Chapter 1

Introduction
Introduction

Contents

General introduction............................................................................................................. 12
History .................................................................................................................................. 12
Evolution ............................................................................................................................... 14
Chemokine receptors ........................................................................................................... 14
  CC chemokine receptors .................................................................................................... 14
  CXC chemokine receptors ................................................................................................. 17
  XC-chemokine receptor .................................................................................................... 19
  CX3C-chemokine receptor ............................................................................................... 19
  "Silent" chemokine receptors .......................................................................................... 20
Signal transduction .............................................................................................................. 21
  G-proteins .......................................................................................................................... 22
  Phospholipase C (PLC) ...................................................................................................... 22
  Synaptotagmin .................................................................................................................... 23
  Phosphatidylinositol-3 kinase (PI3K) .................................................................................. 24
  The JAK/STAT pathway ...................................................................................................... 25
  Mitogen activated protein kinase (MAPK) ......................................................................... 25
  Rho GTPases ....................................................................................................................... 25
  G protein-coupled receptor kinases (GRK) and β-arrestin .................................................. 26
  Integrin activation .............................................................................................................. 26
Physiological functions of chemokines .................................................................................. 27
  Migration ............................................................................................................................. 27
  Immune response ............................................................................................................... 27
  Hematopoiesis .................................................................................................................... 28
  Proliferation ....................................................................................................................... 29
  Development ....................................................................................................................... 29
  Angiogenesis ...................................................................................................................... 29
Chemokines in disease ......................................................................................................... 30
  Cancer ................................................................................................................................. 30
  Acquired immunodeficiency syndrome (AIDS) ................................................................ 31
  Allergy ................................................................................................................................. 31
  Autoimmune diseases ........................................................................................................ 32
  Atherosclerosis .................................................................................................................. 32
  Allograft rejection .............................................................................................................. 32
Scope of this thesis .............................................................................................................. 33
Reference List ...................................................................................................................... 34
Chapter 1

General introduction

The chemoa attractant cytokines (chemokines) are small (8-14 kDa) proteins, which are structurally related because of four conserved cysteine residues linked by disulfide bonds. Four different patterns occur. In the CXC chemokines, the two N-terminal cysteines are separated by a single amino acid to form a CXC motif, whereas in the CC chemokines they are adjacent, and form a CC motif. Later two other classes have been described, the C-chemokines, which lack the second cysteine, and the CX3C chemokines, with 3 amino acids between the first two cysteines. Chemokines bind to seven-transmembrane G-protein-coupled receptors, the chemokine receptors. Each of these chemokine receptors has a distinct chemokine and leukocyte specificity. However, the specificities can overlap considerably, because some chemokines can bind multiple chemokine receptors and some chemokine receptors can bind multiple chemokines.

Chemokines and their receptors are important for several physiological functions, but also play a role in major diseases, such as cancer. The research described in this thesis was focused on the role of CXCR4 and CXCR5 in cancer. Furthermore, the signal transduction pathways underlying migration and invasion were studied. In this introduction, I will briefly discuss the history and evolution of chemokines and their receptors. Thereafter, I will present an overview of the chemokine receptors with emphasis on CXCR4 and CXCR5. Since signal transduction is an important issue in this thesis, I will discuss the different pathways induced by chemokines. After an overview of the physiological functions of the chemokines, I will describe their role in diseases. I will start this part by discussing the major role that chemokines and their receptors play in cancer, again emphasizing the roles of CXCR4 and CXCR5.

History

The existence of chemoattractant proteins had been suspected for a long time. Already in the late 19th century Metchnikov predicted the necessity of cell-specific attractant signals. A hundred years later, in 1977, the first chemokine was described, platelet factor 4 (PF4, CXCL4). However, PF4 (CXCL4) was not identified as a chemokine, but as a procoagulant and angiostatic factor that is stored in the α-granules of platelets. In the next ten years, cDNAs for structurally related proteins were cloned, establishing a new gene family before their function was identified.

A landmark in immunology was the identification of the first prototypical chemokine, interleukin-8 (IL-8/CXCL8). CXCL8 was the first chemoattractant found to attract only a subset of leukocytes, namely neutrophils. Later a specific chemoattractant was found for monocytes and T-cells, monocyte chemotactic protein-1 (MCP-1/CCL-2). Initially, most of these proteins were discovered by purification of chemoattractant activity. Later, the search was facilitated by many new methods. Particularly, expressed sequence tag (EST) databases were useful, because the coding sequence of chemokines is sufficiently small to be captured by a single EST. Advances in molecular cloning techniques and availability of bioinformatics-based analyses of nucleotide databases led to an explosive growth of this new family. As the number of family members expanded, a new nomenclature became necessary. In 1992, at the Third International Symposium on Chemotactic Cytokines, the term chemokine, short for "chemotactic cytokine", was accepted.

The chemotaxis towards chemokines was sensitive to pertussis toxin, an inhibitor of G-proteins. This led to the discovery that chemokines bind to seven-transmembrane G-protein-coupled receptors. In 1995, the International Union of the Pharmacology (NC-IUPHAR) established a chemokine receptor nomenclature, based on the subclassification described above in the general introduction. Later, the NC-IUPHAR established a nomenclature for chemokines that paralleled that of the chemokine receptors (table 1).
Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Ligand</th>
<th>Old name ligand</th>
<th>Knockout phenotype</th>
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<tr>
<td>CCR1</td>
<td>CCL3</td>
<td>MIP-1α/LD78</td>
<td>Defect in neutrophil-mediated immune response</td>
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<td>CCL5</td>
<td>RANTES</td>
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<td><strong>CX3C receptor</strong></td>
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<td>Delayed allograft rejection, beneficial effect in atherosclerosis</td>
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</table>
Chapter 1

Evolution

A large number of chemokines and receptors exists with overlapping specificities. Some chemokines can bind multiple chemokine receptors and some chemokine receptors can bind multiple chemokines. The sequences of these promiscuous chemokines differ markedly between species, whereas the monogamous chemokine-receptor pairs have been conserved evolutionarily. The reason these chemokines evolved so rapidly is likely a response to novel micro-organisms that had developed strategies to impede chemokine function in the immune response.

Most, if not all, chemokines probably arose by gene duplication from a single ancestral gene. Most chemokines are clustered in three chromosomal locations, the CC chemokines on 17q11 and the CXC chemokines on 4q21 and 4q12-13. However, some are located elsewhere. These chemokines have highly specific functions and it has been suggested that they are older in evolutionary terms. They also do not share receptors as extensively as the chemokines in the large clusters do. Therefore, the major cluster chemokines are likely generated more recently in evolution. Also the high redundancy in the chemokine family is a very recent phenomenon that is exclusive to higher vertebrates. This is probably necessary for the highly organized adaptive immune system, which is only present in these higher vertebrates.

The division into subclasses is an ancient phenomenon since both CC and CXC chemokines have been found in fish. The chemokine receptor CXCR4 is evolutionary the most conserved of all CXCRs. It has been found in mammals, amphibians, birds and fish, and even in one of the earliest vertebrates, the sea lamprey. Apart from its role in the immune system it is also important in the early development of the central nervous system. The central nervous system is older than the adaptive immune system, and the CXC chemokines and their receptors pre-date the vertebrate immune system. It has therefore been suggested that CXC chemokines were initially involved in processes related to central nervous system development.

Chemokine receptors

Chemokine receptors are seven-transmembrane G-protein coupled receptors. In this thesis we focus on the role of two chemokine receptors, CXCR4 and CXCR5, in metastasis. However, many other chemokine receptors have been implicated in cancer. To understand all possible roles in a complex process as tumor metastasis, it is relevant to consider all aspects of the functions of chemokine receptors. Therefore, I will give an overview of all chemokine receptors. The chemokines and their receptors can be divided into four groups. In addition, chemokine binding proteins exist, which do not signal and are called "silent" receptors. The chemokine receptor nomenclature was created in 1995, and only recently a similar nomenclature for the chemokines was established. The receptors and their ligands with their old and new names are listed in table 1.

CC chemokine receptors

The CC-chemokines generally activate monocytes and not neutrophils. Up to date, 27 CC-chemokines have been discovered and a few splice variants. These CC-chemokines bind to 10 CC-chemokine receptors (CCRs). According to the new chemokine nomenclature, all CC chemokines are named CCL#.

CCR1 was the first CC chemokine receptor identified. It is expressed on monocytes, CD34+ stem cells, dendritic cells, certain peripheral blood lymphocytes and osteoclasts, but not on resting neutrophils. CCR1 binds with high affinity to the inflammatory chemokines CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), murine CCL9 (MIP-1γ), CCL14 (HCC-1), CCL15 (HCC-2), CCL16 (HCC-4) and CCL23 (MPIF-1). CCR2, CCR3 and CCR5 share chemokines with CCR1, but their specificity is different. Since most ligands also bind to other chemokine receptors, CCR1 knockout mice were expected to be viable and to reproduce normally. However, a variety of defects were observed in the innate and acquired immunity. CCR1 appeared to be especially important for the neutrophil-mediated host response. This could be quite different in humans, since the ligands for CCR1 do not have much effect on human neutrophils.

14
Chapter 1

CCR2 binds to CCL2 (MCP-1), CCL7 (MCP-3) and CCL13 (MCP-4) and the mouse CCL12 (MCP-5). It is expressed on monocytes, activated T cells and B cells. CCR2 can homodimerize and heterodimerize with CCR5 and activate different signaling pathways. The recruitment of monocytes/macrophages to sites of inflammation is impaired in these mice. Furthermore, the absence of CCR2 has a beneficial role and results in 50% reduction in atherosclerotic lesions. CCR2 was also found to be an HIV-1 coreceptor in vitro and the presence of a variant allele in human AIDS patients results in a 2- to 4-year delay in disease progression.

CCR3 was found as a chemokine receptor expressed on eosinophils. The chemokines CCL11 (eotaxin), CCL24 (eotaxin-2) and CCL26 (eotaxin-3) are the most potent and selective ligands of CCR3. CCL5, CCL7, CCL8 (MCP-2), CCL13 and CCL15 also bind to CCR3, but to a lesser extent and share the receptor with other CC chemokine receptors. Except for eosinophils, CCR3 is also expressed on basophils, mast cells and Th2 lymphocytes, which are all essential for the development of an allergic response. The CCR3 ligands, CXCL9, CXCL10 and CXCL11, can act as antagonists on CCR3. Normally, these chemokines attract T helper type 1 (Th1) cells, which express CXCR3. By blocking CCR3 they can inhibit T helper type 2 (Th2) influx and thereby change the Th1/Th2 ratio. CCR3 knockout mice are viable, reproduce normally and are healthy under pathogen-free conditions. The recruitment of eosinophils to the lung and skin is severely impaired in the knockouts, but they migrate normally to the draining lymph nodes. As expected, the animals fail to develop airway hyper-responsiveness. Therefore, blocking CCR3 may offer a potential therapy for allergic asthma.

