marrow Transplant 15:1–11

Ponziani V, Mannelli F, Bartalucci N, Gianfaldoni G, Leoni F, Antonioli E, Guglielmelli P, Ciolli S, Bosi A, Vannucchi AM (2008) No role for CXCL12-G801A polymorphism in the development of extramedullary disease in acute myeloid leukemia. Leukemia 22:669–671

Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B, Mackay CR (1998) The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J Clin Invest 101:746–754

Reikvam H, Olsnes AM, Gjertsen BT, Ersvaer E, Bruserud Ø (2009) Nuclear factor-κB signaling – a contributor in leukemogenesis and a target for pharmacological intervention in human acute myelogenous leukemia. Crit Rev Oncog 15:1–41

Riccioni R, Calzolari A, Biffoni M, Senese M, Riti V, Petrucci E, Pasquini L, Cedrone M, Lo-Coco F, Diverio D, Foà R, Peschle C, Testa U (2006) Podocalyxin is expressed in normal and leukemic monocytes. Blood Cells Mol Dis 37:218–225

Rombouts EJ, Pavic B, Löwenberg B, Ploemacher RE (2004) Relation between CXCR-4 expression, Flt3 mutations, and unfavorable prognosis of adult acute myeloid leukemia. Blood 104:550–557

Rosenkilde MM, Schwartz TW (2004) The chemokine system – a major regulator of angiogenesis in health and disease. APMIS 112:481–495

Ryningen A, Wergeland L, Glenjen N, Gjertsen BT, Bruserud Ø (2005) In vitro crosstalk between fibroblasts and native human acute myelogenous leukemia (AML) blasts via local cytokine networks results in increased proliferation and decreased apoptosis of AML cells as well as increased levels of proangiogenic Interleukin 8. Leuk Res 29:185–196

Schönbohn H, Schuler M, Kolbe K, Peschel C, Huber C, Bemb W, Aulitzky WE (1995) Plasma levels of IL-1, TNF alpha, IL-6, IL-8, G-CSF, and IL1-RA during febrile neutropenia: results of a prospective study in patients undergoing chemotherapy for acute myelogenous leukemia. Ann Hematol 71:161–168

Sieg DJ, Hauck CR, Ilic D, Klingbeil CK, Schaefer E, Damsky CH, Schlaepfer DD (2000) FAK integrates growth-factor and integrin signals to promote cell migration. Nat Cell Biol 2:249–256

Spoo AC, Lübbert M, Wierda WG, Burger JA (2007) CXCR4 is a prognostic marker in acute myelogenous leukemia. Blood 109:786–791

Srivastava MD, Ambrus JL (2004) Effect of 1, 25(OH)2 vitamin D3 analogs on differentiation induction and cytokine modulation in blasts from acute myeloid leukemia patients. Leuk Lymphoma 45:2119–2126

Steinbach D, Schramm A, Eggert A, Onda M, Dawczynski K, Rump A, Pastan I, Wittig S, Pfaffendorf N, Voigt A, Zintl F, Gruhn B (2006) Identification of a set of seven genes for the monitoring of minimal residual disease in pediatric acute myeloid leukemia. Clin Cancer Res 12:2434–2441

Strieter RM, Polverini PJ, Kunkel SL, Arenberg DA, Burdick MD, Kasper J, Dzuiba J, Van Damme J, Walz A, Marriott D, Chan S-Y, Roczniak S, Shanafelt AB (1995) The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. J Biol Chem 270:27348–27357

Struyf S, Schutyser E, Gouwy M, Gijsbers K, Proost P, Benoit Y, Opdenakker G, Van Damme J, Laureys G (2003) PARC/CCL18 is a plasma CC chemokine with increased levels in childhood acute lymphoblastic leukemia. Am J Pathol 163:2065–2075

Szczepansky MJ, Szajnik M, Czystowska M, Mandapathil M, Strauss L, Welsh A, Foon KA, Whiteside TL, Boyiadzis M (2009) Increased frequency and suppression by regulatory T cells in patients with acute myelogenous leukemia. Clin Cancer Res 15:3325–3332

Tanaka T, Bai Z, Srinoulprasert Y, Yang BG, Hayasaka H, Miyasaka M (2005) Chemokines in tumor progression and metastasis. Cancer Sci 96:317–322

Tavernier-Tardy E, Cornillon J, Campos L, Flandrin P, Duval A, Nadal N, Guyotat D (2009) Prognostic value of CXCR4 and FAK expression in acute myelogenous leukemia. Leuk Res 33:764–768

Tavor S, Petit I, Porozov S, Avigdor A, Dar A, Leider-Trejo L, Shemtov N, Deutsch V, Naparstek E, Nagler A, Lapidot T (2004) CXCR4 regulates migration and development of human acute myelogenous leukemia stem cells in transplanted NOD/SCID mice. Cancer Res 64:2817–2824

Tavor S, Eisenbach M, Jacob-Hirsch J, Golan T, Petit I, Benzion K, Kay S, Baron S, Amariglio N, Deutsch V, Naparstek E, Rechavi G (2008) The CXCR4 antagonist AMD3100 impairs survival of human AML cells and induces their differentiation. Leukemia 22:2151–2158

Tobler A, Moser B, Dewald B, Geiser T, Studer H, Baggiolini M, Fey MF (1993) Constitutive expression of interleukin-8 and its receptor in human myeloid and lymphoid leukemia. Blood 82:2517–2525

Uesato N, Fukui K, Maruhashi J, Tojo A, Tajima N (2006) JTE-607, a multiple cytokine production inhibitor, ameliorates disease in a SCID mouse xenograft acute myeloid leukemia model. Exp Hematol 34:1385–1392

Voermans C, van Heese WP, de Jong I, Gerritsen WR, van Der Schoot CE (2002) Migratory behavior of leukemic cells from acute myeloid leukemia patients. Leukemia 16:650–657

Ward SG, Bacon K, Westwick J (1998) Chemokines and T lymphocytes: more than an attraction. Immunity 9:1–11

Wendelbo Ø, Nesthus I, Sjo M, Ernst P, Bruserud Ø (2004a) Cellular immune responses in multiple myeloma patients with treatment-induced cytopenia early after high-dose chemotherapy and autologous peripheral blood stem cell transplantation. Leuk Res 28:461–468

Wendelbo Ø, Nesthus I, Sjo M, Paulsen K, Ernst P, Bruserud Ø (2004b) Functional characterization of T lymphocytes derived from patients with acute myelogenous leukemia and chemotherapy-induced leukopenia. Cancer Immunol Immunother 53:740–747

Wenger RH, Stiehl DP, Camenisch G (2005) Integration of oxygen signaling at the consensus HRE. Sci STKE 306:re12

Wimmer A, Khaldoyanidi SK, Judex M, Serobyan N, Discipio RG, Schraufstatter IU (2006) CCL18/PARC stimulates hematopoiesis in long-term bone marrow cultures indirectly through its effect on monocytes. Blood 108:3722–3729

Xie K (2001) Interleukin-8 and human cancer biology. Cytokine Growth Factor Rev 12:375–391

Yi H, Zhen Y, Jiang L, Zheng J, Zhao Y (2006) The phenotypic characterization of naturally occurring regulatory $CD4^{+}CD25^{+}$ T cells. Cell Mol Immunol 3:189–195

Zahn S, Zwirner J, Spengler HP, Götze O (1997) Chemoattractant receptors for interleukin-8 and C5a: expression on peripheral blood leukocytes and differential regulation on HL-60 and AML-193 cells by vitamin D3 and all-trans retinoic acid. Eur J Immunol 27:935–940

Zebisch A, Czernilofsky AP, Keri G, Smigelskaite J, Sill H, Troppmair J (2007) Signaling through RAS-RAF-MEK-ERK: from basics to bedside. Curr Med Chem 14:601–623

Zeng Z, Samudio IJ, Munsell M, An J, Huang Z, Estey E, Andreeff M, Konopleva M (2006) Inhibition of CXCR4 with the novel RCP168 peptide overcomes stroma-mediated chemoresistance in chronic and acute leukemias. Mol Cancer Ther 5:3113–3121

Zeng Z, Shi YX, Samudio IJ, Wang RY, Ling X, Frolova O, Levis M, Rubin JB, Negrin RR, Estey EH, Konoplev S, Andreeff M, Konopleva M (2009) Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML. Blood 113:6215–6224

CXCR4 in Clinical Hematology

Gary Calandra, Gary Bridger, and Simon Fricker

Contents

Abstract Pharmacological manipulation of CXCR4 has proven clinically useful for mobilization of stem and progenitor cells and in several preclinical models of disease. It is a key component in the localization of leukocytes and stem cells. For patients with multiple myeloma and non-Hodgkin's Lymphoma, treatment with plerixafor, an inhibitor of CXCL12 binding to CXCR4, plus G-CSF mobilizes stem cells for autologous transplantation to a greater degree than the treatment with G-CSF alone, and in some cases when patients could not be mobilized with cytokines, chemotherapy, or the combination. Stem cells from healthy donors mobilized with single agent plerixafor have been used for allogeneic transplantation in acute myelogenous leukemia (AML) patients, although this is still in the early phase of clinical

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development. Plerixafor is also undergoing evaluation to mobilize tumor cells in patients with AML and chronic lymphocytic leukemia (CLL) to enhance the effectiveness of chemotherapy regimens. Plerixafor's effect on neutrophils may also restore circulating neutrophil counts to normal levels in patients with chronic neutropenias such as in WHIMs syndrome. Other areas where inhibition of CXCR4 may be useful based upon preclinical or clinical data include peripheral vascular disease, autoimmune diseases such as rheumatoid arthritis, pulmonary inflammation, and HIV.

1 Introduction

The receptor for CXCL12, CXCR4, has been known only since the early 1990s (reviewed in Murdoch 2000). The first clinical application of CXCR4 inhibition was for the treatment of HIV infection in the late 1990s (Hendrix et al. 2004). Plerixafor, an inhibitor of CXCR4, suppressed the replication of HIV viruses that use CXCR4 for entry and caused a concomitant dose-dependent leukocytosis that included CD34 cells. These observations quickly led to its use in hematology. Plerixafor was approved in 2008 to mobilize $CD34^+$ cells for autologous transplantation of multiple myeloma (MM) and non-Hodgkins lymphoma (NHL) patients. During these studies, other potential hematological uses have emerged and will be discussed in this chapter. In addition to these hematologic uses, preclinical models suggest potential utility in other areas including repair for cardio-vascular diseases such as peripheral vascular disease.

2 Mechanism of Action

Hematopoietic stem cells (HSC) can be found in the circulation, suggesting a continual egress from the bone marrow (BM) into the blood (Levesque and Winkler 2008; Papayannopoulou 2004; Pelus 2008). This process is coordinated by multiple factors that regulate the interactions of HSC with their BM niche. These factors include cytokines and growth factors such as SCF, c-kit ligand, and Granulocyte Colony Stimulating Factor (G-CSF) (Nervi et al. 2006); parathyroid hormone (Adams et al. 2007; Ballen 2007); prostaglandins (Hoggatt et al. 2009; North et al. 2007); neuronal signals (Katayama et al. 2006; Spiegel et al. 2008); adhesion molecules such as VLA-4 (Papayannopoulou 2004, 2000); and chemokines including CXCL12 (Broxmeyer et al. 2008; Dar et al. 2006; Lapidot et al. 2005; Lapidot and Petit 2002). The role of pharmacological mobilizing agents is to promote HSC egress and mobilization into the peripheral circulation. Haematopoietic growth factors, either alone or in combination with chemotherapy, have become the preferred method for HSC mobilization, with the preferred cytokine being G-CSF reviewed in Nervi et al. (2006), Levesque and Winkler (2008), Lapidot and Petit (2002).

HSCs can be mobilized by chemokines such as CXCL2 and CXCL8 in which mobilization occur very rapidly compared with G-CSF mobilization (Pelus and Fukuda 2006). The chemokine CXCL12 and its receptor CXCR4 play an important role in the regulation of hematopoiesis (Nagasawa et al. 1998; Murdoch 2000), and are important components of the mechanism of retention of stem cells in the BM. The expression of CXCL12 and CXCR4 is upregulated in the partially hypoxic BM environment; CXCL12 can up-regulate expression of VLA-4, and mediate the survival, proliferation, and migration of HSCs (Broxmeyer 2008; Dar et al. 2006; Lapidot et al. 2005; Lapidot and Petit 2002). Expression of CXCR4 is reduced on G-CSF mobilized peripheral blood (PB) HSC possibly by proteolytic cleavage (Lapidot and Petit 2002) or at the level of expression (Semerad et al. 2005). These cumulative data support a pivotal role for the CXCL12/CXCR4 axis in stem cell homing and retention.

