Cellular Trafficking in Haematological and Immunological Disorders

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Voor mijn ouders,
Voor Guido,
Voor Bente.

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General Introduction

General introduction

To ensure tissue homeostasis, immune cells are constantly on the move throughout the body; as such, these cells fight infections, replace dead cells and form new cellular networks. This busy trafficking is orchestrated by locally produced chemokines, which interact with chemokine receptors expressed by migrating cells. Importantly, malignant cells seem to exploit the same pathways, which ultimately lead to tumour metastasis and accompanying angiogenesis. Furthermore, chemokine/chemokine receptor interactions will direct cells to tissues in inflammatory processes and during acute and chronic Graft-versus-Host (GvH) reactions occurring after transplantation. Unrevealing the chemokines and receptors involved in these processes might, therefore, help us to design new strategies for the treatment of (malignant) diseases as discussed in this thesis.

Chemokine structure and function

Chemokines belong to a family of over 40 small (8-14 kDa) proteins, which can be divided into 4 subgroups according to the number and spacing of cysteines (C): C, CC, CXC and CX3C. These chemo-attractants are important for a wide range of biological events, including embryogenesis, wound healing, angiogenesis, B- and T cell development, leukocyte homeostasis, lymphoid organ development, pro- and anti-tumour responses and inflammatory processes[1]. Functionally, they can be separated into constitutively expressed or inducible chemokines. Constitutively expressed chemokines coordinate the development of B- and T cells as well as homeostatic leukocyte travelling, both necessary for optimal immune surveillance. Inducible chemokine expression is elicited by locally released stimuli like inflammatory mediators (i.e. IFN-γ and TNF-α), microbial products or trauma[2]. The expression of these chemokines is of short duration and disappears upon resolution of the inducing trigger[3;4].

One particular chemokine can act through different receptors. Likewise, one chemokine receptor can have several ligands. This elaborate network of chemokines and their receptors relies on the complex regulation of chemokine receptor expression. An overview of all chemokines and their receptors is given in Table 1.

Chemokines produced by pathogen-infected cells present in peripheral tissues such as skin or gut, diffuse to the surface of underlying vascular endothelial cells where they bind to glycosaminoglycan (GAG) sugar residues. This leads to accumulation of chemokines at the luminal side of the blood vessel. Passing cells, expressing the appropriate chemokine receptor, will roll over the endothelial wall, a selectin-mediated process known as tethering, which will reduce their speed. Chemokine/chemokine receptor binding will subsequently result in firm adhesion of the cells to the endothelial wall and finally to extravasation into the underlying tissue as depicted in Figure 1[4].

Chemokine	Expression	Corresponding receptor(s)	Tissue specificity (if applicable)
CCL1	Inducible	CCR8	
CCL2	Inducible	CCR2	
CCL3	Inducible	CCR1, CCR5	
CCL4	Inducible	CCR5	
CCL5	Inducible	CCR1,CCR3,CCR5	
CCL7	Inducible/Constitutive	CCR1, CCR2, CCR3	
CCL8	Inducible	CCR1, CCR2, CCR3, CCR5	
CCL11	Inducible	CCR3, CCR5	
CCL13	Inducible	CCR1, CCR2, CCR3	
CCL14	Constitutive	CCR1, CCR5	Spleen, BM, Liver, muscle, gut
CCL15	Constitutive	CCR1, CCR3	Liver, small intestine, colon, lung
CCL16	Inducible/Constitutive	CCR1, CCR2, CCR3, CCR5	Liver, thymus, spleen
CCL17	Constitutive	CCR4	Thymus
CCL19	Constitutive	CCR7	Thymus, LN
CCL20	Inducible/Constitutive	CCR6	LN, liver, appendix
CCL21	Constitutive	CCR7	LN
CCL22	Inducible/Constitutive	CCR4	
CCL23	Constitutive	CCR1	Lung, liver, BM, placenta
CCL24	Inducible	CCR3	
CCL25	Constitutive	CCR9	Small intestine
CCL26	Inducible	CCR3	Heart, lung, ovary
CCL27	Constitutive	CCR10	Skin, placenta, thymus, gonads
CCL28	Constitutive	CCR3, CCR10	Colon, lung, breast, salivary glands
CXCL1	Inducible	CXCR1, CXCR2	
CXCL2	Inducible	CXCR2	
CXCL3	Inducible	CXCR2	
CXCL4	Inducible	CXCR2, CXCR3	
CXCL5	Inducible	CXCR2	
CXCL6	Inducible	CXCR1, CXCR2	
CXCL7	Inducible	CXCR1, CXCR2	
CXCL8	Inducible	CXCR1, CXCR2	
CXCL9	Inducible	CXCR3	
CXCL10	Inducible	CXCR3	
CXCL11	Inducible/Constitutive	CXCR3, CXCR7	Pancreas, liver, thymus, spleen, lung
CXCL12	Constitutive	CXCR4, CXCR7	BM
CXCL13	Constitutive	CXCR5	Liver, spleen, LN, gut
CXCL16	Inducible	CXCR6	Lymphoid organs, spleen
XCL1	Inducible	XCR1	Spleen, thymus, intestine
XCL2	Inducible	XCR1	
CX3CL1	Membrane bound	CX3CR1	