CCR4 is predominantly expressed on Th2 lymphocytes and is upregulated upon activation. CCL17 (TARC) and CCL22 (MDC) are the only specific and high affinity ligands. CCR4 knockout mice develop normally and show no overt morphological or behavioral defects in the unstressed state. The splenocytes of the knockout mice did not migrate towards CCL17 and CCL22 as expected, but surprisingly they also failed to move towards CCL3. No explanation has been found for this yet. CCR4 deletion had no effect on Th2-dependent airway inflammation. However, CCR4 knockout mice showed an unexpected resistance to the lethal effects of the endotoxin LPS in two models of LPS-induced shock. Furthermore, airway hyper-responsiveness was markedly diminished in CCR4 knockout mice during chronic allergy.

CCR5 was originally identified as a receptor for CCL3, CCL4 (MIP-1B) and CCL5. Two months later, five groups simultaneously identified this receptor as a co-receptor for macrophage-tropic human immunodeficiency virus-1 (HIV-1). Shortly thereafter, a 32 base pair deletion was identified in CCR5. This 32-base-pair deletion within the coding region results in a frame shift, and consequently in a severely truncated receptor. This receptor does not reach the cell surface and does not support membrane fusion or infection by macrophage- and dual-tropic HIV-1 strains. Normally, CCR5 is expressed on monocytes and macrophages, but also on certain T cells, dendritic cells and hematopoietic progenitor cells. Since the 32-base-pair deletion in CCR5 did not have any effect in humans, CCR5 knockout mice were also expected to be healthy. However, subtle defects have been found in stressed mice. They have a partial defect in macrophage function and down-modulation of the T cell-dependent immune response was somewhat impaired.

CCR6 is the only known receptor for CCL20 (MIP3α/LARC). Furthermore, beta-defensins appeared to be functional ligands for CCR6. Defensins are anti-microbial factors that contribute to host defense by disrupting the cytoplasmic membrane of microorganisms. Their chemotactic role might be to recruit dendritic and T cells to the site of microbial invasion, linking the innate and adaptive immunity. Similar to defensins some chemokines, including the specific ligand for CCR6, CCL20, have been reported to have anti-microbial effects. CCR6 is expressed on certain dendritic cells and memory T cells and B cells, but not on NK cells, monocytes or granulocytes. The Langerhans-type dendritic cell only reacts to CCL20 and CCR6 has therefore a specific role in the homing of these cells to the skin. CCR6 knockout mice have an impaired humoral immune response to orally administered antigen and to certain viruses.
Chapter 1

The cDNA of CCR7 was already cloned at the time that the IL8 receptors, the first chemokine receptors, were identified.\(^{55}\) However, it took almost five years, before its two highly specific ligands, CCL19 (ELC/MIP-3\(^\beta\)) and CCL21 (SLC/6-C-kin), were identified.\(^{95-98}\) CCL19 and CCL21 are similar in their effects on CCR7, but CCL21 does not induce receptor internalization, whereas CCL19 does.\(^{69}\) CCR7 has a major role in the homing of B lymphocytes, T lymphocytes and dendritic cells.\(^{70,71}\) Naïve and Th1 cells express CCR7 and home to the T cell area, whereas activated Th2 cells lack CCR7 expression and form rings at the periphery of these areas. CCR7-negative effector memory T cells circulate in the periphery. The CCR7-positive central memory T cells express lymph-node homing receptors and lack immediate effector function, but differentiate into CCR7-negative effector cells upon secondary stimulation.\(^{72}\) On dendritic cells CCR7 is upregulated upon maturation and supports the emigration from the peripheral tissues to T cell areas of the lymph nodes.\(^{33,74}\) These important physiological roles of CCR7 are also apparent in the CCR7 knockout mice.\(^{75}\) The mice have profound alterations in all secondary lymphoid organs and a delayed immune response. CCR7 is also found on several carcinomas and melanomas and seems to play a role in lymph node metastasis.\(^{76-78}\)

**CCR8** binds to human chemokines CCL1 (I-309) and CCL16 (LEC/HCC-4).\(^{79-81}\) The murine homologue of CCL1, TCA-3, was discovered even earlier than CCL2, which is regarded as the first discovered CC-chemokine.\(^{82,83}\) However, it took ten years to identify its receptor, which has 71% identity to human CCR8.\(^{84}\) The chemokines CCL4 and CCL17 have also been described as ligands for CCR8, but this was denied by others.\(^{85,86}\) Furthermore, CCR8 binds to some viral chemokines. Viral macrophage inflammatory protein-I (vMIP-I) appeared to be a strong agonist, whereas viral macrophage inflammatory protein-II (vMIP-II) and viral MCV-encoded chemokine-I (vMCC-I), also known as MC148, act as strong antagonists.\(^{87-89}\) CCR8 is mainly expressed on Th2 cells and a role for CCR8 in allergic inflammation had been suggested.\(^{90}\) One group indeed reported that CCR8-deficient mice have an impaired Th2 response with a decrease in eosinophil recruitment in models of allergic airway inflammation.\(^{91}\) However, two other groups also generated CCR8 knockout mice and did not see the increase in Th2 cytokines and eosinophil recruitment.\(^{82,93}\) The administration of anti-CCL1 antibodies also failed to inhibit allergic inflammation suggesting that CCR8 does not participate in allergic diseases.\(^{93}\)

A specific agonist for CCR9, previously known as the orphan receptor GPR-9-6, is CCL25 (TECK).\(^{94-96}\) Confusingly, the chemokine binding protein D6 has also been described as CCR9, but this receptor does not signal upon ligand binding.\(^{97}\) Two splice variants of CCR9 exist. CCR9A contains 12 additional amino acids at its N terminus compared with CCR9B. Most strikingly, CCL25 acts with higher potency on CCR9A than on CCR9B.\(^{94}\) CCR9 is mainly expressed on small intestinal intraepithelial lymphocytes, pre-pro-B cells and the majority of thymocytes, but not on cutaneous lymphocytes, more mature B cells, natural killer cells, monocytes, eosinophils, basophils and neutrophils.\(^{96,99}\) CCR9 knockout mice have normal B cell development, intrathymic T-cell development and thymocyte selection. However, the number of γδTCR+ cells was decreased in small intestine but increased in large intestine.\(^{99,100}\) CCR9 appeared to have a major role in the localization of T cells to the small intestinal epithelium during an immune response to oral antigen.\(^{101,102}\) Furthermore, only dendritic cells from Peyer's patches can target those activated T cells that express α4β7 and CCR9 to the small intestine.

**CCR10** is the specific receptor for CCL27 (CTACK/Eskine/ILC), and CCL28 (MEC).\(^{103-105}\) CCR10 is expressed on circulating and tissue plasmablasts and plasma cells that secrete the immunoglobulin IgA.\(^{106}\) The presence of CCL28 in gastrointestinal tissues, the salivary gland, mammary gland and trachea may provide a mechanism for the localization of the IgA antibody secreting cells to the organs of the secretory IgA immune system. CCR10 is also expressed on a subset of blood-derived skin-homing memory T cells, which are CCR7-negative and therefore called "effector" memory T cells.\(^{107,110}\) These cells are specifically attracted by CCL27, which supports entry into cutaneous sites and is upregulated upon inflammation. Antibodies against CCL27 causes impaired lymphocyte recruitment to the skin leading to suppression of allergen-induced skin inflammation. Furthermore, the expression of CCR10 on malignant melanoma cells could account for the high incidence of skin metastases.\(^{78}\)
CXC chemokine receptors

In the CXC-chemokines the two N-terminal cysteines are separated by a single amino acid to form a CXC motif. The CXC chemokines can be further subclassified based on the presence of a glutamic acid-leucine-arginine (ELR) motif, N-terminal to the first cysteine. The ELR+ chemokines act primarily on neutrophils and are angiogenic. Up to date, 16 CXC-chemokines have been discovered, which bind to 6 CXCRs. According to the new chemokine nomenclature, all CXC chemokines are named CXCL#.9

CXCR1 cDNA was isolated in 1991 as the receptor for interleukin-8 (IL-8/CXCL8).111 CXCR1 and CXCR2 are the only receptors that bind to the chemokines with the ELR motif. In addition to CXCL8, also CXCL6 (GCP-2) binds to CXCR1.112 Both CXCL6 and CXCL8 have a basic residue at the sixth position after the second cysteine. This cysteine is required for their binding to CXCR1, but not to CXCR2. CXCR1 was first detected on neutrophils, but is also expressed by monocytes, T lymphocytes, natural killer cells, and dendritic cells.109;111;113;114 Despite abundant evidence that CXCL8 is important in acute inflammation115, the different functions of CXCR1 and CXCR2 are still not clear. The main reason is that this can not be studied in rodents, since a mouse homologue of CXCR1 has not been found and the rat CXCR1 is not expressed in neutrophils, but in macrophages.116

CXCR2 is the second receptor for CXCL8 and was therefore called IL8Rb.117 This receptor binds to CXCL8 and all other CXC chemokines with the ELR motif, that is CXCL1 (MGSA-α/GRO-α), CXCL2 (MGSA-β/GRO-β), CXCL3 (MGSA-γ/GRO-γ), CXCL5 (ENA-78), CXCL6 and CXCL7 (NAP-2).118-120 CXCR2 is expressed on neutrophils, but also on monocytes, T lymphocytes, natural killer cells, mast cells and dendritic cells.109;111;113;114;121 CXCR2 knockout mice appear healthy. However, a massive expansion of neutrophils and B-cells throughout the hematopoietic system was observed in these mice, when kept under normal conditions, but not in a germ-free environment.115;122;133 Probably, these mice have problems with the elimination of microorganisms and react by producing more of the cytokines that increase neutrophil production. Furthermore, CXCR2 knockout mice show impaired recruitment of neutrophils to sites of acute inflammation, where a rapid and early recruitment is necessary.115;124 It should be noted, however, that mice are poor models for CXCL8 function, since no mouse homologues for human CXCL8 and the other CXCL8 receptor, CXCR1, have been found.