Plerixafor is a selective inhibitor of the chemokine receptor CXCR4 (Hatse 2002; Schols et al. 1997). It has been shown to inhibit CXCL12 ligand binding and CXCL12-mediated processes including G-protein activation and downstream signaling processes such as intracellular calcium flux and receptor internalization (Hatse et al. 2002; Fricker et al. 2006). Significantly, although able to inhibit these processes, plerixafor does not in itself stimulate any of these activities, indicating that it is an inhibitor of CXCR4 function and does not display agonist activity. However, it has been reported that AMD3100 at high concentrations of $10^{-7} - 10^{-5}$ M can be a weak agonist against wild-type CXCR4 and also to stimulate a constitutively active CXCR4 mutant (N119S) (Zhang et al. 2002).

Mechanistic studies have demonstrated that plerixafor is a tight binding, slowly reversible inhibitor of CXCR4 (Hatse et al. 2002; Fricker et al. 2006). Site-directed mutagenesis of amino acid residues on the CXCR4 receptor have identified the negatively charged amino acids Asp171 on transmembrane region (TM) IV and Asp262 on TM VI as major binding site interactions (Gerlach et al. 2001) (Fig. 1).

3 Preclinical Models of Mobilization

The ability of plerixafor to mobilize HPC and HSC has been shown in a number of animal species, including mice, dogs, and monkeys. Mobilization of HPC by a single subcutaneous injection of plerixafor to C3H/HeJ mice was dose-dependent and more rapid than in man, and is able to mobilize HPCs in strains in which G-CSF is known to be a poor mobilizer (Broxmeyer et al. 2005). The murine HSCs were mobilized from BM (Martin et al. 2006) and had potential for long-term engraftment (Broxmeyer et al. 2005). Significantly, for future clinical studies, the combination of G-CSF and plerixafor was synergistic in both model systems.

Administration of plerixafor in dogs caused a generalized leukocytosis with increases in neutrophils, lymphocytes, and monocytes, and rapid mobilization of both HSC and HPC. The plerixafor-mobilized cells were collected by leukaphereis

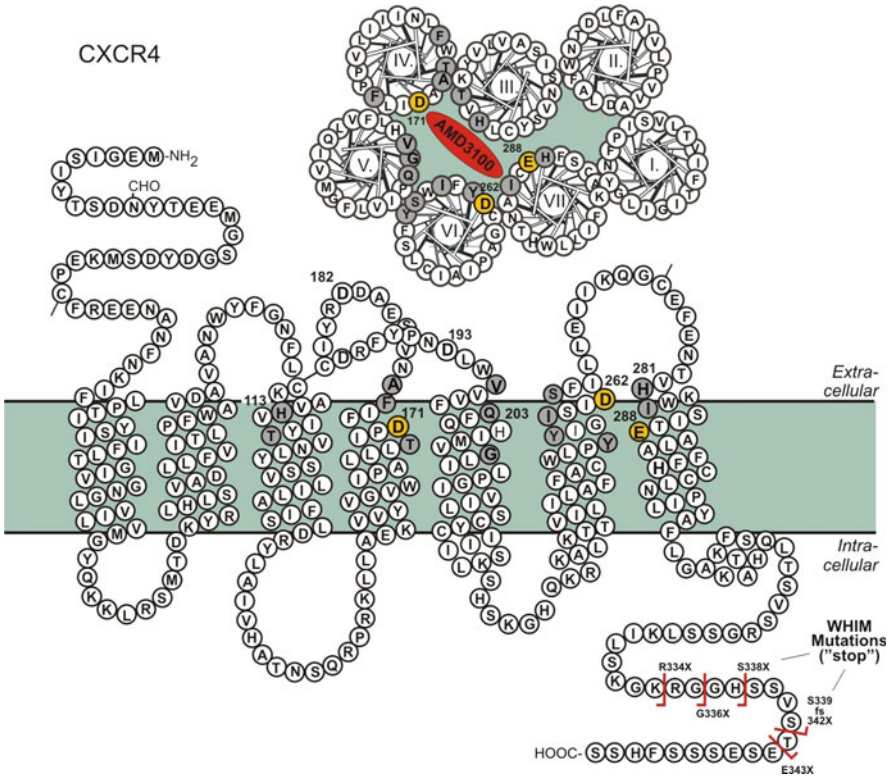

Fig. 1 Binding of plerixafor (AMD3100) to CXCR4 is mediated by Asp171, Asp262. Positions of CXCR4 truncation mutations in the C-terminus responsible for WHIM syndrome are shown

for both autologous and allogeneic transplantation. Durable long-term engraftment was observed in both settings (Burroughs et al. 2005).

Plerixafor also mobilized HSC with the long-term BM repopulating capacity in rhesus macaques (Larochelle et al. 2006). Furthermore, phenotypic analysis of the mobilized $CD34^+$ cells showed that the plerixafor-mobilized cells possessed intrinsic characteristics different from those of HSC mobilized with G-CSF. Significant differences were also seen in the genes expressed by G-CSF-, plerixafor-, and G-CSF+ plerixafor-mobilized $CD34^+$ cells (Donahue et al. 2009).

3.1 Additional Potential Uses Suggested by Preclinical Models

CXCR4 is widely expressed (Murdoch 2000; Nagasawa et al. 1998) and has been implicated in a variety of disease states (De Clercq 2003). Plerixafor has had a positive effect on the outcome in several experimental animal models of disease,

supporting the potential for alternative therapeutic uses of CXCR4 antagonists. This is reflected in the number of CXCR4 inhibitors under investigation, such as 4F-benzoyl-TN14003 (a modified peptide) (Abraham et al. 2009), CTCE-9908 (SDF-1 analog), BTK140, TN14003 (peptide antagonists), and MDX-1388 (antibody), (Burger and Peled 2009) which have progressed at least into phase I clinical trials.

Plerixafor was first identified as a potent inhibitor of HIV infection (Schols et al. 1997; De Clercq et al. 1994) acting through the co-receptor CXCR4 (Bleul et al. 1996; De Clercq 2003). Efficacy of plerixafor in HIV patients has been demonstrated (Hendrix et al. 2004).

Plerixafor reduced the severity of the disease and reduced leukocyte infiltration to the inflamed joint in two models of collagen-induced rheumatoid arthritis (De Klerck et al. 2005; Matthys et al. 2001). Plerixafor was also found to decrease airway hyperreactivity and resistance in a cockroach allergen-induced model of asthma in mice (Lukacs et al. 2002).

Plerixafor can also mobilize circulating angiogenic cells and endothelial progenitor cells (Capoccia et al. 2006; Shepherd et al. 2006). Plerixafor was able to accelerate the restoration of blood flow in models of hindlimb ischemia in diabetic and C57BL/6 mice. Furthermore, systemic administration of plerixafor-mobilized $CD34^+$ cells accelerated restoration of blood flow (Jiao et al. 2006; Capoccia et al. 2006).

CXCR4 has been shown to be expressed on cancers of hematologic origin and on various solid tumors (Balkwill 2004; Burger and Kipps 2006; Juarez and Bendall 2004), and has been hypothesized to play a role in a number of aspects of tumor biology, including angiogenesis, metastasis, and survival. Plerixafor *in vitro* inhibited CXCL12-induced migration and proliferation of a number of tumor cell lines including ovarian (IGROV) (Scotton et al. 2002), lymphoma (Namalwa) (Paul 2002), glioblastoma (U87), and medulloblastoma (Daoy) cells (Rubin et al. 2003). Plerixafor was also shown to inhibit tumor growth in *in vivo* models of non-Hodgkin's lymphoma (Paul 2002), glioblastoma, and medulloblastoma (Rubin et al. 2003), and was synergistic with BCNU in a model of glioblastoma (Redjal et al. 2006). Treatment with plerixafor enhanced the antitumor effect of cytarabine (AraC) in a transgenic mouse model of acute promyelocytic leukemia (APL) (see Sect. 5.2.2).

4 Clinical Uses of Inhibition of CXCR4 in Hematologic Diseases

4.1 The Present Indications

Mobilization and collection of PB $CD34^+$ cells for autologous stem cell transplantation in cancer patients are accomplished by administration of growth factors such as G-CSF with or without chemotherapy (Bensinger et al. 2009). Plerixafor in combination with G-CSF is an alternative to chemotherapy-based mobilization regimens and has been recently licensed in the US and Europe following

completion of two randomized phase III trials that compared plerixafor plus G-CSF to G-CSF alone in patients with MM and NHL. A pilot study (Dugan et al. 2010) to assess the safety of adding plerixafor to a chemotherapy-based mobilization regimen has recently been completed. A number of clinical trials are under way to evaluate the efficacy of this use.

4.1.1 Plerixafor Alone for Stem Cell Mobilization

Plerixafor alone (240 mcg/kg) administered subcutaneously (SC) to healthy volunteers results in an increase in PB $CD34^+$ cells, which peaked at 8–9 h post dose (Liles et al. 2003; Hubel et al. 2004). The half-life was approximately 4–5 h. Patients with MM and NHL were not studied pharmacodynamically in the same detail as healthy volunteers but the results appeared similar for $CD34^+$ cell increase for two time points in a pilot trial (Devine et al. 2004). There was up to 15-fold increase of $CD34^+$ cells in healthy volunteers (Liles et al. 2003; Hubel et al. 2004) and somewhat lower in cancer patients with MM and NHL (Devine et al. 2004), likely due to the prior effects of chemotherapy on BM progenitors.

In the phase I and II studies, when plerixafor alone was used, the 240 mcg/kg SC dose was optimal in normal volunteers (Hubel et al. 2004). Recently, the plerixafor-alone dose has been revisited by several groups (Lemery et al. 2007). SC doses of 320, 400, and 480 mcg/kg in healthy volunteers have shown higher yields and a more prolonged duration of peak $CD34^+$ levels than those with 240 mcg/kg, although the peak fold increase in CD34 was not significantly improved. However, the higher doses may also be associated with more gastrointestinal side effects (diarrhea).

A study (Devine et al. 2008) evaluated the ability of plerixafor alone to mobilize allogeneic donors. This group has extended these studies to determine whether plerixafor (alone) given intravenously (IV) would be better than given subcutaneously (SC). [In treatment studies of HIV patients, plerixafor was given by continuous IV infusion (Hendrix et al. 2004). For reasons of convenience and potential safety, this route was not used in previous hematology studies.] Preliminary data (Rettig et al. 2008) suggest that the time to peak mobilization is earlier with IV dosing and that the peak of $CD34^+$ cells may be higher at the same dose (SC to IV). At present, IV dosing is experimental and the allogeneic use is unapproved.

The Devine study proved that cells mobilized by plerixafor have good engraftment capacity. This was confirmed in an autologous mobilization study (Flomenberg et al. 2010) in which MM patients were mobilized with plerixafor alone, transplanted, and engrafted well.

4.1.2 Plerixafor Plus G-CSF to Mobilize Stem Cells

Plerixafor (240 mcg/kg) administered SC to healthy volunteers following a 5-day regimen of G-CSF (10 mcg/kg) resulted in different pharmacodynamics than that

with plerixafor alone. The peak of mobilization occurred between 10 and 14 hr. The increase in the PB CD34 count compared to a G-CSF baseline was approximately a median of threefold (Liles et al. 2005). The median fold increase in cancer patients is comparable (Flomenberg et al. 2005a), but there is significant variation depending upon the baseline CD34 count following G-CSF, again likely reflecting the status of the marrow in response to prior chemotherapy.

These data suggest that G-CSF has caused expansion of cells within the BM, which are released upon administration of plerixafor via inhibition of CXCR4. Although the precise mechanism has not been fully elucidated, a component of cell mobilization by G-CSF is regulation of CXCL12 and CXCR4 within the BM, as described earlier in Sect. 3. This suggests that a combination of two different mechanisms can result in enhanced mobilization, releasing a larger number of cells into the circulation.