Table 1. Overview of all known chemokines and their corresponding receptors.
This table is constructed based on published data[3-5].

Chemokines exert their effect by binding to their corresponding seven-transmembrane G-protein coupled chemokine receptors (Figure 2). Upon binding, the G-protein becomes activated and dissociates into Gα, Gβ and Gγ subunits. Depending on the type of G-protein in question, several α-subunits can be involved, leading to different signal transduction cascades[6] (which are still incompletely understood),

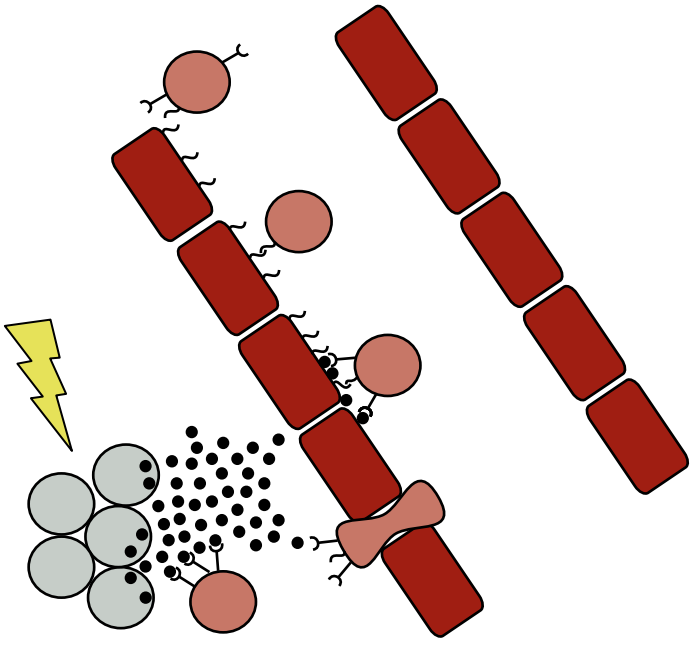

Figure 1. Migration of cells in response to chemokines produced in infected tissue.
Chemokines produced by cells present in peripheral tissues, diffuse to the surface of underlying vascular endothelial cells, leading to their accumulation. Tethering cells, expressing the appropriate chemokine receptor, will reduce their speed and chemokine/chemokine receptor binding will subsequently result in firm adhesion of the cells to the endothelial wall and finally to extravasation into the underlying tissue.

all involved in different stages of cell movement. Together with the α-subunits, the β- and γ-subunits can activate the phospholipase C (PLC) pathway, resulting in intracellular calcium mobilisation and the production of diacylglycerol (DAG). This activates Rap1 guanine nucleotide-nucleotide exchange factor (calDAG-GEF) and triggers the Rap1 enzyme to mediate rapid integrin activation and subsequent cell polarisation[6-8].

Another, ill defined, cascade that leads to integrin activation, is that of phosphatidylinositol 3-kinase (PI3K). PI3K activation induces phosphatidylinositol-3,4,5-triphosphate which, in turn, binds cytohesin-1 resulting in firm adhesion by activated integrins.

Movement of cells requires cell polarisation, the formation of a wide pseudopod at the leading edge and a tail-like structure (uropod) at the end[9;10]. The extension of the pseudopod requires F-actin formation. This is initiated by the activation of DOCK2 (dedicator of cytokinesis 2) which, in turn, induces RAC (RAS-related C3 botulinum substrate) to form F-actin. The precise mechanism of the shortening of the uropod is still unknown but might be induced by the stimulation of RHO-A (RAS homologue A) by the α12 or α13 subunit. RHO-H, in turn, is described to have the opposite effect: it negatively regulates integrin avidity.