CXCR3 is expressed on the majority of memory and activated T cells, primarily on Th0 and Th1 cells.125;126;127 CXCR3 is also expressed on B cells and natural killer cells. The receptor binds to three ELR-negative chemokines, CXCL9 (Mig), CXCL10 (IP-10) and CXCL11 (I-TAC).126-128 Exceptionally, the CC-chemokines CCL11 and CCL13 also bind to CXCR3, but with lower affinity and without activating the receptor. As described above, these chemokines are ligands for CCR3, which is expressed on Th2 cells.129 By blocking CXCR3 they can inhibit Th1 influx and thereby enhance the polarization of T cell recruitment, i.e. the ratio between Th1 and Th2 cells. Furthermore, the mouse chemokine CCL21 is a ligand for CXCR3, whereas the human CCL21 is not.128;130 This shows again that there are differences between the human and mouse chemokine system. Deletion of CXCR3 in mice leads to a marked decrease in the speed and severity of allograft rejection in vivo and a substantial delay in the onset of type 1 diabetes.131;132

CXCR4 was first identified using a monocyte library as a leukocyte-derived seven-transmembrane domain receptor (LESTR).133 Two years later, the same orphan receptor was discovered to be an essential co-receptor for T-tropic HIV entry into CD4-expressing cells.134,135 It was called fusin, because it facilitated fusion of the AIDS virus with the membrane. In 1993 its only ligand CXCL12 (SDF-1) was already cloned from a cDNA library from a bone marrow stromal cell line.136 It was found to support the proliferation of bone marrow B-cell progenitors in the presence of interleukin-7.136 The CXCL12 gene is alternatively spliced to form two variants which differ by 4 amino acids at the C-terminus, but their function is indistinguishable. CXCL12 appeared to be a highly efficacious lymphocyte and monocyte chemoattractant, which unlike other chemokines is expressed constitutively in a broad range of tissues.135-137 In 1996, this chemokine was found to block the T-tropic HIV entry by binding to fusin/LESTR, which was now renamed CXCR4.137;138 At that time its importance in physiological processes was still unknown. CXCR4 is unusually widely
Chapter 1

expressed, namely on T cells, B cells, monocytes, macrophages, dendritic cells, several neuronal cells, endothelial cells, hematopoietic progenitors, platelets and even on some epithelial cells.14,15,16-15

Disruption of the genes of either CXCR4 or its ligand CXCL12 is embryonically lethal.14,15,16 The mice die in uterus and display profound defects in many organs. At embryonic day 11 CXCR4-deficient embryos were still indistinguishable from wild-type littermates, but at day 18 half of the CXCR4-deficient mice had died. The mice exhibited dysplasia of the ventricular septum of the heart. Furthermore, profound defects were visible in cerebellar development. Cells from the external granule layer (EGL) of the cerebellum prematurely migrated into the internal granule layer (IGL).14,15 CXCL12 not only retains granule cells in the EGL, but promotes the proliferation of the granule precursor cells in the EGL together with Sonic hedgehog.14 The CXCR4 receptor is also involved in vascular development in the gastrointestinal tract, but not the vasculature of other organs, such as the brain and the heart.14 The initial capillary network is formed normally in CXCR4- and CXCL12-deficient mice, but remodeling is impaired. Therefore, the larger branches of the vessels are missing. This has, however, no consequences for the development of the gastrointestinal organs.

CXCR4 and its ligand CXCL12 have been implicated in the homing of hematopoietic precursors to the bone marrow. However, CXCR4- and CXCL12-deficient mice have only defects in B cell lymphopoiesis and myelopoiesis and not in T cell lymphopoiesis.14,15,16,17 B-cell lymphopoiesis is blocked in the pro-B-cell stage. This is not due to a defect in homing to specific growth-supportive B cell niches, but due to a deficiency in commitment or proliferation. Cells of the myeloid lineage develop normally in the fetal liver, but fail to colonize the bone marrow. Normally CXCR4 is also highly expressed in the thymus, particularly by immature CD4+ CD8+ T cells. However, no defect was observed in T cell lymphopoiesis. When thymuses from CXCR4-embryos were implanted into T cell-deficient mice, the knockout thymocytes developed normally and efficiently populated the peripheral lymphoid organs of the recipient, showing that CXCR4 is not necessary for their maturation and emigration.

The colonization of the gonads by primordial germ cells, the progenitors of sperm and oocytes, is also impaired in CXCL12 knockout mice, but the initial migration through tissues is unaltered.14,15 The authors suggest that proliferation of the primordial germ cells in the gonads is again normal. In zebrafish CXCR4b knockouts are healthy, probably because the other CXCR4, CXCR4a, can take over. However, CXCL12 has an essential role in the guiding of the primordial germ cells.15,16

Since no viable knockout mice are available, the function of CXCR4 in the adult is hard to establish. However, it is suggested that CXCR4 is necessary for human stem cell engraftment.15 Furthermore, CXCL12 is important for the activation of CD4+ T cells.154 CXCL12 costimulates CD4+ T cells in the presence of anti-CD3 antibody or antigen, resulting in upregulation of the expression of activation markers, proliferation, and cytokine production. In this thesis we focus on the role of CXCR4 in cancer. The receptor is expressed by many lymphomas and carcinomas and appears to be essential for metastasis (see also Chemokines in disease: Cancer and chapter 2 and 6 of this thesis).78,155-158 Because CXCR4 is also a co-receptor for HIV-1 several peptide and non-peptide inhibitors, such as AMD3100, ALX40-4C and T22, have been developed for the treatment of AIDS.161-163 Possibly, these CXCR4 inhibitors can also be used for the treatment of cancer.

In 1992, CXCR5 was discovered as a G-protein coupled receptor expressed in Burkitt's lymphoma cells.154 CXCR5 is mainly expressed on peripheral blood B cells, but only on a fraction of cord blood and bone marrow B cells.156,157 A small subset of peripheral blood memory T cells also expresses CXCR5. In secondary lymphoid organs the majority of T-helper cells are CXCR5-positive in order to localize them to the B cell area where they can provide B-cell help. Moreover, CXCR5 is expressed in the granule and Purkinje cell layer of the cerebellum. CXCL13 (BCA-1/BLC) is the only known ligand for CXCR5 and is constitutively expressed in secondary lymphoid organs and liver.157,158 In the spleen, CXCL13 is present in B-cell rich areas in the outer regions of the white pulp. In lymph nodes CXCL13 is also expressed in the B-cell follicles. This chemokine is expressed by resident stromal cells, the follicular dendritic cells, which are localized in these follicles. In Peyer's patches CXCL13 is most strongly expressed in germinal centers, where B cells undergo somatic mutation and affinity maturation. Due to the expression of this chemokine naive B
lymphocytes localize efficiently to the B-cell areas of secondary lymphoid organs. Once a B cell has bound enough antigen, it is redirected to the boundary of the B cell and T cell areas. The B cell expresses more chemokines, which attract specific T-helper cells. Furthermore, after antigen encounter, helper T cells downregulate CCR7 and upregulate CXCR5, also allowing them to enter the boundary of the B cell and T cell areas. At this boundary the antigen-presenting B cells can encounter the antigen-specific T cells.

As expected, mice lacking CXCR5 have a severe defect in normal B cell migration and localization. This impaired migration causes a rise in the number of B cells in peripheral blood. The mice have no inguinal lymph nodes and they lack germinal centers in the spleen. However, transferred CXCR5 

\footnote{176} B cells are found in inguinal lymph nodes of the wild-type recipient. This suggests that the lack of these lymph nodes is not only due to impaired migration, but that CXCR5 plays a role in their development. Furthermore, the CXCR5 knockout mice only have a few Peyer's patches, whereas the lamina propria of the intestinal mucosa was not altered. Surprisingly, after antigen challenge the immunoglobulin levels in these mice are normal. In CXCR5 

\footnote{177} double knockout mice all peripheral lymph nodes are lacking, suggesting that CXCR5 and CCR7 are both necessary for lymphoid organ formation and organization.

Treatment of B cells with pertussis toxin, which blocks Gi proteins, inhibits the chemotaxis towards CXCL13. However, chemotaxis and Ca\textsuperscript{2+} signaling of CXCR5-transfected HEK293 cells appears to be pertussis toxin insensitive, whereas Erk activation is G protein-dependent. Indeed, in the CXCR5-transfected colon carcinoma cell line CT-26, migration towards CXCL13 is also pertussis toxin-insensitive (chapter 7 of this thesis). Probably, the specificity of G-protein coupling can differ between cell types. In addition to the Burkitt's lymphomas, in which it was originally identified, CXCR5 is also expressed in other lymphomas. In chapter 7, results are described that suggest a role for CXCR5 in proliferation and metastasis of carcinomas.

**CXCR6** is the receptor for CXCL16, which is its only ligand. In addition to the chemokine domain, CXCL16 also has a mucin-like stalk, as previously found only in the CX3C chemokine fractalkine (CX3CL1). CXCL16 is expressed by antigen-presenting cells, whereas CXCR6 is expressed by specific subsets of T lymphocytes, but not B cells, monocytes or dendritic cells. This expression pattern suggests a role in promoting interactions between dendritic cells and T cells, and thus in supporting antigen presentation. Mice in which the gene for CXCR6 was replaced by EGFP were healthy and fertile. It has been suggested that CXCR6 plays a role in the recruiting T lymphocytes to sites of inflammation. Moreover, its ligand CXCL16 may play an additional role in the clearing of bacteria since it can support binding and phagocytosis of Gram-negative and Gram-positive bacteria.

**XC-chemokine receptor**

In the XC-chemokines the first and third cysteines are missing and therefore only one disulfide bridge is formed. These chemokines also have a longer C-terminal tail compared to the other chemokines. Up to date, 2 XC-chemokines have been discovered. According to the new chemokine nomenclature, the two XC chemokines are named XCL\#.\footnote{9}

**XCR1** is the only known chemokine receptor of the XC class. It binds to both XC chemokines, XCL1 (SCM-1alpha/lymphotactin) and XCL2 (SCM-1beta). XCR1 is expressed on T cells, B cells and neutrophils. XCR1 has a role in the regulation of T cell proliferation. XCL1 acts as a negative regulator of proliferation of helper T cells and even induces apoptosis of these cells, whereas it positively regulates the proliferation of cytotoxic T cells. It has been suggested to use XCL-1 in combination with interleukin-2 (IL-2) for cancer therapy. A vaccine with allogeneic tumor cells, which express XCL1 and IL-2, can induce an anti-tumor immune response.

**CX3C-chemokine receptor**

In the CX3C-chemokine the two N-terminal cysteines are separated by three amino acids to form a CX3C motif. Up to date, only one CX3C-chemokine has been discovered, which binds to CX3CR1. According to the new chemokine nomenclature, this chemokine is called CX3CL1.\footnote{9}

CX3CL1 (fractalkine/neurotactin), the ligand of CX3CR1, is one of the two known chemokines that is membrane-bound. As discussed above, the other membrane-bound
chemokine with a mucin-like stalk is CXCL16. CCL1 is expressed on activated endothelial cells, dendritic cells, mast cells and neurons. In contrast to other chemokines that can induce cell adhesion by activating integrins, CCL1 can induce firm adhesion to the membrane-bound chemokine itself, independent of integrins. CCL1 requires pertussis toxin-sensitive G proteins for the induction of migration, but not for the induction of adhesion. CCL1 is expressed on monocytes, neutrophils, natural killer cells, T lymphocytes and in several organs, including the brain. CCL1 can also act as a co-receptor for HIV-1. CCL1 knockout mice showed no overt developmental or morphological abnormalities. The absence of CCL1 confers protection from cardiac transplant rejection and has a beneficial effect on atherosclerotic plaque formation. Consistently, patients with a common polymorphism in CCL1 have a decreased incidence of coronary artery disease. In contrast, HIV-infected patients with the same polymorphism progressed to AIDS more rapidly. The exact role of CCL1 in the pathology of AIDS is still unclear.