Using the 240 mcg/kg SC dose of plerixafor with G-CSF, two different times of collection via apheresis have been studied (Flomenberg et al. 2005a). When apheresis was initiated 6 h post-plerixafor administration on day 5 of G-CSF, the fold difference between patients harvested post G-CSF alone and the same patients harvested post G-CSF plus plerixafor was a median of threefold. In the Phase III studies, in which one group of patients received 5 days of G-CSF alone and the other received 5 days G-CSF plus plerixafor on the evening of day 4 approximately 10–11 h before apheresis on day 5, the fold increase in PB CD34 counts was also a median of threefold (DiPersio et al. 2009a, b), suggesting that these contrasting schedules provide comparable collection results. This is perhaps not surprising, given the long duration of peak CD34 mobilization after plerixafor (Liles et al. 2005). Given the variation of optimal collection times within transplant centers, a flexible dosing schedule is desirable. Thus, a study exploring plerixafor administration at 5 pm on the evening prior to collection is currently in progress.

The pharmacokinetics of plerixafor when combined with G-CSF for mobilization of cancer patients (Stewart et al. 2009) was similar to healthy volunteers.

4.1.3 Efficacy of Plerixafor Plus G-CSF for Mobilization of Stem Cells

The key phase II study (proof of principle) was conducted in patients with MM and NHL (Flomenberg et al. 2005a). Patients were in first or second complete or partial remission, with no prior attempted mobilization. Patients were mobilized with either a G-CSF (10 mcg/kg) alone regimen or with a plerixafor (240 μg/kg) plus G-CSF regimen, underwent apheresis, rested for 13 days, and then mobilized with the opposite regimen. Apheresis was for up to 4 days or until at least 5×10^6 $CD34^+$ cells/kg were collected. Transplantation was performed with the cells collected by using plerixafor plus G-CSF and the other cells remained as backup. When individual patients were compared to themselves, they collected more on the plerixafor plus G-CSF regimen in fewer days. Among the mobilized patient given G-CSF alone, there were nine patients who failed on the collection of two million cells/kg (poor mobilizers), but were successfully mobilized with plerixafor plus

G-CSF. The median $CD34^+$ cell increase for the entire population was threefold with the addition of plerixafor. However, the range was large with just above onefold (the best mobilizers) to 50-fold (the poor mobilizers). This study formed the basis for the comparative phase III studies, which used essentially the same entry criteria.

In phase III studies, plerixafor plus G-CSF was studied in patients with MM (DiPersio et al. 2009a) and NHL (DiPersio et al. 2009b) as first line treatment compared to G-CSF alone. [The choice of comparative regimen and the design was dictated by FDA rules for having a comparative drug(s) that was (were) approved for the indication.]

In the MM trial, the primary end point was the proportion of patients collecting more than or equal to 6×10^6 $CD34^+$ cells/kg in two or fewer apheresis days. A significantly greater proportion of patients in the plerixafor group reached the primary end point than those in the other group, 106 or 148 (71.6%) vs. 53 of 154 (34.4%), respectively ($p < 0.001$).The median number of apheresis days required to collect more than or equal to 6×10^6 $CD34^+$ cells/kg was 1.0 day in the plerixafor group compared to 4.0 days in the other group ($p < 0.001$). The largest collection of cells per day was greatest on day 1 but at least beneficial to day 4. Days to neutrophil and platelet engraftment were similar and the grafts were durable. Survival at 12 months post-transplant was comparable between the arms, and a long-term follow up study is ongoing.

In the NHL trial, the primary end point was the proportion of patients able to mobilize more than or equal to 5×10^6 $CD34^+$ cells/kg in equal to or less than 4 apheresis days. A significantly greater proportion of patients in the plerixafor group (59.3%) achieved this compared to the other group (19.6%), $p < 0.001$. The time to reach more than or equal to 5×10^6 $CD34^+$ cells was significantly shorter in the plerixafor group, $p < 0.001$. More plerixafor-treated patients (135/150; 90.0%) underwent transplantation after initial mobilization than the other group (82/148; 55.4%), $p < 0.001$. Both groups had similarly successful transplantation, engraftment, and durability at 1 year.

The decreased efficacy in the control group appeared to be due to the lack of PB mobilization of $CD34^+$ cells by G-CSF. Both groups had equal numbers of PB $CD34^+$ cells on day 4 of G-CSF treatment. On day 5 (10–11 h after the study treatment – plerixafor or placebo), plerixafor-treated patients had significantly higher median PB $CD34^+$ cell count (36.2 cells/μl) than placebo-treated patients (13 cells/ul), $p < 0.001$. Since plerixafor does not stimulate $CD34^+$ cell expansion, it is reasonable to conclude that there were equal number of cells available to be released on day 5. However, as previously discussed, administration of plerixafor appears to allow improved mobilization of the remaining cells residing in the marrow.

4.1.4 Plerixafor for Poor Mobilizers

An obvious benefit of plerixafor in MM and NHL patients is the ability to collect sufficient cells in patients who would otherwise not proceed to transplant, the

so-called "poor mobilizers" who have previously failed other mobilizing regimens. Because of the data collected in poor mobilizers from prior studies (Tricot et al. 2010; Flomenberg et al. 2005a), a program ("compassionate") was set up for adult and pediatric patients with a wide range of diseases and proven poor mobilizer status. In an assessment of approximately the first 200 patients (Calandra et al. 2008), a subgroup of 115 patients having MM, NHL, or HD was evaluated for ability to collect equal to or more than 2×10^6 $CD34^+$ cells/kg during mobilization with plerixafor plus G-CSF. The success rate was greater than 66% overall and was higher for patients previously failing chemotherapy mobilization than for cytokine mobilization in the NHL and MM groups but not the HD group. By disease, the overall rate of success was 60.3% (38/63) for NHL, 77.1% (27/35) for MM, and 88.2% (15/17) for HD. Side effects were similar to other studies as discussed hereafter. Overall, more than 75% of the patients were transplanted. Engraftment times and durability were similar to the other trials.

In the NHL phase III trial (Micallef et al. 2009), there were 62 patients (10 from the plerixafor group and 52 from the G-CSF alone group) who entered a rescue protocol because of failure to collect 2 M $CD34^+$ cells/kg on-study. These patients had at least 7 days rest between the two studies and then received G-CSF daily (10 mcg/kg) plus plerixafor starting the evening of day 4 of G-CSF and were apheresed starting the AM of day 5 with up to 4 days of apheresis. Four of the 10 patients (40%) from the plerixafor group and 33/52 (63%) from the placebo group mobilized equal to or more than 2×10^6 $CD34^+$ cells/kg. Transplanted patients had similar engraftment to the primary phase III study and were durable at the 12 months follow up. These results are very similar to those from the compassionate therapy program (Calandra et al. 2008) and from other studies with proven poor mobilizers (Fowler et al. 2009) and predicted poor mobilizers (Stiff et al. 2009).

Although not approved for mobilization of HD patients, plerixafor has been shown useful as primary treatment in such patients (Cashen et al. 2008), as well as in poor mobilizers (Calandra et al. 2008).

4.1.5 Safety of Plerixafor

The safety of plerixafor has been generally acceptable. For the US package circular description of adverse events, those during use of plerixafor plus G-CSF were compared to control (G-CSF alone) from phase III studies (DiPersio et al. 2009a, b). The most common adverse reactions ($\geq$10%) reported in patients who received plerixafor in conjunction with G-CSF regardless of causality and more frequent with plerixafor that placebo during human stem cell mobilization were diarrhea, nausea, fatigue, injection site reactions, headache, arthralgia, dizziness, and vomiting. Although paresthesias were noted in earlier studies (Liles 2003), the frequency was similar between the plerixafor plus G-CSF group and the G-CSF alone group in phase III.

4.1.6 Assessment of Cancer Cell Contamination

Other important safety issues include whether tumor cells are mobilized along with $CD34^+$ cells from MM, NHL, and HD patients. Tricot et al. (2010) used flow cytometry with ability to detect 1/100 tumor cells in MM patients and found none from 10 prior poor mobilizers who were then mobilized with plerixafor plus G-CSF. Fruehauf et al. (2010) used DNA probes in seven MM patients who were not poor mobilizers and who were being mobilized with G-CSF plus plerixafor. While low numbers of tumor cells were found post G-CSF dosing in some patients, there was no increase when plerixafor was then administered. Gazitt et al. (2007) studied a small number of NHL patients and did not find evidence of tumor cell mobilization. In phase III studies, there is similar survival in the plerixafor plus G-CSF group compared to the G-CSF alone group at 12 months (DiPersio et al. 2009a, b). Overall survival will be evaluated for 4 years post-transplant in both phase III studies. These results are in stark contrast to some patients with AML who were mobilized in the Compassionate Use Program and had large contamination of PB with tumor cells (Zeng et al. 2009).

4.1.7 Characterization of Human Cells Mobilized with Plerixafor +/− G-CSF

Studies (Broxmeyer et al. 2005; Hess et al. 2007) of the SCID Hu mouse repopulating frequency of cells obtained from humans treated with plerixafor with and without G-CSF have shown an equal or higher frequency than cells obtained with G-CSF alone. Fruehauf et al. (2006) found that plerixafor plus G-CSF mobilized $CD34^+$ cells express significantly higher levels of genes previously suggested to promote engraftment compared to G-CSF-mobilized $CD34^+$ cells. In MM and NHL patients (Fruehauf et al. 2009), there was a significant increase in primitive $CD34^+$/CD38− cells based on intra-patient comparisons of all patients after the administration of G-CSF plus plerixafor compared to G-CSF alone.

Mobilization with plerixafor in combination with G-CSF for autologous transplantation, or as a single agent in allogeneic donors, provides a graft with an increased T-lymphocyte content compared to a G-CSF mobilized product and may have positive effects on overall survival (Devine et al. 2008; Holtan et al. 2007).

Blum et al. (2009) measured CD133+ cells after healthy subjects were given plerixafor alone at doses of 240, 320, and 400 mcg/kg SC. There was an average 24-fold increase at 6 h post administration, regardless of dose. By multiple other markers, there was no evidence for systematic activation of inflammation. Regarding the original proposed use, plerixafor suppresses the replication of CXCR4-using strains of HIV when administered via 10-day continuous infusion (Hendrix et al. 2004). More recently, a second-generation orally bioavailable CXCR4 inhibitor, AMD070 (Stone et al. 2007), also exhibited anti-HIV activity, but interestingly appears to be less potent in mobilizing $CD34^+$ cells than plerixafor (Moyle et al. 2009).

4.2 Potential Future Indications

4.2.1 Congenital Neutropenias: (Example: WHIM Syndrome)

WHIM syndrome is a rare immunodeficiency disease characterized by warts, hypogammaglobulinemia, bacterial infections, and myelokathexis, in which patients experience severe chronic neutropenia, lymphopenia, and hypercellular BM, attributed to a defect in the release of neutrophils with an accompanying apoptosis of mature myeloid cells, a condition termed myelokathexis (Gorlin 2000). In the majority of WHIM patients, the disease has been linked to autosomal dominant inherited mutations in the chemokine receptor CXCR4, causing truncations in the C-terminus (Hernandez et al. 2003; Gulino 2003; Gulino et al. 2004). In some families with myelokathexis, the specific heterozygous mutations R334X, G336X, S338X, S339fs342X, E343X have been identified (Kawai et al. 2005; Kawai and Malech 2009) as shown in Fig. 1.

The functional consequence of mutations within the receptor appear to be related to a dysfunction in signaling, whereby WHIM neutrophils have enhanced chemotactic responsiveness to CXCL12 caused by impaired desensitization and internalization of CXCR4, resulting in abnormal retention within the marrow (Gulino et al. 2004; Balabanian et al. 2005; Kawai et al. 2005; Lagane et al. 2008). This molecular mechanism is supported by the identification of two unrelated WHIM patients with full clinical characteristics of the disease that do not exhibit detectable mutations within CXCR4 (Balabanian et al. 2008). Patient cells remained hyperresponsive to CXCL12, attributed to a dysregulation of the normal attenuation of CXCR4 function by the GPCR kinase, GRK3. Overexpression of GRK3 in patient neutrophils and fibroblasts restored normal internalization, desensitization, and chemotaxis in response to CXCL12, suggesting that patients with or without CXCR4 mutations share a common dependency on CXCL12 signaling, and WHIM syndrome is a disease of functional hyperresponsiveness of CXCR4.

In a model of WHIM syndrome myelokathexis, human CD34 cells transfected with WHIM R334X mutant CXCR4 transplanted into NOD/SCID mice gave rise to neutrophils with decreased release from the marrow and increased apoptosis (Kawai et al. 2007). However, when the transfected cells were cultured to form neutrophils *in vitro*, apoptosis was comparable to controls, suggesting that mutation and activation of the receptor does not directly cause enhanced apoptosis and the observed increases are secondary to the failure of marrow release.

As the binding site for plerixafor is on the extracellular regions between TM, IV, VI, and VII, remote from the intracellular C-terminal truncations responsible for WHIM syndrome (see Fig. 1), plerixafor is able to inhibit CXCL12-mediated cell migration of leukocytes from WHIM patients with wild-type or mutated receptors (Balabanian et al. 2005). These collective observations support the possibility that CXCR4 antagonists may be a suitable treatment for WHIM syndrome.

4.2.2 Expanding the Application of Mobilizing Agents in Cancer Treatment: Improving the Effectiveness of Chemotherapy to Treat Hematological Malignancies

Growth and differentiation of most types of hematopoietic cells *in vivo* require direct contact with stromal cells, providing a supportive environment (Allen and Dexter 1984; Dorshkind 1990; Koller et al. 1999). Since leukemic cells originate from their normal counterparts and also reside within the BM, it is likely that stromal cells influence the proliferation and apoptosis of leukemic cells (Konopleva et al. 2009). This interaction may play a role in the resistance of residual, post-chemotherapy AML blasts to additional chemotherapeutic agents, a problem that remains a major hurdle in the treatment of AML. Stromal cells were shown to prevent spontaneous or induced apoptosis in AML (Bendall et al. 1994; Garrido et al. 2001; Milojkovic et al. 2004), ALL (Manabe et al. 1992) and CLL cells (Panayiotidis et al. 1996). Recent data demonstrated that MS-5 stromal cells prevented apoptosis in HL-60 cells and in primary AML blasts via modulation of Bcl-2 family proteins (Konopleva et al. 2002). There was also an association between increased Bcl-2 expression levels in stroma-supported AML blasts *in vitro*, with resistance to chemotherapy *in vivo*. In a prospective study of childhood B-ALL (Kumagai et al. 1996), the high recovery of ALL blasts in stroma-supported cultures predicted a lower 4-year event-free survival rate (50% vs. 91%).

Candidate molecules for providing this supportive milieu are cytokines, adhesive ligands, and chemokines. As described in Sect. 3, CXCL12 and CXCR4 play an important role in maintaining HSC in the BM microenvironment. It is therefore conceivable that they play a similar role for leukemic cells. Significantly elevated CXCR4 levels are detected on leukemic cells from patients with B cell chronic lymphocytic leukemia (B-CLL) (Mohle et al. 1999), B-ALL (Bradstock et al. 2000; Dialynas et al. 2001; Shen et al. 2001), and AML (Voermans et al. 2002). Leukemic cells expressing CXCR4 are highly responsive to CXCL12, with cells demonstrating CXCL12-induced calcium flux, integrin-mediated adhesion, chemotaxis, and migration. Moreover, the autocrine secretion of CXCL12 by blood-derived adherent nurse-like cells in CLL protects leukemic B-cells from spontaneous apoptosis (Burger et al. 1999, 2000). Peptide inhibitors of CXCR4 were able to antagonize these effects (Burger et al. 2005). CXCR-4 expression is significantly higher in fetal liver tyrosine kinase-3 (Flt3)/internal tandem duplication (ITD) AML than in Flt3/wild-type (wt) AML (Rombouts et al. 2004). Over-expression of constitutively activated ITD-Flt3 mutants in Ba/F3 cells dramatically enhanced migration toward CXCL12 (Fukuda et al. 2005), suggesting that the FL/Flt3 axis regulates trafficking of AML cells to the BM niche. CXCR4 expression is associated with a poor outcome in B-CLL (Ishibe et al. 2002), pre-B ALL (Crazzolara et al. 2001), and AML patients (Rombouts et al. 2004; Konoplev et al. 2007; Spoo et al. 2007). CXCR4 also mediates homing and engraftment of pre-B ALL (Shen et al. 2001) and AML (Tavor et al. 2004) cells to the BM of NOD/SCID mice. These collective observations suggest that CXCL12/CXCR4 interactions are involved in the maintenance of leukemic cells within the BM and contribute to their resistance to

chemotherapy-induced apoptosis. Disrupting this interaction by administration of CXCR4 inhibitors during chemotherapy represents a novel strategy for targeting leukemic cells within their BM microenvironment (Burger and Peled 2009). In a mouse model of leukemia (APL), AMD3100 mobilized leukemic cells from the BM into the PB and increased their sensitivity to Ara-C, significantly improving overall survival compared to mice treated only with Ara-C (Nervi et al. 2009). Similarly, a second generation CXCR4 antagonist AMD3465 increased the sensitivity of FLT-3 mutated cells to the FLT-3 inhibitor sorafenib (Zeng et al. 2009). In models of ALL, CXCR4 antagonists mobilized leukemic cells into the PB and prevented engraftment (Juarez et al. 2007).

5 Conclusions

Major progress has been made in a short time to elucidate the mechanisms of stem cell mobilization, including the role of CXCR4. While CXCR4 was originally a target (and still is) to treat HIV infection, the major focus of therapy involving this receptor is now in hematology. There is a proven role for CXCR4 in the mobilization of stem cells for autologous transplant of MM and NHL patients. Trials are ongoing to determine whether plerixafor will enhance chemotherapy for AML, and perhaps, CLL. Whether inhibition of CXCR4 will be useful in the treatment of solid tumors is unknown, but trials are planned. Preclinical models show potential for other areas of disease, but as yet no clinical trials have begun. There are many potential opportunities and challenges for inhibition of CXCR4 in human diseases.

References

Abraham M, Beider K, Wald H, Weiss ID, Zipori D, Galun E, Nagler A, Eizenberg O, Peled A (2009) The CXCR4 antagonist 4F-benzoyl-TN14003 stimulates the recovery of the bone marrow after transplantation. Leukemia 23:1378–1388

Adams GB, Martin RP, Alley IR, Chabner KT, Cohen KS, Calvi LM et al (2007) Therapeutic targeting of a stem cell niche. Nat Biotechnol 25:238–243

Allen TD, Dexter TM (1984) The essential cells of the hemopoietic microenvironment. Exp Hematol 12:517–521

Balabanian K, Lagane B, Pablos J et al (2005) WHIM syndromes with different genetic anomalies are accounted for by impaired CXCR4 desensitization to CXCL12. Blood 105:2449–2457

Balabanian K, Levoye A, Klemm L (2008) Leukocyte analysis from WHIM syndrome patients reveals a pivotal role for GRK3 in CXCR4 signaling. J Clin Invest 118:1074–1084

Balkwill F (2004) The significance of cancer cell expression of the chemokine receptor CXCR4. Semin Cancer Biol 14:171–179

Ballen K (2007) Targeting the stem cell niche: squeezing blood from bones. Bone Marrow Transplant 39:655–660

Bendall LJ, Daniel A, Kortlepel K et al (1994) Bone marrow adherent layers inhibit apoptosis of acute myeloid leukemia cells. Exp Hematol 22:1252–1260

Bensinger W, DiPersio JF, McCarty JM (2009) Improving stem cell mobilization strategies: future directions. Bone Marrow Transplant 43:181–195

Bleul C, Farzan M, Choe H et al (1996) The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature 382:829–833

Blum A, Childs R, Smith A, Patibandla S, Zalos G, Samsel L, McCoy JP, Calandra G, Csako G, Cannon RO III (2009) Targeted antagonism of CXCR-4 mobilizes progenitor cells under investigation for cardiovascular disease. Cytotherapy 11(8):1016–1019

Bradstock KF, Makrynikola V, Bianchi A et al (2000) Effects of the chemokine stromal cell-derived factor-1 on the migration and localization of precursor-B acute lymphoblastic leukemia cells within bone marrow stromal layers. Leukemia 14:882–888

Broxmeyer HE (2008) Chemokines in hematopoiesis. Curr Opin Hematol 15:49–58

Broxmeyer H, Orschell C, Clapp D, Hangoc G, Cooper S, Plett PA, Liles WC, Li X, Graham-Evans B, Campbell TB, Calandra G, Bridger G, Dale D, Srour E (2005) Rapid mobilization of murine and human hematopoietic stem and progenitor cells, with AMD3100, a CXCR4 antagonist. J Exp Med 201(8):1307–1318

Burger JA, Kipps TJ (2006) CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. Blood 107:1761–1767

Burger JA, Peled A (2009) CXCR4 antagonists: targeting the microenvironment in leukemia and other cancers. Leukemia 23:43–52

Burger JA, Burger M, Kipps TJ (1999) Chronic lymphocytic leukemia B cells express functional CXCR4 chemokine receptors that mediate spontaneous migration beneath bone marrow stromal cells. Blood 94:3658–3667

Burger JA, Tsukada N, Burger M et al (2000) Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. Blood 96:2655–2663

Burger M, Hartmann T, Krome M et al (2005) Small peptide inhibitors of the CXCR4 chemokine receptor (CD184) antagonize the activation, migration, and antiapoptotic responses of CXCL12 in chronic lymphocytic leukemia B cells. Blood 106:1824–1830

Burroughs L, Mielcarek M, Little MT, Bridger G, Macfarland R, Fricker S et al (2005) Durable engraftment of AMD3100-mobilized autologous and allogeneic peripheral-blood mononuclear cells in a canine transplantation model. Blood 106:4002–4008

Calandra G, McCarty J, McGuirk J, Tricot G, Crocker SA, Grove B, Badel K, Dye A, Bridger G (2008) AMD 3100 plus G-CSF can successfully mobilize $CD34^+$ cells from non-Hodgkin's lymphoma, Hodgkin's disease and multiple myeloma patients previously failing mobilization with chemotherapy and/or cytokine treatment: compassionate use data. Bone Marrow Transplant 41(4):331–338

Capoccia BJ, Shepherd RM, Link DC (2006) G-CSF and AMD3100 mobilize monocytes into the blood that stimulate angiogenesis *in vivo* through a paracrine mechanism. Blood 108:2438–2445

Cashen A, Lopez S, Gao F, Calandra G, MacFarland R, Badel K, DiPersio J (2008) A Phase II study of Plerixafor (AMD3100) plus G-CSF for autologous hematopoietic progenitor cell mobilization in patients with Hodgkin Lymphoma. Biol Blood Marrow Transplant 24 (11):1253–1261

Crazzolara R, Kreczy A, Mann G et al (2001) High expression of the chemokine receptor CXCR4 predicts extramedullary organ infiltration in childhood acute lymphoblastic leukaemia. Br J Haematol 115:545–553

Dar A, Kollet O, Lapidot T (2006) Mutual, reciprocal SDF-1/CXCR4 interactions between hematopoietic and bone marrow stromal cells regulate human stem cell migration and development in NOD/SCID chimeric mice. Exp Hematol 34:967–975

De Clercq E (2003) The bicyclam AMD3100 story. Nat Rev Drug Discov 2:581–587

De Clercq E, Yamamoto N, Pauwels R, Balzarini J, Witvrouw M, De Vreese K et al (1994) Highly potent and selective inhibition of human immunodeficiency virus by the bicyclam derivative JM3100. Antimicrob Agents Chemother 38:668–674

De Klerck B, Geboes L, Hatse S, Kelchtermans H, Meyvis Y, Vermeire K et al (2005) Pro-inflammatory properties of stromal cell-derived factor-1 (CXCL12) in collagen-induced arthritis. Arthritis Res Ther 7:R1208–R1220

Devine S, Flomenberg N, Vesole D, Liesveld J, Weisdorf D, Badel K, Calandra G, DiPersio J (2004) Rapid mobilization of $CD34^+$ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-Hodgkin's lymphoma. J Clin Oncol 22:1095–1102

Devine SM, Vij R, Rettig M, Todt L, McGlauchlen K, Fisher N, Devine H, Link DC, Calandra G, Bridger G, Westervelt P, DiPersio JF (2008) Rapid mobilization of functional donor hematopoietic cells without G-CSF using AMD3100, an antagonist of the CXCR4/SDF-1 interaction. Blood 112(4):990–998

Dialynas DP, Shao L, Billman GF, Yu J (2001) Engraftment of human T-cell acute lymphoblastic leukemia in immunodeficient NOD/SCID mice which have been preconditioned by injection of human cord blood. Stem Cells 19:443–452