"Silent" chemokine receptors

The "silent" chemokine receptors lack the important DRY sequence in the third intracellular loop, which is required for signaling. Binding to chemokines therefore does not elicit signals. The function of the "silent" receptors is mostly unknown. They have been suggested to function as decoy receptors or as transporters or presenters of chemokines.

DARC (Duffy antigen receptor for chemokines) has become of particular interest, because of its role in malaria. Plasmodium parasites use DARC to enter the red blood cell. Most West Africans and African Americans lack DARC on their erythrocytes and appear to be resistant to malaria. DARC binds to basic CC chemokines as well as CXC chemokines with the ELR motif. In addition to erythrocytes, DARC is also expressed by endothelial cells of post-capillary venules and by Purkinje cells of the cerebellum. DARC was thought to be an intravascular "sink" for chemokines. However, in DARC knockout mice chemokines disappear more rapidly from the plasma than in wild-type mice. Furthermore, the knockout mice show an exaggerated inflammatory response with increased granulocyte accumulation in lung and liver. The exact function of DARC expression on endothelial cells and its role in the inflammatory immune response still needs further investigation.

The chemokine binding protein D6 had previously been renamed CCR9 and CCR10. However, so far no signaling was demonstrated after ligand binding. Therefore, D6 is not included in the CCR# nomenclature. High affinity ligands for D6 are murine CCL2, CCL3, CCL4 and CCL8. With lower affinity also the CC chemokines CCL5, CCL7, CCL11, CCL13 and CCL14 bind to D6. Binding of a chemokine to D6 does not lead to calcium response and chemotaxis, but it leads to efficient internalization and degradation of the ligand. D6 might act as a scavenger receptor in lymphatic vessels, to prevent excessive diffusion of inflammatory chemokines.

CCX-CKR, the human homologue of the bovine orphan receptor PPR1 was designated CCR11 by Schweickart et al. However, the effects they saw upon binding of CCL2, CCL8 and CCL13 were not due to the orphan receptor PPR1. It appeared that the chemokine receptor CCR2 was upregulated in the transfected cells. Another group found that the same orphan receptor binds to CCL19, CCL21 and CCL25, but no signaling function was identified. Therefore, this receptor does not qualify for a CCR# designation and also belongs to the "silent" receptors.

No ligands for human chemokine receptor (HCR) have been reported, but it was suggested to belong to the "silent" receptors. HCR is expressed on neutrophils, macrophages, monocytes, T cells and on dendritic cells during a very small window in their maturation process. Knockout mice for HCR have a defect in contact hypersensitivity (A. Mantovani, chemokine conference, Paris, 2003).
Signal transduction

Chemokine receptors are seven-transmembrane G-protein-coupled receptors. They have long been known to signal via pertussis toxin-sensitive G-proteins. However, this is not the only important signal. In this thesis I primarily focus on the function of the G-proteins. We investigated the role of Go and the small G-proteins in lymphoma migration and invasion (chapter 3). We also investigated the necessary signals for the activation of the β2 integrin LFA-1. In chapter 4 the role of the Gβγ dimer in the migration and invasion of a T cell hybridoma is described. The role of Go in carcinoma metastasis is described in chapter 6. In chapter 5 we show that a special effector of calcium, synaptotagmin, is involved in the migration and invasion of the T cell hybridoma. The different roles of the many involved signal transducers, which can be activated by chemokine receptors, are discussed below. An overview of signal transduction molecules activated by chemokine receptors is depicted in Fig. 1.

![Diagram of signal transduction pathways](Image)

**Fig. 1.** Schematic overview of the complex signaling pathways involved in chemokine receptor stimulation. Dotted lines are drawn to depict the activation of other effector molecules, which are not discussed in the text.
Chapter 1

G-proteins

The classical view of chemokine receptor signaling requires the activation of heterotrimeric guanine-nucleotide-binding regulatory proteins (G-proteins). G-proteins consist of α-, β- and γ-subunits, the latter two forming a non-dissociable complex. All subunits belong to families of different gene products. The α-subunits can be divided into four classes: Gαi, Gαs, Gαq and G12/G13. Pertussis toxin catalyzes the ADP-ribosylation of C-terminal cysteine residues of the Gα subunits of the G1 class. This modification prevents the interaction between Gα subunits and the receptor, thereby blocking activation of the G-protein. Only CXCR5-induced migration has been described to be insensitive to pertussis toxin. Some studies have shown that chemokine receptors can also couple to other classes of G-proteins. Furthermore, chemokine receptor/G-protein pairing may be cell-specific. In chapter 3, we demonstrate a crucial role for Gα proteins in the invasion and in vivo migration of a T cell hybridoma, dependent on signaling by the CXCR4 chemokine receptor.

The Gα subunits contain a single guanine-nucleotide binding site and possess an intrinsic GTPase activity. G-proteins are inactive in the GDP-bound stage. Binding of a chemokine to its receptor changes the receptor conformation unmasking previously hidden G-protein binding sites. The interaction with the activated receptor drives the guanine-nucleotide exchange resulting in GTP binding to the α-subunit. This leads to a conformational change in the Gα subunit and therefore a lower affinity for the Gβγ dimer. The complex dissociates and both the Gα and the Gβγ subunits can bind to several second messengers. The conformation of the free Gβγ dimer is identical to that in the trimer, suggesting that in the intact trimer the Gα subunit inhibits the interaction of Gβγ with its effectors. The Gα subunits of the four classes have different functions.

Adenylyl cyclase is inhibited by Gα, but activated by Gα. Gαα directly stimulates phospholipase Cβ (PLCb) and activates RhoA. The G12/G13α subunits can also activate RhoA and it is suggested that they regulate Na+/H+ exchange.

The Gβγ dimer can interact with many effectors. Most importantly for migration towards chemokines is its interaction with PLCβ and phosphatidylinositol-3 kinase γ (PI3Kγ). However, Gβγ has also been reported to bind to certain positive ion channels and a number of kinases. In yeast, Gβγ has been shown to bind to members of the small G proteins of the Rho family. Furthermore, an important function of Gβγ together with phosphatidylinositol 4,5-bisphosphates (PIP2) is the recruitment of G-protein-coupled receptor kinases (GRKs) to the membrane (see below). This leads to desensitization and internalization of the chemokine receptors. The overall function of the Gβγ dimers in migration is not clear. On the one hand, they stimulate migration, because of their coupling to second messengers such as PLCβ and PI3Kγ, but on the other hand they inhibit migration, by coupling to GRKs and inducing desensitization. For two chemokine receptors, CCR2 and CXCR2, it has been shown that inhibition of Gβγ activity results in reduced migration. In chapter 4 we show that inhibition of Gβγ promoted CXCL12-induced migration of a T cell lymphoma and resulted in sustained CXCL12-dependent invasion. This was due to a block in desensitization. Moreover, the T cell migration towards CXCL12 was dependent on PI3Kγ, but after blocking the Gβγ subunits, the cells migrated independently of PI3Kγ.

Phospholipase C (PLC)

Chemokines induce a rapid rise in intracellular Ca2+ concentration. This is mainly due to the activation of phospholipase C (PLC). The PLC isozymes can be divided into three types, PLCβ, PLCγ and PLCδ. Both the α-subunit of Gα and the Gβγ dimer can interact with phospholipase Cβ (PLCb), but in a different region. PLCγ is activated by protein tyrosine kinases and it has been suggested that the small GTPase RhoA can activate PLCδ. Furthermore, a raise in Ca2+ can activate all PLCs, but PLCδ is more sensitive to Ca2+ than the others. Upon chemokine activation, PLCβ is activated via Gβγ dimers and/or Gαα, but PLCs from the other classes might also be activated via indirect pathways. Contradictory results have been published on the requirement of PLC for cell migration and the isozyme involved. It probably depends on the cell type and on the chemokine receptor. PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphates (PIP2) to inositol trisphosphate (IP3) and diacylglycerol (DAG). DAG induces translocation of protein kinase C (PKC) to the membrane, where it is activated. The other product of PIP2 hydrolysis, IP3, binds to receptors located on the endoplasmatic reticulum, which stores Ca2+. 

22
Four IP₃ receptors form a Ca²⁺ channel, which opens upon binding of IP₃. This leads to a rapid increase in the concentration of cytosolic Ca²⁺. Ca²⁺ influences many molecules and processes. In this thesis, the role is described of one special Ca²⁺ effector, synaptotagmin, in CXCL12-induced migration (chapter 5).

**Synaptotagmin**

SNAREs (soluble N-ethyl maleimide-sensitive factor (NSF) attachment protein receptors) are located on the membranes of both vesicles (v-SNARE) and the target membrane (t-SNARE) and mediate vesicle fusion with the target membrane. SNAREs bind to each other to form a very stable four-stranded coiled-coil core complex, which docks the vesicle to the membrane. More than a hundred SNARE proteins from diverse organisms have been discovered. In neurons, vesicles loaded with neurotransmitter are docked to the membrane and remain quiescent until an action potential arrives. Through voltage-activated channels the influx of Ca²⁺ is triggered, which activates the calcium-sensor synaptotagmin resulting in fusion of the vesicle. Synaptotagmins are 65 kDa transmembrane proteins with a short extracellular domain. The cytoplasmic part consists of two calcium binding domains, C2A and C2B. Synaptotagmins undergo Ca²⁺-dependent oligomerization, but the structure of these oligomers is still unclear. Thirteen isoforms of synaptotagmin have been identified. The neuron-specific synaptotagmin 1 and 2 have a low affinity for Ca²⁺. Other synaptotagmins are more ubiquitously expressed and can be activated by lower concentrations of Ca²⁺, in the micromolar range.

Three models have been proposed for how synaptotagmin operates as a Ca²⁺ sensor to induce fusion. The first model is depicted in Fig. 2. In the absence of Ca²⁺, SNAREs, synaptotagmin and phosphatidylinositol(4,5)bisphosphate (PIP₂) pre-assemble in a ring-like structure between the membranes that have to fuse. When the concentration of Ca²⁺ rises, synaptotagmins oligomerize and the Ca²⁺-binding loops of the C2 domains penetrate into PIP₂ rafts in the plasma membrane. Together with other factors this leads to dilatation of the neck of a fusion pore, resulting in secretion. Synaptotagmin can bind to SNAREs in the absence of Ca²⁺. Another possibility is that synaptotagmin inhibits the disassembly of the SNAREs and, in response to Ca²⁺, changes its interaction with the SNAREs and thereby facilitates fusion. In the third model, synaptotagmin binds to the SNARE complex after Ca²⁺ binding. It might twist the complex and thereby pull the two membranes closer together so that they can fuse. The models are not mutually exclusive and a combination of elements of the three models may occur.

![Fig. 2. A model for synaptotagmin function during exocytosis. A schematic view of docked vesicle fusion regulated by synaptotagmin. The v-SNARE, synaptobrevin, associates with the t-SNAREs, SNAP25 and syntaxin. The C2B-domain of synaptotagmin weakly binds to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) in the target membrane. When the concentration of Ca²⁺ rises, synaptotagmins oligomerize and the Ca²⁺-binding loops of the C2 domains penetrate into PIP₂ rafts in the plasma membrane. Together with other factors this leads to dilatation of the neck of a fusion pore, resulting in secretion. Other factors clearly have roles in exocytosis but are not considered here. From: Nat.Rev.Mol.Cell Biol. 3:498-508 (2002)²³¹](image-url)
In addition to its role in regulated exocytosis, which leads to neurotransmitter release, synaptotagmin is also involved in endocytosis. Synaptotagmin binds to PIP$_2$ and both can bind to the adaptor protein complex AP-2. AP-2 mediates the assembly of clathrin coats onto the fused membrane, which deform the membrane into a vesicle. This vesicle is pinched off the membrane and endocytosed.

Synaptotagmin homologues have also been found in plants and worms and arose early in evolution before nerve cells existed. Therefore, in early eukaryotes their function can not have been the regulation of neurotransmitter release in the central nervous system, and they must have had a different and probably more general function. Indeed, synaptotagmin-3 and -7 have been implicated in regulated insulin secretion in pancreatic cells. Synaptotagmin-6 is involved in the release of degradative enzymes from the acrosome of sperm cells necessary to penetrate the oocyte. Furthermore, the repair of membrane defects by exocytosis of lysosomes is mediated by synaptotagmin-7. In chapter 5 of this thesis we show that synaptotagmin also plays a role in chemotaxis.

**Phosphatidylinositol-3 kinase (PI3K)**

The phosphatidylinositol-3 kinase (PI3K) family can be divided into four classes. The class I, enzymes consist of a p85 regulatory subunit and a p110 catalytic subunit. The p110$_\alpha$ and p110$_\beta$ catalytic subunits are expressed ubiquitously, but p110$_\delta$ mainly in leukocytes. PI3K enzymes are constitutively active, but normally located in the cytoplasm and not at the membranes that contain its lipid substrate PIP$_2$. PI3K activity is induced in cells by translocation of the enzyme to these membranes. The class II PI3Ks are translocated after tyrosine phosphorylation of the p85 subunit, since the phosphorylated p85 binds to SH2 domains in membrane proteins. The class I$_\beta$ PI3K catalytic subunit p110$\gamma$ lacks the binding site for p85, but instead binds to a p101 regulatory subunit. Free G$\gamma$ dimers that are released from Go after activation of G-protein coupled receptors, but are still attached to the membrane, bind to p101 and thus bring the p110$\gamma$ close to the membrane and induce PI3K$\gamma$ activity. The function and activation of the class II and III PI3K families is largely unknown. However, the class II PI3K-C2$\alpha$ can be activated by the chemokine receptor CCR2, and this is inhibited by pertussis toxin.

Phosphatidylinositol-3 kinases (PI3Ks) phosphorylate the third position of the inositol ring of phosphoinositides. The products are phosphatidylinositol(3)monophosphate (PI(3)P), phosphatidylinositol(3,4)bisphosphate (PI(3,4)P$_2$) and phosphatidylinositol(3,4,5)trisphosphate (PI(3,4,5)P$_3$). Two phosphatases, PTEN and SHIP, can dephosphorylate 3' phosphoinositides and thus counteract the PI3Ks. PI3K$\gamma$ is considered to be the most important PI3K necessary for migration in response to chemokines. However, chemotaxis of PI3K$\gamma$-/- cells is never completely abrogated, but is inhibited by 50 to 90%, depending on the chemokine and cell type, suggesting that other PI3Ks are also important. In chapter 4 we show that in some cases cells can even migrate independently of PI3K. P110$\alpha$ and p110$\delta$ are essential for development, since knockout mice are embryonic lethal. The p110$\gamma$ and p110$\delta$ knockout mice are viable. Antigen receptor signaling in B and T cells was impaired in the p110$\delta$ knockout mice, whereas the inflammatory response was impaired in the p110$\gamma$ knockouts.

Cells can respond to very shallow chemoattractant gradients. The small difference in chemokine concentration between the front and the rear of the cell is amplified intracellularly into a very steep gradient of signaling molecules. PI3Ks and its products are key players in this process and accumulate rapidly at the front of the cell, whereas the phosphatases PTEN and SHIP accumulate at the lateral sides and the rear of the cell. This has been shown for the amoebae Dictyostellium and for neutrophils.

The products of the PI3Ks interact with different effectors. Protein kinase B (PKB/AKT) and phosphoinositide-dependent protein kinase 1 (PDK1) are key serine/threonine kinases which bind to PI(3,4)P$_2$ and PI(3,4,5)P$_3$. PDK1 can phosphorylate PKB/AKT and PKC, whereas PKB activates several transcription factors. Via the Rho GTPases Rac and Cdc42, PI3K can also coordinate the organization of the cytoskeleton, which is important for movement. A third group of effectors is the Tec family of tyrosine kinases, which are activated upon phosphorylation by Src kinases. Their binding to PI(3,4,5)P$_3$ results in recruitment to the membrane and therefore brings them in closer vicinity to the Src kinases. The PI(3,4,5)P$_3$-activated Tec kinases, such as Btk and Itk, then phosphorylate PLC$_\gamma$, leading to enhanced PLC activity and Ca$^{2+}$ mobilization.
The JAK/STAT pathway

Cytokine and growth receptors are known to dimerize, or even form oligomers, upon binding of ligand. This receptor aggregation leads to the activation of associated tyrosine kinases, the Janus kinases (JAK). By co-immunoprecipitation it was demonstrated that the chemokine receptors CCR2, CCR5, CXCR2 and CXCR4 can also dimerize. To show that dimers exist in intact living cells, fluorescence or bioluminescence resonance energy transfer (FRET or BRET) analysis has been used. Thus, oligomers of G-protein-coupled receptors have been demonstrated in yeast and oligomers of CXCR4 and CCR5 in transfected HEK cells. Chemokine receptors have been shown to induce JAK signaling. However, further research is necessary to find out whether dimerization of the receptor is necessary and whether G proteins are involved. Four members of the JAK family are known, JAK1, JAK2, JAK3 and Tyk2, which are activated in response to different cytokines. After ligand binding, the activated JAKs phosphorylate tyrosine residues of the receptor. This provides docking sites for STAT (signaling transducer and activator of transcription) transcription factors, but also for PLCs. The STATs are phosphorylated by JAK and form dimers by SH2-phosphotyrosine interactions. These dimers become activated after the recruitment to the receptor and translocate to the nucleus where they act as transcription factors. The JAK-STAT pathway is mainly implicated in regulating cellular growth and differentiation, but is also important for chemotaxis.

Mitogen activated protein kinase (MAPK)

The family of mitogen-activated protein kinases (MAPKs) consists of four members. The extracellular signal-regulated kinases 1 and 2 (Erk-1 and Erk-2) have a molecular weight of 42 and 44 kDa. The stress activated protein kinases (SAPK) consist of a 38 kDa protein, p38 and the c-Jun N-terminal kinase (JNK). All MAPKs can be activated via the Gαβγ dimer and Gαq. JNKs can also be activated via Gα12 and Gα13. The MAPK family is activated by the phosphorylation of tyrosine or threonine residues. They activate transcription factors, but also protein kinases, which phosphorylate heat shock proteins. All four members of the MAPK family can be activated by chemokine receptors, but the pathways may differ between cell types and chemokine receptors. CCL2, CXCL8 and CX5CL1 induce phosphorylation of all MAPK family members. They are all necessary for CX3CL1-mediated adhesion, whereas only Erk is necessary for CCL2-mediated adhesion and only p38 MAPK for CCL2-induced migration. Binding of CCL11 to CCR3 can activate Erk, and this is dependent on p38 MAPK. Both members of the MAPK family are necessary for migration. Many contradictory results have been published regarding MAPK phosphorylation after CXCR4 stimulation. Sometimes only Erk activation was observed, whereas in other cases also p38 was phosphorylated. Erk and p38 have been implicated in migration, based on effects of inhibitors, but in other cases these inhibitors had no effect. CXCR3 and CXCR5 have only been shown to activate Erk and this is probably not essential for migration. Furthermore, Erk activation after stimulation with CXCL13 is G protein-dependent, in contrast to migration towards CXCL13, which is independent of G proteins.

Rho GTPases

The small GTPases of the Rho family coordinate the dynamic organization of the actin cytoskeleton that is crucial for cell polarization and motility. Many members of the Rho GTPases have been identified, and of these RhoA, Cdc42 and Rac1 have been studied most extensively. Activation of Cdc42 leads to the formation of filopodia. These are thin extensions that make contact with the area in front of the moving cell. In migrating cells, a lamellipodium is formed at the leading edge and this requires Rac1. Movement also involves contraction of the tail and this requires RhoA. The Rho GTPases cycle between an active GTP-bound state and an inactive GDP-bound state. They can exchange nucleotides and hydrolyze GTP at a slow rate, but this is greatly enhanced when catalyzed by Guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs).

Active GTP-bound Rho GTPases interact with multiple downstream effectors. For example, Rac1 and Cdc42 can activate PAK kinases and stimulate actin polymerization via the Arp2/3 complex. RhoA can activate Rock, Dia kinases and P(4)P-5 kinase. Chemokines can activate Rho, Rac and Cdc42, but their effect on migration and adhesion differs between cell types and chemokine receptors. RhoA is involved in adhesion and therefore also in migration of adherent
Chapter 1

cells. In a T cell lymphoma, we showed that for migration towards high concentrations of CXCL12, which is independent of the adhesion molecule LFA-1, RhoA is not necessary. In contrast, RhoA is required for LFA-1-dependent migration towards a low concentration of CXCL12, as well as for CXCR4- and LFA-1-dependent invasion of these cells in vivo (Chapter 3 of this thesis). The GTPase Cdc42 is essential for migration towards C-chemokines as well as CXC-chemokines. In a T cell lymphoma, expression of an activator (GEF) of Rac, Tiam-1, induced invasion. Furthermore, T cells of Rac2-deficient mice do not migrate, underscoring the importance of the Rac GTPases.