DiPersio JF, Stadtmauer EA, Nademanee A, Micallef IN, Stiff PJ, Kaufman JL, Maziarz RT, Hosing C, Früehauf S, Horwitz M, Cooper D, Bridger G, Calandra G, for the 3102 Investigators (2009a) Plerixafor and G-CSF versus placebo and G-CSF to mobilize hematopoietic stem cells for autologous stem cell transplantation in patients with multiple myeloma. Blood 113:5720–5726

DiPersio JF, Micallef IN, Stiff PJ, Bolwell BJ, Maziarz RT, Jacobsen E, Nademanee A, McCarty J, Bridger G, Calandra G, for the 3101 Investigators (2009b) Phase 3 prospective randomized double blind placebo-controlled trial of plerixafor (AMD3100) plus G-CSF versus placebo plus G-CSF for autologous stem cell mobilization and transplantation in patients with non-Hodgkin's lymphoma. J Clin Oncol 28:4767–4773

Donahue RE, Jin P, Bonifacino AC, Metzger ME, Ren J, Wang E et al (2009) Plerixafor (AMD3100) and granulocyte colony-stimulating factor (G-CSF) mobilize different $CD34^+$ cell populations based on global gene and microRNA expression signatures. Blood 114:2530–2541

Dorshkind K (1990) Regulation of hemopoiesis by bone marrow stromal cells and their products. Annu Rev Immunol 8:111–137

Dugan MJ, Maziarz RT, Bensinger WI, Nademanee A, Liesveld J, Badel K, Dehner C, Gibney C, Bridger G, Calandra G (2010) Safety and preliminary efficacy of plerixafor (Mozobil™) in combination with chemotherapy and G-CSF: an open-label, multicenter, exploratory trial in patients with multiple myeloma and non-Hodgkin’s lymphoma undergoing stem cell mobilization. Bone Marrow Transplant. 45:39–47

Flomenberg N, Devine SM, DiPersio JF, Liesveld JL, McCarty JM, Rowley SD, Vesole DH, Badel K, Calandra G (2005a) The use of AMD3100 plus G-CSF for autologous hematopoietic progenitor cell mobilization is superior to G-CSF alone. Blood 106(5):1867–1874

Flomenberg N, Comenzo RL, Badel K, Calandra G (2010) Plerixafor (Mozobil) alone to mobilize hematopoietic stem cells from multiple myeloma patients for autologous transplantation. Biol Blood Marrow Transplant Published on-line January 11, 2010

Fowler CJ, Dunn A, Hayes-Lattin B, Hansen K, Hansen L, Lanier K, Nelson V, Kovacsovics T, Leis J, Calandra G, Maziarz RT (2009) Rescue from failed growth factor and/or chemotherapy HSC mobilization with G-CSF and Plerixafor (AMD3100): an institutional experience. Bone Marrow Transplant 43:1–9

Fricker SP, Anastassov V, Cox J, Darkes MC, Grujic O, Idzan SR et al (2006) Characterization of the molecular pharmacology of AMD3100: a specific antagonist of the G-protein coupled chemokine receptor, CXCR4. Biochem Pharmacol 72:588–596

Fruehauf S, Seeger T, Maier P, Li L, Weinhardt S, Laufs S, Wagner W, Eckstein V, Bridger G, Calandra G, Wenz F, Zeller WJ, Goldschmidt H, Ho A (2006) The CXCR4 antagonist AMD3100 releases a subset of G-CSF-primed peripheral blood progenitor cells with specific gene expression characteristics. Exp Hematol 34:1052–1059

Fruehauf S, Ehninger G, Hubel K, Topaly J, Goldschmidt H, Ho AD, Muller S, Moos M, Badel K, Calandra G (2010) Mobilization of peripheral blood stem cells for autologous transplant in

non-Hodgkin’s lymphoma and multiple myeloma patients by plerixafor and G-CSF and detection of tumor cell mobilization by PCR in multiple myeloma patients. Bone Marrow Transplant 45:269–275

Fruehauf S, Veldwijk MR, Seeger T, Schubert M, Laufs S, Topaly J, Wuchter P, Dillmann F, Eckstein V, Wenz F, Goldschmidt H, Ho AD, Calandra G (2009) A combination of granulocyte colony-stimulating factor (G-CSF) and AMD3100 (plerixafor) mobilizes more primitive peripheral blood progenitor cells than G-CSF alone: results of a European phase II study with AMD3100. Cytotherapy 11:992–1001

Fukuda S, Broxmeyer HE, Pelus LM (2005) Flt3 ligand and the Flt3 receptor regulate hematopoietic cell migration by modulating the SDF-1alpha(CXCL12)/CXCR4 axis. Blood 105:3117–3126

Garrido SM, Appelbaum FR, Willman CL, Banker DE (2001) Acute myeloid leukemia cells are protected from spontaneous and drug-induced apoptosis by direct contact with a human bone marrow stromal cell line (HS-5). Exp Hematol 29:448–457

Gazitt Y, Freytes CO, Akay C, Badel K, Calandra G (2007) Improved mobilization of peripheral blood $CD34^+$ cells and dendritic cells by AMD3100 plus granulocyte-colony-stimulating factor in Non-Hodgkin's lymphoma patients. Stem Cells Dev 16(4):657–666

Gerlach LO, Skerlj RT, Bridger GJ, Schwartz TW (2001) Molecular interactions of cyclam and bicyclam non-peptide antagonists with the CXCR4 chemokine receptor. J Biol Chem 276:14153–14160

Gorlin R, Gelb B, Diaz G et al (2000) WHIM syndrome: an autosomal dominant disorder. Am J Med Genet 91:368–376

Gulino A (2003) WHIM Syndrome: a genetic disorder of leukocyte trafficking. Curr Opin Allergy Clin Immunol 3:443–450

Gulino A, Moratto D, Sozzani S et al (2004) Altered leukocyte response to CXCL12 in patients with warts hypogammaglobulinemia, infections, myelokathexis (WHIM) syndrome. Blood 104:444–452

Hatse S, Princen K, Bridger G, De Clercq E, Schols D (2002) Chemokine receptor inhibition by AMD3100 is strictly confined to CXCR4. FEBS Lett 527:255–262

Hendrix C, Collier A, Lederman M, Schols D, Pollard R, Brown S, Jackson JB, Coombs R, Glesby M, Flexner C, Bridger G, Badel K, MacFarland R, Henson G, Calandra G (2004) Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection. J Acquir Immune Defic Syndr 37(2):1253–1262

Hernandez P, Gorlin R, Lukens J (2003) Mutations in the chemokine receptor gene CXCR4 are associated with WHIM syndrome, a combined immunodeficiency disease. Nat Genet 34:70–73

Hess DA, Bonde J, Craft TC, Wirthlin L, Hohm S, Lahey R, Todt LM, Dipersio JF, Devine SM, Nolta JA (2007) Human progenitor cells rapidly mobilized by AMD3100 repopulate NOD/SCID mice with increased frequency in comparison to cells from the same donor mobilized by granulocyte colony stimulating factor. Biol Blood Marrow Transplant 13:398–411

Hoggatt J, Singh P, Sampath J, Pelus LM (2009) Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation. Blood 113:5444–5455

Holtan SG, Porrata LF, Micallef IN et al (2007) AMD3100 affects autograft lymphocyte collection and progression-free survival after autologous stem cell transplantation in non-Hodgkin lymphoma. Clin Lymphoma Myeloma 7:315–318

Hubel K, Liles C, Broxmeyer HE, Rodger E, Wood B, Cooper S, Hangoc G, MacFarland R, Bridger GJ, Henson G, Calandra G, Dale DC (2004) Leukocytosis and mobilization of $CD34^+$ hematopoeitic progenitor cells by AMD3100, a CXCR4 antagonist. Support Cancer Ther 1 (3):165–172

Ishibe N, Albitar M, Jilani IB et al (2002) CXCR4 expression is associated with survival in familial chronic lymphocytic leukemia, but CD38 expression is not. Blood 100:1100–1101

Jiao C, Fricker S, Schatteman GC (2006) The chemokine (C-X-C motif) receptor 4 inhibitor AMD3100 accelerates blood flow restoration in diabetic mice. Diabetologia 49:2786–2789

Juarez J, Bendall L (2004) SDF-1 and CXCR4 in normal and malignant hematopoiesis. Histol Histopathol 19:299–309

Juarez J, Dela Pena A, Baraz R et al (2007) CXCR4 antagonists mobilize childhood acute lymphoblastic leukemia cells into the peripheral blood and inhibit engraftment. Leukemia 21:1249–1257

Katayama Y, Battista M, Kao WM, Hidalgo A, Peired AJ, Thomas SA et al (2006) Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. Cell 124:407–421

Kawai T, Malech H (2009) WHIM Syndrome: congenital immune deficiency disease. Curr Opin Hematol 16:20–26

Kawai T, Choi U, Whiting-Theobald N et al (2005) Enhanced function with decreased internalization of carboxy-terminus truncated CXCR4 responsible for WHIM syndrome. Exp Hematol 33:460–468

Kawai T, Choi U, Cardwell L et al (2007) WHIM syndrome myelokathexis reproduced in the NOD/SCID mouse xenotransplant model engrafted with healthy human stem cells transduced with C-terminus–truncated CXCR4. Blood 109:78–84

Koller MR, Oxender M, Jensen TC et al (1999) Direct contact between $CD34^{+}$lin- cells and stroma induces a soluble activity that specifically increases primitive hematopoietic cell production. Exp Hematol 27:734–741

Konoplev S, Rassidakis GZ, Estey E et al (2007) Overexpression of CXCR4 predicts adverse overall and event-free survival in patients with unmutated FLT3 acute myeloid leukemia with normal karyotype. Cancer 109:1152–1156

Konopleva M, Konoplev S, Hu W et al (2002) Stromal cells prevent apoptosis of AML cells by up-regulation of anti-apoptotic proteins. Leukemia 16:1713–1724

Konopleva M, Tabe Y, Zeng Z et al (2009) Therapeutic targeting of microenvironmental interactions in leukemia: mechanisms and approaches. Drug Resist Updat 12:103–113

Kumagai M, Manabe A, Pui CH et al (1996) Stroma-supported culture in childhood B-lineage acute lymphoblastic leukemia cells predicts treatment outcome. J Clin Invest 97:755–760

Lagane L, Chow K, Balabanian K et al (2008) CXCR4 dimerization and β-arrestin-mediated signaling account for the enhanced chemotaxis to CXCL12 in WHIM syndrome. Blood 112:34–44

Lapidot T, Petit I (2002) Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. Exp Hematol 30:973–981

Lapidot T, Dar A, Kollet O (2005) How do stem cells find their way home? Blood 106:1901–1910

Larochelle A, Krouse A, Metzger M, Orlic D, Donahue RE, Fricker S et al (2006) AMD3100 mobilizes hematopoietic stem cells with long-term repopulating capacity in nonhuman primates. Blood 107:3772–3778

Lemery SJ, Hsieh MM, Smith A, Rao S, Khuu HM, Donohue T, Karpovich JM, Cook L, Goodwin R, Boss C, Calandra G, Geller N, Tisdale J, Childs R (2007) A pilot study evaluating the $CD34^{+}$ cell mobilizing activity and adverse events associated with escalating doses of AMD3100 in health volunteers. ASH Annual Meeting Abstracts 110:3278

Levesque JP, Winkler IG (2008) Mobilization of hematopoietic stem cells: state of the art. Curr Opin Organ Transplant 13:53–58

Liles WC, Broxmeyer HE, Rodger E, Wood B, Hubel K, Cooper S, Hangoc G, Bridger G, Henson G, Calandra G, Dale D (2003) Mobilization of hematopietic progenitor cells in healthy volunteers by AMD3100, a CXCR4 antagonist. Blood 102:2728–2730

Liles W, Rodger E, Broxmeyer H, Dehner C, Badel K, Calandra G, Christensen J, Wood B, Price T, Dale D (2005) Augmented mobilization and collection of $CD34^{+}$ hematopoietic cells from normal human volunteers stimulated with granulocyte-colony-stimulating factor by single-dose administration of AMD3100, a CXCR4 antagonist. Transfusion 45:295–300

Lukacs NW, Berlin A, Schols D, Skerlj RT, Bridger GJ (2002) AMD3100, a CxCR4 antagonist, attenuates allergic lung inflammation and airway hyperreactivity. Am J Pathol 160:1353–1360

Manabe A, Coustan-Smith E, Behm FG et al (1992) Bone marrow-derived stromal cells prevent apoptotic cell death in B-lineage acute lymphoblastic leukemia. Blood 79:2370–2377

Martin C, Bridger GJ, Rankin SM (2006) Structural analogues of AMD3100 mobilise haematopoietic progenitor cells from bone marrow *in vivo* according to their ability to inhibit CXCL12 binding to CXCR4 *in vitro*. Br J Haematol 134:326–329

Matthys P, Hatse S, Vermeire K, Wuyts A, Bridger G, Henson GW et al (2001) AMD3100, a potent and specific antagonist of the stromal cell-derived factor-1 chemokine receptor CXCR4, inhibits autoimmune joint inflammation in IFN-gamma receptor-deficient mice. J Immunol 167:4686–4689

Micallef IN, Stiff PJ, DiPersio JF, Maziarz RT, McCarty JM, Bridger G, Calandra G (2009) Successful stem cell re-mobilization using plerixafor (Mozobil) plus G-CSF in patients with non-Hodgkin's lymphoma (NHL): results from the plerixafor NHL phase 3 study rescue protocol. Biol Blood Marrow Transplant 15:1578–1586

Milojkovic D, Devereux S, Westwood NB et al (2004) Antiapoptotic microenvironment of acute myeloid leukemia. J Immunol 173:6745–6752

Mohle R, Failenschmid C, Bautz F, Kanz L (1999) Overexpression of the chemokine receptor CXCR4 in B cell chronic lymphocytic leukemia is associated with increased functional response to stromal cell-derived factor-1 (SDF-1). Leukemia 13:1954–1959

Moyle G, DeJesus E, Boffito M, Wong RS, Gibney C, Bade K, MacFarland R, Calandra G, Bridger G, Becker S, for the X4 Antagonist Concept Trial (XACT) study team (2009) Proof of activity with AMD11070, an orally bioavailable inhibitor of CXCR4-tropic HIV-1 virus. Clin Infect Dis 48(6):798–805

Murdoch C (2000) CXCR4: chemokine receptor extraordinaire. Immunol Rev 177:175–184

Nagasawa T, Tachibana K, Kishimoto T (1998) A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection. Semin Immunol 10:179–185

Nervi B, Link DC, DiPersio JF (2006) Cytokines and hematopoietic stem cell mobilization. J Cell Biochem 99:690–705

Nervi B, Ramirez P, Rettig M et al (2009) Chemosensitization of acute myeloid leukemia (AML) following mobilization by the CXCR4 antagonist AMD3100. Blood 113:6206–6214

North TE, Goessling W, Walkley CR, Lengerke C, Kopani KR, Lord AM et al (2007) Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. Nature 447:1007–1011

Panayiotidis P, Jones D, Ganeshaguru K et al (1996) Human bone marrow stromal cells prevent apoptosis and support the survival of chronic lymphocytic leukaemia cells *in vitro*. Br J Haematol 92:97–103

Papayannopoulou T (2000) Mechanisms of stem-/progenitor-cell mobilization: the anti-VLA-4 paradigm. Semin Hematol 37:11–18

Papayannopoulou T (2004) Current mechanistic scenarios in hematopoietic stem/progenitor cell mobilization. Blood 103:1580–1585

Paul S, Mancuso P, Rabascio C, Gobbi A, Capillo M, Pruneri G, Martinelli G, Fricker S, Bridger G, Bertolini F (2002) In vitro and preclinical activity of the novel AMD3100 CXCR4 antagonist in lymphoma models. 44th Annual Meeting of the American Society of Hematology: Blood

Pelus LM (2008) Peripheral blood stem cell mobilization: new regimens, new cells, where do we stand. Curr Opin Hematol 15:285–292

Pelus LM, Fukuda S (2006) Peripheral blood stem cell mobilization: the CXCR2 ligand GRObeta rapidly mobilizes hematopoietic stem cells with enhanced engraftment properties. Exp Hematol 34:1010–1020

Redjal N, Chan JA, Segal RA, Kung AL (2006) CXCR4 inhibition synergizes with cytotoxic chemotherapy in gliomas. Clin Cancer Res 12:6765–6771

Rettig MP, Shannon WD, Richey J, Holt M, McFarland K, Lopez S, Gabriel J, DiPersio JF (2008) Characterization of human $CD34^+$ hematopoietic stem cells following administration of G-CSF or plerixafor. ASH Abstract 3476

Rombouts EJ, Pavic B, Löwenberg B, Ploemacher RE (2004) Relation between CXCR-4 expression, Flt3 mutations, and unfavorable prognosis of adult acute myeloid leukemia. Blood 104:550–557

Rubin JB, Kung AL, Klein RS, Chan JA, Sun Y, Schmidt K et al (2003) A small-molecule antagonist of CXCR4 inhibits intracranial growth of primary brain tumors. Proc Natl Acad Sci USA 100:13513–13518

Schols D, Este JA, Henson G, De Clercq E (1997) Bicyclams, a class of potent anti-HIV agents, are targeted at the HIV coreceptor fusin/CXCR-4. Antiviral Res 35:147–156

Scotton CJ, Wilson JL, Scott K, Stamp G, Wilbanks GD, Fricker S et al (2002) Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer. Cancer Res 62:5930–5938

Semerad CL, Christopher MJ, Liu F, Brenton S, Simmons PJ, Winkler I, Leveneque J-P, Chappel J, Ross FP, Link DC (2005) G-CSF potently inhibits osteoblast activity and CXCL12 mRNA expression in the bone marrow. Blood 106:3020–3027

Shen W, Bendall LJ, Gottlieb DJ, Bradstock KF et al (2001) The chemokine receptor CXCR4 enhances integrin-mediated *in vitro* adhesion and facilitates engraftment of leukemic precursor-B cells in the bone marrow. Exp Hematol 29:1439–1447

Shepherd RM, Capoccia BJ, Devine SM, Dipersio J, Trinkaus KM, Ingram D et al (2006) Angiogenic cells can be rapidly mobilized and efficiently harvested from the blood following treatment with AMD3100. Blood 108:3662–3667

Spiegel A, Kalinkovich A, Shivtiel S, Kollet O, Lapidot T (2008) Stem cell regulation via dynamic interactions of the nervous and immune systems with the microenvironment. Cell Stem Cell 3:484–492

Spoo AC, Lübbert M, Wierda WG, Burger JA (2007) CXCR4 is a prognostic marker in acute myelogenous leukemia. Blood 109:786–791

Stewart DA, Smith C, MacFarland R, Calandra G (2009) Pharmacokinetics and pharmacodynamics of plerixafor in patients with non-Hodgkin's lymphomas and multiple myeloma. Biol Blood Marrow Transplant 15:39–46

Stiff P, Micallef I, McCarthy P, Magalhaes-Silverman M, Weisdorf D, Territo M, Badel K, Calandra G (2009) Treatment with plerixafor in non-Hodgkin's lymphoma and multiple myeloma patients to increase the number of peripheral blood stem cells when given a mobilizing regimen of G-CSF: implications for the hard to mobilize patients. Biol Blood Marrow Transplant 15:249–256

Stone N, Dunaway S, Flexner C, Tierney C, Calandra G, Becker S, Cao Y, Wiggins I, Conley J, MacFarland R, Park J, Lalama C, Snyder S, Kallungal B, Klingman K, Hendrix C (2007) Multiple dose escalation study of the safety, pharmacokinetics, and biologic activity of oral AMD070, a selective CXCR4 receptor inhibitor, in human subjects (ACTG A5191). Antimicrob Agents Chemother 51(7):2351–2358

Tavor S, Petit I, Porozov S et al (2004) CXCR4 regulates migration and development of human acute myelogenous leukemia stem cells in transplanted NOD/SCID mice. Cancer Res 64:2817–2824

Tricot G, Cottler-Fox MH, Calandra G (2010) Safety and efficacy assessment of plerixafor in patients with multiple myeloma proven or predicted to be poor mobilizers including assessment of tumor cell mobilization. Bone Marrow Transplant 45:63–68

Voermans C, van Heese WP, de Jong I et al (2002) Migratory behavior of leukemic cells from acute myeloid leukemia patients. Leukemia 16:650–657

Zeng Z, Shi Y, Samudio I et al (2009) Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML. Blood 113:6215–6224

Zhang WB, Navenot JM, Haribabu B, Tamamura H, Hiramatu K, Omagari A et al (2002) A point mutation that confers constitutive activity to CXCR4 reveals that T140 is an inverse agonist and that AMD3100 and ALX40-4C are weak partial agonists. J Biol Chem 277:24515–24521

Immunobiology of Heparin-Induced Thrombocytopenia

Per Morten Sandset

Contents

Abstract Heparin-induced thrombocytopenia (HIT) is a potentially life-threatening adverse drug reaction that may develop in certain patients exposed to heparin and is caused by antibodies with specificity for chemokine CXCL4 (formerly known as platelet factor 4)/heparin complexes. Rapid diagnosis and intervention is key to prevent severe thrombotic complications. The immunobiology of HIT is atypical as the immune reaction most often involves rapid generation of immunoglobulin class G within 5–14 days after heparin exposure, and apparently lacks memory as patients may be reexposed to heparin. This report reviews clinical presentation, diagnostic issues, and immunobiology of HIT.

Abbreviations

HIT Heparin-induced thrombocytopenia
Ig Immunoglobulin

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1 Introduction

Heparin-induced thrombocytopenia (HIT) is a potentially life-threatening adverse drug reaction that may develop in certain patients exposed to heparin and is caused by antibodies with specificity for chemokine CXCL4 (formerly known as platelet factor 4)/heparin complexes (Arepally and Ortel 2006; Greinacher 2009; Warkentin 2007b). Such antibodies may activate platelets followed by activation of coagulation, and are clinically associated with arterial and venous thrombosis despite low levels of circulating platelets. Rapid diagnosis is key to proper management of such patients to avoid severe thrombotic complications, and most important is switching from heparin to non-heparin anticoagulants. Diagnosis is difficult and is based on both the clinical presentation and detection of pathogenic HIT antibodies. This chapter will briefly review clinical and diagnostic aspects and focus on the immunobiology of HIT.

2 Clinical Characteristics

HIT typically features a relative fall in platelet count by >50% on heparin anticoagulation (Greinacher et al. 2005; Warkentin et al. 2003), but the fall in platelet count may be less, for example, by 30–40%, in some patients. The appropriate "baseline" platelet count is probably not the preoperative, pre-heparin value, but rather the peak postoperative platelet count that precedes the fall, indicating HIT (Warkentin et al. 2003).

Thrombotic complications develop in 20–50% of the patients with HIT (Greinacher et al. 2005; Wallis et al. 1999; Warkentin 2007a). Most often HIT-associated thrombosis occurs when platelet count has decreased by approximately 50%, but thrombosis can occur before the fall in platelets by 1–2 days in one-third of the patients. Thrombosis may occur in any vascular bed and frequently occurs at sites of vascular injury. Venous thrombosis is the dominant thrombotic phenotype, but arterial thrombosis is common in patients with arteriosclerosis. Other, but rare complications include microvascular-dependent thrombosis responsible for skin necrosis, adrenal hemorrhagic necrosis, and anaphylactoid reactions, for example, fever/chills, tachycardia, hypotension, dyspnea, and cardiac arrest, after intravenous bolus injections of unfractionated heparin. Unusual thrombotic events are more often associated with HIT.

HIT usually develop 5–14 days after exposure to prophylactic or therapeutic heparin anticoagulation ("typical onset HIT") (Warkentin and Kelton 2001b). In patients previously exposed to heparin within the last month and rarely up to 3 months, reexposure to heparin may trigger HIT after only 1 day of treatment ("rapid onset HIT") because of heparin-dependent antibodies still being present in the patient's plasma (Warkentin and Kelton 2001b). In some cases, HIT may develop days or even a few weeks after exposure to heparin, and is triggered by antibodies

that activate platelets independently of heparin ("delayed onset HIT") (Warkentin and Kelton 2001a).

Unfractionated heparin is more likely to cause HIT than fractionated (low molecular weight) heparins (Warkentin et al. 1995). The frequency of HIT is highest after surgery and much lower in medical patients, which is possibly explained by higher number of CXCL4 molecules and generation of proinflammatory cytokines in the surgical setting. HIT can be identified in approximately 3% of patients after cardiac and orthopedic surgery (Pouplard et al. 1999; Schenk et al. 2007), and in 0.5–1% in medical patients (Warkentin et al. 2000).