**G protein-coupled receptor kinases (GRK) and β-arrestin**

Chemokine receptors are rapidly desensitized and internalized. This process is mediated by G-protein coupled receptor kinases (GRKs) and β-arrestins. Seven members of the GRK family have been identified. Two rhodopsin kinases are known, GRK1 and GRK7, two β-adrenergic receptor kinases (GRK2 and GRK3) and three members of the GRK4 subfamily (GRK4, GRK5 and GRK6). The arrestin family consists of four members, β-arrestin-1, β-arrestin-2 and two visual arrestins. After receptor stimulation G-proteins are activated, resulting in free Gβγ dimers. The free Gβγ together with PIP2, which are both in the membrane, bind GRKs which are thus translocated to the plasma membrane. The GRK phosphorylates the receptor, which increases the affinity for β-arrestin. The binding of β-arrestin to the receptor leads to desensitization because it prevents interaction with G-proteins. β-Arrestin also acts as an adaptor protein that targets the receptors to clathrin-coated pits for internalization. However, internalization can also take place independent of β-arrestins. Furthermore, β-arrestin can also initiate signals by acting as a scaffold protein to recruit Src family tyrosine kinases, MAPKs and other proteins. Actually, overexpression of β-arrestin enhances the migration towards CXCL12 and CCL5 and the migration of β-arrestin-deficient lymphocytes towards CXCL12 is impaired. In contrast, G-protein activation is attenuated after overexpression of β-arrestin, whereas it is enhanced in β-arrestin-deficient cells. Further studies are required to solve this apparent contradiction. The coupling to different GRKs can be important, since GRK6-deficient lymphocytes are impaired in migration, whereas GRK5-deficient lymphocytes are not. In chapter 4 of this thesis we show that blocking the function of the Gβγ dimer results in increased migration and sustained invasion, which is due to a block in desensitization. Indeed, less GRK2 is recruited to the chemokine receptor CXCR4. In this case less GRK2 does not lead to reduced migration, suggesting that GRK2 is not essential for migration.

**Integrin activation**

Cell adhesion molecules of the integrin superfamily are transmembrane proteins that consist of an α- and a β-subunit, which form a stable dimer. Most integrins bind to extracellular matrix components, but others can also bind counterreceptors on other cells mediating cell-cell adhesion. They mediate signal transduction through the cell membrane in two directions. Binding of ligands to the extracellular domain of the heterodimer leads to the activation of certain signaling molecules inside the cell, such as ILK, FAK and ZAP-70. This is called outside-in signaling. Inside-out signaling changes the avidity or affinity of the integrin and leads to cell adhesion. Mainly chemokines, but also growth factors or cytokines can activate signal transduction pathways that lead to inside-out signaling. By a change of the three-dimensional conformation of the integrins the affinity is increased. Higher avidity is due to the clustering of integrins. Together this leads to firm adhesion. Chemokines can activate several signal transduction pathways that lead to the activation of β1, β2 and β7 integrins. In chapter 3 we show that Gi and Gq proteins, myosin and the small GTPases RhoA and Cdc42 are required for the CXCL12-induced activation of the β2 integrin LFA-1. Other signaling molecules have also been shown to be involved in the activation of integrins by chemokines. PI3K can be activated directly by the Gβγ dimer of G-proteins or via H-Ras. The PI3K product PIP3 activates cytoplasm-1, which is an important activator of LFA-1. Another potential signaling molecule, capable of mediating chemokine-induced activation of integrins, is the small GTPase Rap1. Rap1 stimulates association of the protein RAPL with LFA-1, which leads to modulation of both the avidity and affinity of the integrin.
Physiological functions of chemokines

Chemokines play a major role in many diseases, including cancer and AIDS. In this thesis I focus on the role of chemokines in cancer. A thorough understanding of this role is only possible with a profound knowledge of the normal physiological function of the chemokines. Below these functions are briefly discussed.

Migration
An important role of chemokines is the induction of movement of cells. They attract leukocytes to sites of inflammation, but also direct movement of cells to and within lymphoid organs. Furthermore, as described in the previous section, chemokine-induced signals activate cell adhesion molecules, in particular integrins, which are necessary for movement into tissues. The emigration of a leukocyte through the endothelial wall involves multiple steps. An inactive leukocyte circulates in the blood and may roll on the endothelial wall via interactions with selectins, but will not stop. When a cell encounters a chemokine it will activate β1 and/or β2 integrins, which can then bind to their ligands on the endothelial cell surface. This response is extremely fast, and is achieved by rapid induction of a high-affinity integrin conformation, but also by clustering of the integrins. The adherent cell starts crawling along the vessel wall and finally through the endothelial barrier.

This directional migration depends on the polarization of the cell, induced by the chemokine. The response of a cell to a chemotactic stimulus is the extension of protrusions in the direction of migration. Actin filaments push the cell membrane in an outward direction, which results in the formation of large broad lamellipodia or spike-like filopodia. As mentioned before, PI3K and its products are localized at the front of the cell, the so-called leading edge. They can bind to and activate GEFs that regulate the activity of the Rho GTPases Rac and Cdc42. Rac stimulates the formation of lamellipodia by activating WAVE proteins. Cdc42 activation leads to the formation of filopodia, but Cdc42 also affects the polarity of the cell by localization of the microtubule-organizing center. The Rho GTPase RhoA is active at the rear and the sides of the cell and suppresses Rac activity, thereby preventing protrusions at sites other than at the front. Integrins on the cell bind to the extracellular matrix, and the binding sites can serve as traction sites for migration as the cell moves forward over them. The cytoplasmic tail of the integrins interacts with several proteins, such as α-actinin, talin, focal adhesion kinase and other proteins.

Certain cells use proteases to cleave extracellular matrix components for easier access. However, if these proteases are inhibited, cancer cells can change their morphology and move in an amoeboid way independent of these proteases. The next step in movement is the contraction of the cell by actinomysin, which is primarily regulated by activated RhoA. Finally, the integrins at the rear of the cell detach from the substrate so that the cell can move forward. These contraction and detachment steps are most obvious in slow-moving fibroblasts and less clear in fast-moving cells, such as lymphocytes. Furthermore, the migratory behavior of a cell depends highly on the environment.

Immune response
Chemokines not only attract cells and induce their movement, but they are also regulators of the immune response. They link the innate immune response, the first line of defense, to the adaptive immune response, which is characterized by specificity and memory. When a microorganism enters the body, it encounters macrophages and dendritic cells that capture it. Lipopolisaccharides (LPS) on the surface of bacteria bind to toll-like receptors (TLRs) on the macrophages and dendritic cells. This triggers the release of chemokines such as CCL3, CCL4, CCL5, CXCL8 and CXCL10. These chemokines attract immature dendritic cells which express CCR1, CCR5, CCR6 and TLRs. Bacteria, LPS and inflammatory cytokines induce the maturation and differentiation of the dendritic cells. This results in the down-regulation of their original chemokine receptors and the upregulation of CCR7. The cells synthesize large amounts of inflammatory chemokines that attract other cells including additional immature dendritic cells. The ligand for CCR7, CCL21, is expressed on the endothelium of the afferent lymphatic vessels and attracts the antigen-loaded matured dendritic cells. They migrate to the T cell area in the lymph node in response to CCL21, but also CCL19. They produce CCL18 and CCL19, to attract naive T
cells. Dendritic cells interact rapidly with many T cells, which scan for the presented antigen. Activated T cells downregulate CCR7 and upregulate CXCR3 and therefore are attracted into the inflamed tissues.\textsuperscript{310} Other attracted T cells upregulate CXCR5. The stromal cells in the B cell area express CXCL13 that attracts the CXCR5-expressing T cells to the edge of the B cell area, where they provide help to antigen-primed B cells. Some T cells get only partially activated and keep their CCR7 expression. Therefore, they stay in the lymph nodes and are temporarily arrested in their development. During a secondary response these central memory cells can complete differentiation into effector cells.

Naïve T cells migrate to lymphoid tissues, and memory and effector T cells to non-lymphoid tissues. The activated T cell preferentially migrates to the place where the antigen was first encountered. If a T cell is primed by a dendritic cell derived from Peyer's patches, it upregulates CCR9 and the integrin α4β7.\textsuperscript{102} Therefore the T cells will be directed to the small intestine, which contains large amounts of CCL25. In contrast, skin-homing cells express CCR4.\textsuperscript{107} CCL17 is indeed upregulated in inflamed skin and attracts these cells. A small group of skin-homing helper T cells also expresses CCR10.\textsuperscript{107,106} These so called "effector" memory T cells migrate to the skin, because skin keratinocytes are the only cells that express CCL27. They reside in the skin until they encounter antigen during a secondary response. Thus, chemokines together with adhesion molecules direct the homing of T cells in a tissue-selective manner.

All CCR5 ligands, but also CXCL12, costimulate T cells in the presence of anti-CD3 antibody or antigen.\textsuperscript{154,311} Moreover, micromolar doses of CCL5 induce T cell proliferation and cytokine production even in the absence of antigen. The physiological relevance of this is doubtful.\textsuperscript{312} However, if chemokines bind to proteoglycans, the local chemokine levels presented to the receptor are increased.\textsuperscript{313} Micromolar levels of soluble chemokines might be comparable to surface-bound aggregates of chemokines. This antigen-independent activation of T cells has important implications for autoimmunity, because high CCL5 levels are present in the attacked tissues.\textsuperscript{314} This leads to enhanced proliferation and cytokine production by bystander T cells.

**Hematopoiesis**

Except for the direction of leukocytes to the right location during the immune response, chemokines are also important for their development.\textsuperscript{315} Hematopoiesis originates in the early yolk sac. As embryogenesis proceeds, this function is taken over by the fetal liver and after birth by the bone marrow. The hematopoietic stem cell is multipotent and differentiates first to a myeloid or a lymphoid progenitor. Myeloid progenitors differentiate further to granulocytes, monocytes, macrophages, platelets and erythrocytes. Lymphoid progenitors are the precursors of B and T cells.

Differentiation requires homing of progenitors to specialized environments. CXCR4 and its ligand CXCL12 have been implicated in the homing of hematopoietic precursors to the bone marrow. However, CXCR4- and CXCL12-deficient mice have only defects in B cell lymphopoiesis and myelopoiesis and not in T cell lymphopoiesis.\textsuperscript{15} The precursors appear in increased numbers in the blood, indicating that the retention in fetal liver and bone marrow is defective. Normally, B cells leave the bone marrow if they gain immunoglobulins on their surface. In this stage they are less responsive to CXCL12 despite relatively high levels of CXCR4.\textsuperscript{316} For the migration towards the highly specialized B cell areas in secondary lymphoid organs the surface expression of CXCR5 becomes important.\textsuperscript{179} CXCL12 is also expressed in the thymus, but T cell lymphopoiesis is not impaired in the CXCR4 and CXCL12 knockout mice.\textsuperscript{15} The early progenitor T cells are probably attracted to the thymus by another chemokine, CCL25.\textsuperscript{317} In the next stage, T cells move to the remainder of the cortex, where they undergo positive selection. In this stage, CCR9, the receptor for CCL25, is even more highly expressed. Selected thymocytes move to the medulla, from where mature T cells migrate to the peripheral blood. Many chemokines are expressed in the medulla, including CCL17, CCL19, CCL21, CCL22 and CXCL16.\textsuperscript{315} The matured thymocytes first upregulate CCR4 and encounter self-antigens for negative regulation. Only cytotoxic T cells also express CXCR6, suggesting a more specific role for CXCR6. However, CXCR6-deficient mice are not defective in T cell lymphopoiesis.\textsuperscript{179} Finally, the mature T cells loose CCR9 and CXCR4 expression, upregulate CCR7 and leave the thymus.\textsuperscript{316,319} Although all these chemokine receptors seem very important, CCR4-, CCR7- and CCR9-deficiency does not severely affect T cell development.\textsuperscript{38,75,96} Studies with the G-protein inhibitor pertussis toxin, however, suggest that T
cell development does depend on chemokines. It seems likely that the multiple chemokines have redundant functions, so that effects can only be expected in double- or triple- knockout mice.