3 Diagnostic Issues

3.1 Clinical Scoring System for HIT

The diagnosis of HIT is based both on clinical characteristics and on the detection of antibodies against CXCL4/heparin as part of a clinicopathological syndrome. Thrombocytopenia is key to the diagnosis, but thrombocytopenia is not specific for HIT, as many patients have other potential causes for a reduction in platelets. Moreover, specific antibodies against CXCL4/heparin are found in nearly all patients with HIT, but detection of such antibodies are not specific, and many patients have antibodies in the absence of HIT. Laboratory testing also has the disadvantage of taking too long time in the acute setting, which means that a decision to switch anticoagulant treatment must be based on clinical characteristics of the patient.

A clinical scoring system for HIT, labeled the 4 T's clinical scoring system, has recently been developed (Warkentin and Heddle 2003). The 4 T's scoring system is based on four criteria: the platelet count, that is, thrombocytopenia; the timing of platelet count reduction in relation to heparin; the absence or presence of thrombosis; and the absence or presence of other cause(s) for thrombocytopenia (Table 1). The scoring is independent of laboratory evaluation and classifies the patients in three different categories of probability, that is, low ($\leq$3 points), intermediate (4–5 points), and high (6–8 points) probability for HIT. A low probability 4 T's score has been found to rule out HIT in most patients (high negative predictive value), but a high score was not always strongly predictive of HIT (low specificity), indicating that patients with HIT cannot be identified by clinical diagnosis alone (Lo et al. 2006; Pouplard et al. 2007). In one study, use of the 4 T's score in combination with laboratory testing (particle gel immunoassay) was found to increase the sensitivity and specificity for the diagnosis of HIT, and an algorithm for the diagnosis and management of HIT was proposed (Pouplard et al. 2007). One study has found that careful clinical evaluation may significantly increase cost-effectiveness of the clinical management of HIT (Patrick et al. 2007).

Table 1 The 4 T's clinical scoring system for heparin-induced thrombocytopenia (Warkentin and Heddle 2003)

Parameter ("4 T's")	Points (0, 1, or 2 for each category – maximum possible score = 8)		
	0	1	2
Thrombocytopenia – platelet count	<30% fall or nadir $<10 \times 10^9$/L	30–50% fall or nadir 10–19 $\times 10^9$/L	>50% fall and nadir >20 $\times 10^9$/L
Timing of platelet count decrease	Fall <4 days without recent exposure	Uncertain onset days 5–10, (missing count), or onset >10 days; or onset ≤1 day in case of heparin exposure 30–100 days ago	Clear onset days 5–10; or onset ≤1 day in case of heparin exposure <30 days ago
Thrombosis or other sequelae	None	Progressive or recurrent thrombosis; non-necrotizing (erythematous) skin lesions; suspected thrombosis (not proven)	New thrombosis (confirmed); skin necrosis; acute systemic reaction after intravenous heparin bolus injection
Other cause of thrombocytopenia	Definite	Possible	None apparent

≤3 points: low probability for HIT; 4–5 points: medium probability; and 6–8 points: high probability

3.2 Laboratory Evaluation of HIT

In principle, there are two types of laboratory assays that are sensitive to detect clinically relevant HIT antibodies. The first class of assays is enzyme-linked immunosorbant assay (ELISA) that detects the binding of HIT antibodies to CXCL4/heparin complexes (e.g., Asserachrom-HPIA, Diagnostica Stago, Asnière, France) or to polyvinyl sulfonate – CXCL4 complexes (e.g., HAT-GTI, Brookfield, WI, USA). These assays are well standardized and easy to perform, and have very high (approximately 99%) sensitivity and negative predictive values for HIT (Greinacher et al. 2007). The specificity and positive predictive values are, however, poor (approximately 50–90%) since many patients on heparin may develop nonpathogenic antibodies against CXCL4 (Greinacher et al. 2007; Warkentin et al. 2005). Since most nonpathogenic antibodies are weakly positive, that is, low optical density (typically <0.8–1.0), and most pathogenic antibodies are strongly positive, the optical density should be considered when these assays are employed in the diagnosis of HIT.

The other class of assays detects HIT antibodies of the immunoglobulin (Ig) G isotype via their capacity to activate platelets from healthy donors by cross-linking

of FcγIIa receptors in the presence of CXCL4 and heparin. The tests are positive if a maximal activation is recorded in the presence of therapeutic concentrations of heparin, for example, 0.1–0.5 U/mL of unfractionated heparin, and with no activation at heparin concentrations >10 U/mL. Platelets must be carefully prepared from several healthy donors as not all donors have activatable platelets. Moreover, many agonists can activate platelets and care must be taken to avoid nonspecific activation. The platelets are mixed with patient's plasma and heparin and then followed over time to see if antibodies in the patient's plasma activate the platelets. The serotonin release assay investigates the release of ^{14}C-serotonine (Sheridan et al. 1986), whereas the heparin-induced platelet activation assay is based on the optical assessment of platelet aggregation in microtiter plate wells (Greinacher et al. 1991). Both assays have very high sensitivity (approximately 99%) and fairly high specificity (95–99%) for HIT (Warkentin et al. 2005).

The conventional immunoassays and the platelet activation assays are time-consuming to perform. Moreover, the platelet activation assays require preparation of platelets from healthy controls. Use of radioactive isotopes, requirements for special equipment, and lack of experienced staff limits the utility and feasibility of these methods to a few highly specialized reference laboratories. Recently, a particle immunoassay (H/PF4-PaGIA, Diamed SA, Cressier sur Morat, Switzerland) was developed. In this assay, ready-to-use polystyrene beads are coated with CXCL4/heparin complex that reacts with antibodies in plasma. This assay allows the detection of HIT antibodies in <1 h after blood collection and may therefore be used as a rapid screening method. The performance of the assay has been evaluated and found to be intermediate of the immunoassays and the platelet activation assays (Eichler et al. 2002), and the assay has been validated for use in combination with clinical probability testing as part of a diagnostic algorithm (Pouplard et al. 2007).

4 Treatment of HIT

A comprehensive guideline to the management of HIT was recently published (Warkentin et al. 2008). This report provides detailed recommendations based on present knowledge, and should be consulted in cases of suspected HIT. The key issues in the management are immediate discontinuation of heparin treatment, initiation of alternative anticoagulation, and delayed anti-vitamin K therapy until normal platelet count.

Discontinuation of heparin is mandatory to remove HIT antigens found on CXCL4 bound to heparin. Since HIT is a prothrombotic condition associated with increased thrombin generation, discontinuation of heparin alone may significantly enhance thrombotic lesions, unless anticoagulation is prolonged. Therefore, alternative anticoagulation, usually in therapeutic doses, is needed in most patients. Several such anticoagulants are now available and include direct thrombin inhibitors and indirect factor Xa inhibitors. The direct thrombin inhibitors include *lepirudin*, a recombinant hirudin (leech anticoagulant), that irreversibly inhibits

thrombin, *bivalirudin*, a recombinant hirudin derivative, and *argatroban*, a synthetic inhibitor, that are reversible inhibitors of thrombin. All drugs need monitoring using activated partial thromboplastin time or specialized assays (e.g., ecarin clotting time or chromogenic assay). The indirect factor Xa inhibitors include *danaparoid*, an antithrombin- and heparin co-factor II-dependent heparinoid with mostly anti-factor Xa activity, and *fondaparinux*, a synthetic pentasaccharide identical to the high-affinity pentasaccharide of antithrombin responsible for antithrombin-dependent inhibition of factor Xa. All drugs must be given either intravenously or subcutaneously. Argatroban is mainly eliminated in the liver, whereas all the other drugs show renal elimination. Lepirudin poses a special problem related to antigenicity and secondary antibody development. Only argatroban, danaparoid, and lepirudin are variously licensed for the treatment of HIT in different countries, whereas treatment with bivalirudin and fondaparinux is still anecdotal (Warkentin et al. 2008). Recently, synthetic, oral, direct inhibitors of either thrombin, for example, dabigatran etixilate, or factor Xa, for example, rivaroxaban, have been developed. These drugs most probably do not need monitoring of anticoagulant effect and have shown promising results in large, randomized, clinical trials of patients with acute venous thrombosis (Buller et al. 2008; Schulman et al. 2009). These drugs have great potential to replace and greatly simplify established treatment of HIT in the future.

Initiation of anti-vitamin K therapy is normally associated with a rapid decrease in protein C activity, which far exceeds the much slower decrease in the vitamin K-dependent clotting factor activities (especially decrease in prothrombin). This mechanism is commonly thought to be responsible for microvascular thrombosis and the "blue toe syndrome" that may develop on anti-vitamin K treatment (O'Keeffe et al. 1992). In patients with HIT, this mechanism coincides with the additional thrombin generation triggered by HIT and may cause severe microvascular thrombosis. Current guidelines therefore advocate to postpone anti-vitamin K treatment until platelet count has recovered, and to administer vitamin K to those who has commenced anti-vitamin K therapy (Warkentin et al. 2008). An alternative option might be to continue alternative anticoagulation in therapeutic doses combined with anti-vitamin K for at least 7 days and until international normalized ratio is within target therapeutic range (2.0–3.0) on two consecutive days, similar to treatment of patients with protein C deficiency. Theoretically, this approach should not be associated with excess thrombin generation and excess risk of thrombosis, but whether this is a safe approach needs validation in future studies.

5 Immunobiology of HIT

The immunobiology of HIT is still not fully understood. It is recognized that HIT antigens are exposed on CXCL4 bound to heparin or other sulfated polysaccharides (Amiral et al. 1992). Serial CXCL4 molecules may then align on these polyanions to build linear multimolecular structures (Rauova et al. 2006). The immunogenicity

of these complexes seems to be influenced by relative size, amount, and stability of the CXCL4/heparin complexes (Greinacher et al. 2008), and unfractionated heparin > low molecular weight heparin > fondaparinux in complex with CXCL4 is immunogenic. Mainly IgG is generated, and HIT IgG then binds to CXCL4 via their F(ab) domains. With the alignment or clustering of multiple CXCL4 molecules on heparin, several IgG molecules may bind to the CXCL4/heparin complexes. Simultaneously, IgG of these complexes may bind to the platelet FcγIIa receptors, resulting in platelet cross-linking and simulating platelet aggregation. Moderate platelet consumption and thrombocytopenia then follows (Kelton et al. 1988). Moreover, platelet cross-linking results in platelet activation and release of procoagulant microparticles. Binding of pathogenic IgG to endothelial cells (Arepally and Ortel 2006) and monocytes (Pouplard et al. 2001) may possibly lead to activation of these cell lines and contribute to the prothrombotic phenotype.

Although there is strong evidence that HIT is mediated by antibodies against CXCL4/heparin, HIT has several atypical features that differ from the classical antigen-induced immune response. First, antibodies are generated as early as 5 days from exposure to heparin (Greinacher et al. 2009; Lubenow et al. 2002; Warkentin and Kelton 2001b). Second, mainly IgG isotype is generated, which is typical for a secondary immune response, and with no antibody switching from IgM isotype, which is typical for a primary immune response (Greinacher et al. 2009; Selleng et al. 2009b; Warkentin et al. 2009). Third, heparin-dependent antibodies remain positive for only a few weeks or months after an episode of HIT (Greinacher et al. 2009; Warkentin and Kelton 2001b). Fourth, patients may be reexposed to heparin months or years after HIT without risk of recurrence (Potzsch et al. 2000; Warkentin et al. 2008), suggesting the lack of an anamnestic immune response. Finally, many patients develop antibodies against CXCL4/heparin but do not develop thrombocytopenia or HIT (Greinacher et al. 2009; Warkentin et al. 2008).

The mechanism(s) of the unusual features of the immune response against CXCL4/heparin are not yet known. Greinacher et al. speculated that the pattern of antibody formation may be compatible with a T-cell-independent immune reaction, which means that production of antibodies in B-cells could be triggered by CXCL4/heparin without the help of T-cells (Greinacher et al. 2009). This immune response is characterized by rapid onset and disappearance and with no memory. Such reactivity has been described for immune reactions against antigens with repetitive epitopes (Zinkernagel and Hengartner 2001), resembling the linear, ridge-like clusters of 100–150 nm size of CXCL4/heparin, in which CXCL4 tetramers expose repetitive epitopes (Greinacher et al. 2006). The single CXCL4 tetramers have a distance of approximately 4–6 nm, similar to the range found to cause T-cell-independent B-cell activation with certain repetitive viral epitopes (Greinacher et al. 2009). Recently, Selleng et al. reported that the anti-CXCL4/heparin immune response was infrequently associated with the formation of specific memory cells (Selleng et al. 2009a). These investigators speculated that CD5+ B-1 B-cells, and not the typical B-2 B-cells, were involved. Such B-cells can be activated independent of T-cell help and can produce some IgG, usually with low affinity for the target antigen (Selleng et al. 2009a).

However, a T-cell-independent B-cell activation should primarily generate IgM antibodies, whereas IgG predominates in HIT. Moreover, a mouse model found that the immune response was T-cell-dependent (Suvarna et al. 2005). Both findings are supportive of a T-cell-dependent activation, and do also suggest previous contact(s) between the immune system and HIT resembling antigens. One possibility is that early exposure to HIT resembling antigens, that is, CXCL4 complexes clustering on non-heparin factors, may induce a T-cell-dependent antibody class switch of B-cells, and that later in life, these B-cells may be rapidly activated when CXCL4 clusters are produced by heparin combined with release of CXCL4 and proinflammatory mediators (Greinacher 2009).

Finally, it should be mentioned that CXCL4 is a 70-amino acid protein that is produced in megakaryocytes and released from the alpha-granules of activated platelets. It binds with high affinity to negatively charged glycosaminoglycans, including heparin, and heparan-, dermatan-, and chondroitin sulfates, which is a key event for the development of HIT (Kowalska et al. 2009). CXCL4 may also bind to thrombomodulin and facilitate protein C activation (Dudek et al. 1997), which could be an anticoagulant effect of CXCL4. CXCL4 is chemotactic for neutrophils, fibroblasts, and monocytes. It does not bind to the chemokine receptors CXCR1 or 2, but it binds with a splice variant of CXCR3, known as CXCR3B, which is found on endothelial cells and on activated T-lymphocytes, and which exerts strong antiproliferative effects (Kowalska et al. 2009). Because of these roles, it is predicted to play a role in wound repair and inflammation (Dimberg 2010).

6 Disclosure of Interests

The author has served on an advisory board and received an honorarium from Mitsubishi Pharmaceuticals.

References

Amiral J, Bridey F, Dreyfus M, Vissoc AM, Fressinaud E, Wolf M, Meyer D (1992) Platelet factor 4 complexed to heparin is the target for antibodies generated in heparin-induced thrombocytopenia. Thromb Haemost 68:95–96

Arepally GM, Ortel TL (2006) Clinical practice. Heparin-induced thrombocytopenia. N Engl J Med 355:809–817

Buller HR, Lensing AW, Prins MH, Agnelli G, Cohen A, Gallus AS, Misselwitz F, Raskob G, Schellong S, Segers A (2008) A dose-ranging study evaluating once-daily oral administration of the factor Xa inhibitor rivaroxaban in the treatment of patients with acute symptomatic deep vein thrombosis: the Einstein-DVT dose-ranging study. Blood 112:2242–2247

Dimberg A (2010) Chemokines in angiogenesis. Curr Top Microbiol Immunol. doi: 10.1007/82_2010_21

Dudek AZ, Pennell CA, Decker TD, Young TA, Key NS, Slungaard A (1997) Platelet factor 4 binds to glycanated forms of thrombomodulin and to protein C. A potential mechanism for enhancing generation of activated protein C. J Biol Chem 272:31785–31792

Eichler P, Raschke R, Lubenow N, Meyer O, Schwind P, Greinacher A (2002) The new ID-heparin/PF4 antibody test for rapid detection of heparin-induced antibodies in comparison with functional and antigenic assays. Br J Haematol 116:887–891

Greinacher A (2009) Heparin-induced thrombocytopenia. J Thromb Haemost 7(Suppl 1):9–12

Greinacher A, Michels I, Kiefel V, Mueller-Eckhardt C (1991) A rapid and sensitive test for diagnosing heparin-associated thrombocytopenia. Thromb Haemost 66:734–736

Greinacher A, Farner B, Kroll H, Kohlmann T, Warkentin TE, Eichler P (2005) Clinical features of heparin-induced thrombocytopenia including risk factors for thrombosis. A retrospective analysis of 408 patients. Thromb Haemost 94:132–135

Greinacher A, Gopinadhan M, Gunther JU, Omer-Adam MA, Strobel U, Warkentin TE, Papastavrou G, Weitschies W, Helm CA (2006) Close approximation of two platelet factor 4 tetramers by charge neutralization forms the antigens recognized by HIT antibodies. Arterioscler Thromb Vasc Biol 26:2386–2393

Greinacher A, Juhl D, Strobel U, Wessel A, Lubenow N, Selleng K, Eichler P, Warkentin TE (2007) Heparin-induced thrombocytopenia: a prospective study on the incidence, platelet-activating capacity and clinical significance of antiplatelet factor 4/heparin antibodies of the IgG, IgM, and IgA classes. J Thromb Haemost 5:1666–1673

Greinacher A, Alban S, Omer-Adam MA, Weitschies W, Warkentin TE (2008) Heparin-induced thrombocytopenia: a stoichiometry-based model to explain the differing immunogenicities of unfractionated heparin, low-molecular-weight heparin, and fondaparinux in different clinical settings. Thromb Res 122:211–220

Greinacher A, Kohlmann T, Strobel U, Sheppard JA, Warkentin TE (2009) The temporal profile of the anti-PF4/heparin immune response. Blood 113:4970–4976

Kelton JG, Sheridan D, Santos A, Smith J, Steeves K, Smith C, Brown C, Murphy WG (1988) Heparin-induced thrombocytopenia: laboratory studies. Blood 72:925–930

Kowalska MA, Rauova L, Poncz M (2009) Role of the platelet chemokine platelet factor 4 (PF4) in hemostasis and thrombosis. Thromb Res. doi:10.1016/j.thromres.2009.11.023

Lo GK, Juhl D, Warkentin TE, Sigouin CS, Eichler P, Greinacher A (2006) Evaluation of pretest clinical score (4 T's) for the diagnosis of heparin-induced thrombocytopenia in two clinical settings. J Thromb Haemost 4:759–765

Lubenow N, Kempf R, Eichner A, Eichler P, Carlsson LE, Greinacher A (2002) Heparin-induced thrombocytopenia: temporal pattern of thrombocytopenia in relation to initial use or reexposure to heparin. Chest 122:37–42

O'Keeffe ST, Woods BO, Breslin DJ, Tsapatsaris NP (1992) Blue toe syndrome. Causes and management. Arch Intern Med 152:2197–2202

Patrick AR, Winkelmayer WC, Avorn J, Fischer MA (2007) Strategies for the management of suspected heparin-induced thrombocytopenia: a cost-effectiveness analysis. Pharmacoeconomics 25:949–961

Potzsch B, Klovekorn WP, Madlener K (2000) Use of heparin during cardiopulmonary bypass in patients with a history of heparin-induced thrombocytopenia. N Engl J Med 343:515

Pouplard C, May MA, Iochmann S, Amiral J, Vissac AM, Marchand M, Gruel Y (1999) Antibodies to platelet factor 4-heparin after cardiopulmonary bypass in patients anticoagulated with unfractionated heparin or a low-molecular-weight heparin: clinical implications for heparin-induced thrombocytopenia. Circulation 99:2530–2536

Pouplard C, Iochmann S, Renard B, Herault O, Colombat P, Amiral J, Gruel Y (2001) Induction of monocyte tissue factor expression by antibodies to heparin-platelet factor 4 complexes developed in heparin-induced thrombocytopenia. Blood 97:3300–3302

Pouplard C, Gueret P, Fouassier M, Ternisien C, Trossaert M, Regina S, Gruel Y (2007) Prospective evaluation of the '4Ts' score and particle gel immunoassay specific to heparin/PF4 for the diagnosis of heparin-induced thrombocytopenia. J Thromb Haemost 5:1373–1379

Rauova L, Zhai L, Kowalska MA, Arepally GM, Cines DB, Poncz M (2006) Role of platelet surface PF4 antigenic complexes in heparin-induced thrombocytopenia pathogenesis: diagnostic and therapeutic implications. Blood 107:2346–2353

Schenk S, El-Banayosy A, Morshuis M, Arusoglu L, Eichler P, Lubenow N, Tenderich G, Koerfer R, Greinacher A, Prohaska W (2007) IgG classification of anti-PF4/heparin antibodies to identify patients with heparin-induced thrombocytopenia during mechanical circulatory support. J Thromb Haemost 5:235–241

Schulman S, Kearon C, Kakkar AK, Mismetti P, Schellong S, Eriksson H, Baanstra D, Schnee J, Goldhaber SZ (2009) Dabigatran versus warfarin in the treatment of acute venous thromboembolism. N Engl J Med 361:2342–2352

Selleng K, Schutt A, Selleng S, Warkentin TE, Greinacher A (2009a) Studies of the anti-platelet factor 4/heparin immune response: adapting the enzyme-linked immunosorbent spot assay for detection of memory B cells against complex antigens. Transfusion 50(1):32–39

Selleng K, Warkentin TE, Sheppard JA, Greinacher A (2009b) Immune mechanisms in heparin-induced thrombocytopenia: no evidence for immunoglobulin M anti-idiotype antibodies. Transfusion 49:1812–1818

Sheridan D, Carter C, Kelton JG (1986) A diagnostic test for heparin-induced thrombocytopenia. Blood 67:27–30

Suvarna S, Rauova L, McCracken EK, Goss CM, Sachais BS, McKenzie SE, Reilly MP, Gunn MD, Cines DB, Poncz M, Arepally G (2005) PF4/heparin complexes are T cell-dependent antigens. Blood 106:929–931

Wallis DE, Workman DL, Lewis BE, Steen L, Pifarre R, Moran JF (1999) Failure of early heparin cessation as treatment for heparin-induced thrombocytopenia. Am J Med 106:629–635

Warkentin (2007a) Clinical picture of heparin-induced thrombocytopenia. In: Warkentin TE, Greinacher A (eds) Heparin-induced thrombocytopenia, 4th ed. Informa Healthcare, New York, USA, pp 21–66

Warkentin TE (2007b) Drug-induced immune-mediated thrombocytopenia–from purpura to thrombosis. N Engl J Med 356:891–893

Warkentin TE, Heddle NM (2003) Laboratory diagnosis of immune heparin-induced thrombocytopenia. Curr Hematol Rep 2:148–157

Warkentin TE, Kelton JG (2001a) Delayed-onset heparin-induced thrombocytopenia and thrombosis. Ann Intern Med 135:502–506

Warkentin TE, Kelton JG (2001b) Temporal aspects of heparin-induced thrombocytopenia. N Engl J Med 344:1286–1292

Warkentin TE, Levine MN, Hirsh J, Horsewood P, Roberts RS, Gent M, Kelton JG (1995) Heparin-induced thrombocytopenia in patients treated with low-molecular-weight heparin or unfractionated heparin. N Engl J Med 332:1330–1335

Warkentin TE, Sheppard JA, Horsewood P, Simpson PJ, Moore JC, Kelton JG (2000) Impact of the patient population on the risk for heparin-induced thrombocytopenia. Blood 96:1703–1708

Warkentin TE, Roberts RS, Hirsh J, Kelton JG (2003) An improved definition of immune heparin-induced thrombocytopenia in postoperative orthopedic patients. Arch Intern Med 163:2518–2524

Warkentin TE, Sheppard JA, Moore JC, Moore KM, Sigouin CS, Kelton JG (2005) Laboratory testing for the antibodies that cause heparin-induced thrombocytopenia: how much class do we need? J Lab Clin Med 146:341–346

Warkentin TE, Greinacher A, Koster A, Lincoff AM (2008) Treatment and prevention of heparin-induced thrombocytopenia: American College of Chest Physicians evidence-based clinical practice guidelines (8th edn). Chest 133:340S–380S

Warkentin TE, Sheppard JA, Moore JC, Cook RJ, Kelton JG (2009) Studies of the immune response in heparin-induced thrombocytopenia. Blood 113:4963–4969

Zinkernagel RM, Hengartner H (2001) Regulation of the immune response by antigen. Science 293:251–253

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