**Proliferation**

CXCL12 was originally isolated from a bone marrow stromal cell line and was found to support the proliferation of B-cell progenitors in the presence of IL-7. Clearly, CXCL12 is a proliferation-enhancing factor for CD34⁺ progenitors and myeloid progenitors. CXCL12 is also highly expressed in tumors. In ovarian and lung cancer a proliferation-enhancing effect of CXCL12 was seen under suboptimal conditions. By producing their own CXCL12 and upregulating its receptor CXCR4, the tumor cells may be able to grow in distant and less favorable sites. CXCL12 triggers proliferation probably via the Erk and Akt pathways, as shown for glioblastoma cells. However, CXCL12 did not influence proliferation of several B and T cell lymphoma cell lines. CXCL12 even induced apoptosis in T cells after 3 days exposure. This was dependent on Fas and Fas ligand expression. The cleavage of CXCL12 by metalloproteases might explain this. CXCL12, cleaved at the fourth serine residue, has lower affinity for CXCR4, cannot attract receptor CXCR4, the tumor cells may be able to grow in distant and less favorable sites. CXCL12 can be myelosuppressive, such as CCL1-3, CCL9-13, CCL15-16, CCL18-21, CCL23-25, CXCL4-6, CXCL9-10 and XCL1. The reason for this is not clear. The balance between proliferation-promoting and -suppressing cytokines and chemokines is important to keep the inflammatory process under control. In tumors and chronic inflammation the balance is shifted towards proliferation.

**Development**

CXCL12 is the only chemokine essential for life, at least in mice. The mice die in utero and display profound defects in gastric vasculogenesis, cerebellar and cardiac development. Furthermore, the mice have defects in bone marrow myelopoiesis, B-cell lymphopoiesis, but not T cell lymphopoiesis. In brain development, CCR1 and CXCR2 also play a role. CXCR2 directs the development of oligodendrocytes and myelination in the spinal cord. CCR1 has been suggested to play a role in the maturation of neurites and synapse formation in the postnatal period. As described above, CCR7 and CXCR5 are required for the development of secondary lymphoid organs.

**Angiogenesis**

The formation of new blood vessels can be divided into three stages: vasculogenesis, which involves the maturation of mesodermal precursor cells in to haemangioblasts; angiogenesis, in which these cells develop into an initial capillary network; and, finally, pruning and remodeling to form a functional circulatory network. The latter process is impaired in CXCR4-knockout mice. Several physiological processes such as embryonic development and wound healing depend on angiogenesis, the formation of new blood vessels. In chronic inflammation and in tumors, angiogenesis is aberrantly upregulated. The formation of blood vessels depends on a balance between angiogenic factors and angiostatic factors, which promote and inhibit neovascularization, respectively. Normally, the turnover of endothelial cells is very slow, but during wound healing it should be rapid, strictly controlled and transient. Quiescent endothelial cells are activated and the basement membrane and proximal extracellular tissues are degraded. The activated endothelial cells migrate, divide and form new capillaries. As soon as the wound has been repaired, the new vessels virtually disappear. CXC chemokines containing the Glu-Leu-Arg (ELR) motif are potent angiogenic factors. In contrast, ELR-negative CXC chemokines are potent angiostatic factors. Cytokines can regulate the balance between angiogenic and angiostatic chemokines. For example, IL-12 and IL-18 induce the angiostatic cytokine IFN-γ, which induces expression of non-ELR chemokines and suppress ELR⁺ chemokines. An exception is CXCL12, which does not contain the ELR-motif, but does play a role in vasculogenesis. Formation of the
large vessels towards the stomach and intestines was defective in knockout embryos.\textsuperscript{147} CXCR4 is strongly expressed by gastrointestinal endothelium in the embryo. CXCL12 is a chemoattractant for endothelial cells and acts as an angiogenic factor.\textsuperscript{336} Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), important mediators of angiogenesis, increase expression of CXCR4 and CXCL12 in endothelial cells.\textsuperscript{306;337} On its turn, CXCL12 can induce expression of VEGF by endothelial cells amplifying the angiogenic process.\textsuperscript{324} The CC chemokines CCL1 and CCL11 can also induce angiogenesis in vivo.\textsuperscript{358;339} However, it is not clear whether the effects of these chemokines are direct or indirect.

**Chemokines in disease**

Chemokines are required for a well-regulated immune response. However, in certain diseases the immune reaction is out of control, leading to aberrant recruitment of leukocytes, triggered by chemokines. A few years ago some chemokine receptors were discovered to act as co-receptors for HIV. More recently, we and others have described the role of the chemokine receptor CXCR4 in cancer metastasis.

**Cancer**

It has been known for more than a decade that tumor cells produce certain chemokines and it is now clear that cancer cells also express chemokine receptors.\textsuperscript{328;340} They control the infiltration of leukocytes, but are also involved in tumor growth and survival, angiogenesis and the promotion of metastasis.

Inhibition of CXCR4 function results in reduced metastasis of breast carcinoma, colon carcinoma and T cell lymphoma, as described for the latter two tumor types in chapters 2 and 6 of this thesis.\textsuperscript{78;159;156} Overexpression of CXCR4 enhances metastasis of melanomas to the lung, and CCR7 overexpression promotes metastasis of melanomas to the lymph nodes.\textsuperscript{77;157} CXCR4 has also been implicated in the metastasis of neuroblastomas, ovarian cancer, prostate cancer, multiple myeloma, melanomas and B cell lymphomas.\textsuperscript{78;159;160;341-343} CCR7 is involved in the dissemination to lymph nodes, not only of melanomas, but also of lung cancer, esophageal cancer, gastric cancer, breast cancer and Hodgkin disease.\textsuperscript{78;76;173;344;345} Other chemokine receptors have been found to be expressed on malignancies, but their role is not established, yet. CCR1 is expressed in ovarian carcinomas, multiple myeloma and hepatomas.\textsuperscript{342;346;347} CCR2 is expressed in follicular lymphomas and myelomas.\textsuperscript{348;349} CCR3 and CCR4 are expressed in T-cell lymphomas.\textsuperscript{350;351} AIDS-patients heterozygous for the 32-deletion in CCR5 as described above, have a lower risk of developing non-Hodgkin lymphomas.\textsuperscript{352} Furthermore, a prolonged disease-free survival in breast cancer was observed in individuals bearing this allele.\textsuperscript{353} CCR6 is expressed in multiple myeloma, pancreatic cancer and B-cell non-Hodgkin lymphomas.\textsuperscript{342;354;355} CCR9 is expressed in T-cell acute lymphocytic leukemia and is associated with metastasis to the gut.\textsuperscript{356;357} Malignant melanomas express high levels of CCR10.\textsuperscript{78} CXCR1 and CXCR2 are expressed in gastric, colon and pancreatic carcinomas.\textsuperscript{358-360} CXCR3 is expressed on malignant B cells, multiple myeloma and melanomas.\textsuperscript{342;361;362} CXCR5 has been implicated in several lymphomas, such as Burkitt’s lymphoma, cutaneous B cell lymphoma, gastric lymphoma and classical Hodgkin disease.\textsuperscript{164;173-175}

In the first publication describing the role of CXCR4 in breast carcinoma metastasis, it was suggested to be involved in the invasion of tissues from the blood.\textsuperscript{362} For the T cell lymphoma we studied, we in fact demonstrated that CXCR4 is required for invasion, as described in chapter 2.\textsuperscript{364} However, for the CT-26 colon carcinoma cell line we studied, CXCR4 can not play a role in invasion (see chapter 6).\textsuperscript{155} Firstly, metastasis of the colon carcinoma is not blocked by pertussis toxin, an inhibitor of CXCR4-induced migration. Secondly, CXCR4 levels were low in vitro, but strongly upregulated in vivo and only several days after lung colonization. Finally, we showed that the CXCR4-deficient cells did colonize the lungs, but did not grow out, suggesting a role in growth and survival. CXCR5 has only been implicated in several lymphomas, which is expected since CXCR5 is primarily expressed on B cells and T helper cells from which these malignancies originate.\textsuperscript{164;173-175} Strikingly, in the CT-26 colon carcinoma we found that CXCR5 levels were also low in vitro, but strongly upregulated in vivo (chapter 7 of this thesis). The role of this chemokine receptor in metastasis remains to be established, but we found that CXCL13 can promote
proliferation of CXCR5-expressing colon carcinoma cells, and that CXCL13 is abundantly present in the metastases. CXCL12 has been shown to stimulate growth of ovarian and small cell lung cancer.\textsuperscript{321,322} In pancreatic tumor cells and melanomas CXCL1 and CXCL8 are overexpressed compared to normal tissue and enhance tumor cell proliferation.\textsuperscript{325} Moreover, the chemokines CXCL1-3 were first discovered as promoters of melanoma growth.\textsuperscript{327} These ELR-motif containing chemokines can also promote angiogenesis, another feature important in the development of a tumor. Overexpression or administration of angiostatic chemokines, such as CXCL9 and CXCL10, inhibited the growth of tumors.\textsuperscript{365,366} The secretion of chemokines by tumor cells might create an environment that allows them to grow. This may be relevant in the primary tumor but in particular for single metastasized tumor cells that invade a tissue that is quite distinct from the tissue of origin.

The expression of chemokines at the site of the tumor also attracts certain leukocytes, mainly macrophages and T cells. Whether the recruitment of leukocytes is favorable for the tumor remains to be seen. Activated macrophages are capable of killing tumor cells and destroy the tumor vasculature.\textsuperscript{367} However, macrophages can also produce growth and angiogenic factors and promote the proliferation of the tumor. Chemokines involved in the attraction of macrophages are mainly CCL2 and CCL5 and their expression by tumor cells correlates with clinical aggressiveness.\textsuperscript{368,369} Cytotoxic T lymphocytes can kill tumor cells and are the target of many immunotherapy strategies. However, the tumor-associated macrophages produce mainly Th2 type cytokines and chemokines, such as CCL22.\textsuperscript{367} This causes an influx of Th2 cells and eosinophils that suppress the Th1 cell-mediated cellular immune response, which could kill the tumor. By manipulating the balance between Th1 and Th2 cytokines, the tumor can thus impede the immune response.

### Acquired immunodeficiency syndrome (AIDS)

Acquired immunodeficiency syndrome (AIDS) is caused by infection with a retrovirus, human immunodeficiency virus type 1 or 2 (HIV-1 or -2).\textsuperscript{370} HIV-2 is the major cause of AIDS in West Africa and is now spreading in India. Elsewhere, however, it is HIV-1 that causes AIDS. It has long been known that HIV binds to CD4 on helper T cells, but it took a long time before it was discovered that HIV also binds to chemokine receptors and that this is required for entry of the virus.

HIV-1 isolates can be divided into two groups: macrophage-tropic (M-tropic) and T cell-tropic (T-tropic) viruses.\textsuperscript{371} CXCR4 was the first chemokine receptor identified as a co-receptor that is required for fusion of the T-tropic virus with the target cell membrane.\textsuperscript{134} The T-tropic viruses, which replicate in lymphocytes, normally emerge later in infection and are associated with the progression to AIDS and accelerated immune destruction. Next, CCR5 was identified as a co-receptor for M-tropic viruses.\textsuperscript{42,46} This is the prevalent virus shortly after infection and during the asymptomatic period. It replicates in primary macrophages and lymphocytes. Later, CCR2, CCR3, CCR8, CXCR6 and CX3CR1 were also found to act as co-receptors for HIV-1. Furthermore, CCR1, CCR2, CCR3, CCR4, CCR5, CXCR4 and CXCR5 are co-receptors for HIV-2.\textsuperscript{372-374} This has been shown in vitro, but in vivo they do not play a major role. In vivo the main co-receptor is CCR5 and in later phases the virus may use other receptors, but primarily CXCR4.\textsuperscript{371,373}

Some individuals remain uninfected with HIV-1 despite multiple high-risk sexual exposures due to the 32-nucleotide deletion in CCR5 as described above.\textsuperscript{48,53} Heterozygotes progress more slowly towards AIDS. CCR2 is also a co-receptor for HIV-1. In individuals with a variant allele of CCR2, disease progression is delayed by two to four years.\textsuperscript{28}

### Allergy

In allergic reactions, macrophages and dendritic cells, that encounter the allergen, produce cytokines, such as TNF-α and IL-1.\textsuperscript{575} These cytokines trigger epithelial cells to produce CCL11, which attracts eosinophils. The mature macrophages and dendritic cells move to the lymph nodes where they activate antigen-specific T helper 2 (Th2) cells. The Th2 cells express CCR3, CCR4 and CCR8 and move to the site of the allergic reaction due to the presence of CCL17, CCL22 and probably also CCL1. Upon contact with the antigen, they produce more cytokines that trigger more chemokine production by the epithelial cells, which leads to more Th2 cell and eosinophil
Chapter 1

infiltrates. In atopic dermatitis the fibroblasts surrounding the allergic area also produce CCL5, which leads to the influx of Th1 cells.

CCL11 was the first chemokine discovered to be important in allergy and it was expected that CCL11 knockout mice could not develop an allergic reaction. However, the allergic response was only partially reduced, possibly due to compensation by other CCR3 ligands such as CCL24 and CCL26. Indeed, allergic reactions were less severe in CCR3 knockout mice, due to impaired eosinophil recruitment.

Autoimmune diseases

Autoimmunity is due to an immune response to a self-antigen. This leads to leukocyte activation and their accumulation at the target site. Since chemokines are involved in these processes, they also play a role in autoimmune diseases.

In multiple sclerosis (MS) the myelin sheath of the oligodendrocytes in the central nervous system is damaged by T cells reactive to e.g. myelin basic protein. The T cells produce IFN-γ (interferon-γ) and TNF-α that stimulate astrocytes and leukocytes to produce chemokines that attract more macrophages, monocytes and T cells. The auto-reactive Th1 cells express CXCR3 and CCR5. Inhibition of the interaction of CXCR3 with its ligand CXCL10, led to reduced experimental autoimmune encephalomyelitis (EAE), a model for MS in mice. However, CCR5 knockout mice are not resistant to EAE. Surprisingly, mice deficient for either CCR1 or CCR2 are resistant to EAE, suggesting a major role for these chemokine receptors.

In rheumatoid arthritis (RA), myeloid and lymphoid cells infiltrate and eventually destroy cartilage and bone in the joints. Also in this autoimmune disease, chemokines recruiting Th1 cells are elevated at the reactive site. Furthermore, angiogenesis is required for the development of the inflammatory synovial tissue. The angiogenic CXCL5, CXCL8 and CXCL12 are overexpressed in the rheumatoid joint. In addition, CXCL12 promotes the survival of the auto-reactive T cells by protection of apoptosis, probably via enhanced IL-2 production.

In type I diabetes the insulin-producing pancreatic beta cells are destroyed by auto-reactive T cells. However, the role of chemokines is not as clear as in MS or arthritis. CXCL10, CCL4 and CCL5 are expressed in the islet tissue, but also in non-autoimmune tissue. In contrast to the other autoimmune diseases, a specific role was found for CCR4 on the autoimmune T cells. Blocking a ligand for CCR4, CCL22, delayed the development of diabetes, whereas overexpression of CCL22 accelerated it.

Atherosclerosis

Atherosclerosis is a chronic inflammatory process of the major arteries and is the underlying cause of heart attacks. It starts with the upregulation of vascular cell adhesion molecule-1 (VCAM-1) on the endothelium by inflammatory stimuli. Monocytes and T cells adhere to VCAM-1 and migrate through the endothelial wall into the arterial intima. The monocytes acquire macrophage characteristics, express scavenger receptors that bind lipoprotein particles and become foam cells. The foam cells and T cells express inflammatory cytokines and amplify the inflammation, which will ultimately lead to the formation of an atherosclerotic plaque. Growth of the lesion, as a result of continued monocyte recruitment, can lead to plaque rupture or thrombotic vessel occlusion. Three chemokine receptors: CCR2, CXCR2 and CX3CR1, appear to be involved. CCR2- and CXCR2-knockout mice develop smaller lesions than wild-type mice in an atherosclerosis-susceptible background. CCR2 has been suggested to promote the adhesion of monocytes to the vessel wall, and CCR2 to be necessary for the subsequent migration. The membrane-bound chemokine CX3CL1 not only acts as a chemoattractant, but it can also induce firm adhesion, independent of integrins. Deletion of CX3CR1 led to reduced monocyte recruitment and consequently to reduced development of atherosclerotic lesions. Deletion of individual chemokine receptors only provided a 50% decrease in atherosclerosis, suggesting that all three are important.

Allograft rejection

In the absence of immunosuppression, organ transplants are often rejected by T cell alloreactivity. The recruitment of natural killer cells and later Th1 cells to the transplant is crucial for the rejection. CXCR3 is expressed on both cell types, and inhibition of CXCR3 or its ligand CXCL10
results in prolonged allograft survival. In CXCR3-deficient mice the allograft rejection is much delayed. A transplant from a CXCL10 knockout mouse showed prolonged survival. CCR5, expressed on Th1 cells, is also important for allograft rejection, since in CCR5 knockout mice, the survival rate was prolonged. Indeed, humans homozygous for the 32-base pair deletion in CCR5 showed prolonged graft survival. CCR1 and CX3CR1 have also been implicated in allograft rejection. Inhibition of ligand interactions with these receptors, however, resulted only in profound effects when an immunosuppressor was administered simultaneously.

Scope of this thesis

At the start of this research project, it was not known that chemokine receptors play a major role in metastasis. The G protein inhibitor, pertussis toxin, had been found to inhibit the dissemination and invasion of the T cell lymphoma we study. CXCR4 is expressed on the T cell lymphoma and CXCL12 is expressed in the major dissemination sites. Therefore, we suspected that CXCR4 played a role in the process of dissemination. Since disruption of the genes of either CXCR4 or its ligand CXCL12 leads to embryonic death, CXCR4-knockout cells were not available. We therefore used a different approach. As described in chapter 2, we expressed CXCL12 fused to an endoplasmic reticulum (ER) retention signal, KDEL, in the T cell lymphoma. This CXCL12-KDEL is retained in the ER by the KDEL receptor. The CXCL12-KDEL also binds to CXCR4, which is consequently also sequestered in the ER, so that the cells have no CXCR4 on their surface. These cells did not migrate towards CXCL12 and did not invade into fibroblast monolayers. Moreover, in mice injected with these cells no metastases were formed. This showed that CXCR4 plays an essential role in metastasis of these cells. The roles of different signaling pathways underlying migration and invasion are described in chapter 3 and 4. In these chapters we focused mainly on the roles of the different subunits of G-proteins and the Rho GTPases. In chapter 3 we describe the role of the Gq protein and the Rho GTPases Cdc42 and RhoA in CXCL12-induced migration, invasion and metastasis. In chapter 4 we focus on the role of the Gβγ dimer of G-proteins. Blocking the function of this dimer resulted in increased migration and sustained invasion, which was due to a block in desensitization. Moreover the cells became insensitive to inhibitors of PI3K. The response to chemokines is extremely rapid. We hypothesized that this involves rapid fusion of vesicles with the membrane. Such rapid fusion is only possible if the vesicles are already docked, similarly as synaptic vesicles in neurons. In neuronal cells synaptotagmin is the calcium sensor that controls fusion of docked vesicles. In chapter 5 we show that migration and invasion of the T cell lymphoma in response to CXCL12 is inhibited when synaptotagmin function is blocked. This suggests that docked vesicle fusion, regulated by synaptotagmin, is required for migration and invasion induced by CXCL12. In chapters 6 and 7 we focus on the metastasis of a colon carcinoma cell line. CXCR4 is not present on the cells in vitro, but is upregulated in vivo. Using the same method as described above for the T cell lymphoma, we show that CXCR4 is essential for the metastasis of the colon carcinoma. However, in contrast to the T cell lymphoma, we show that CXCR4 does not play a role in invasion, but in the outgrowth of micrometastases. In chapter 7 we show that CXCR5 is also upregulated in the colon carcinoma cell line in vivo. Since this receptor is hardly expressed on the surface of the colon carcinoma in vitro, we transfected CXCR5 and determined its function in growth and migration. The role of this chemokine receptor in metastasis remains to be established, but we found that CXCL13 can promote proliferation of CXCR5-expressing colon carcinoma cells, and that CXCL13 is abundantly present in the metastases.
Chapter 1

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Chapter 1

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Chapter 1


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Chapter 1

Section 1

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Chapter 1


Chapter 1


Chapter 1