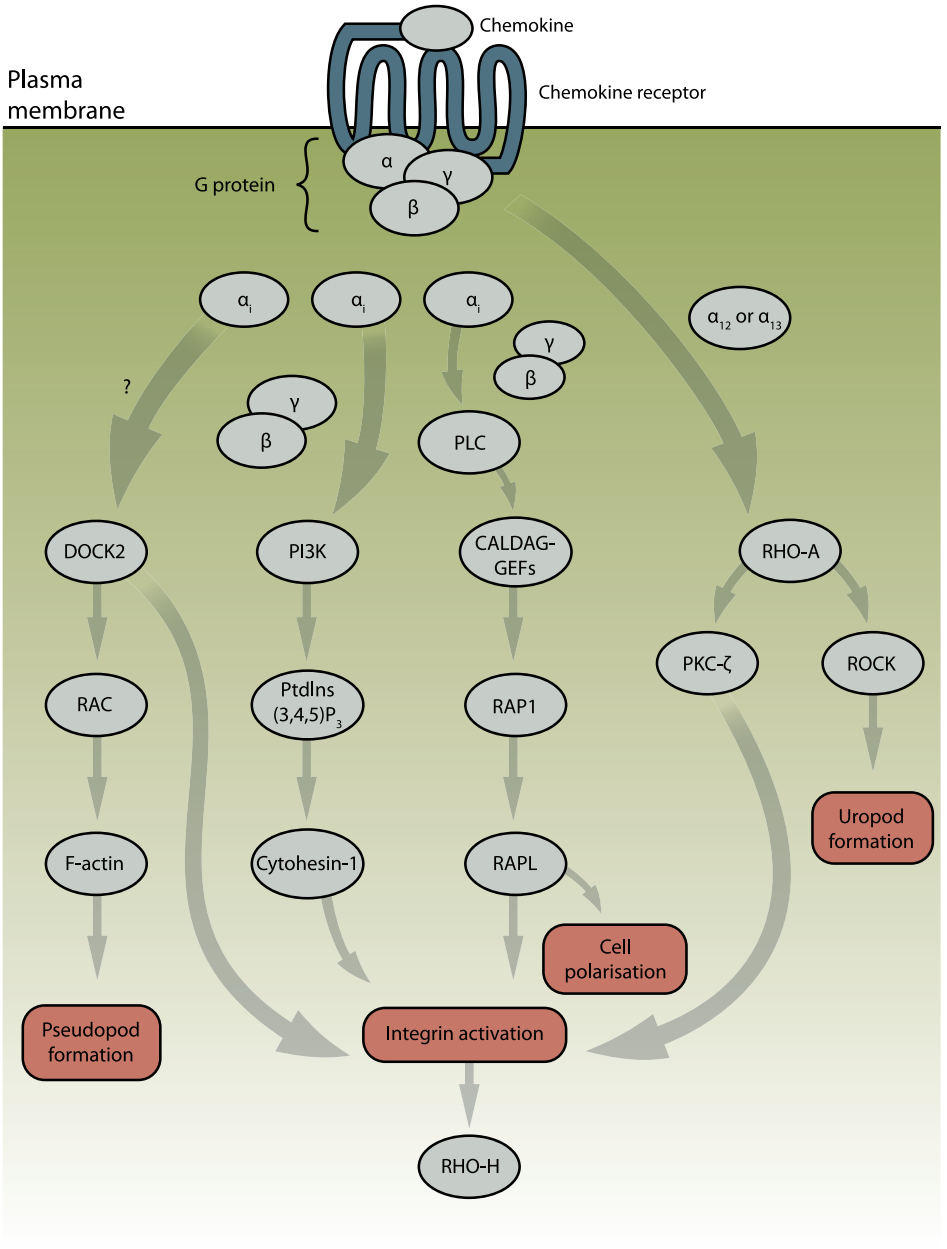

Figure 2. Schematic overview of the different intracellular signalling pathways which may be activated upon chemokine binding to its receptor expressed at the cell surface.
(Adapted from: Kinashi, Nat Rev Immunol 2005; 5: 546-59)

Cell homing in Leukaemia

Besides playing an active role in immune surveillance, chemokines and their receptors are also involved in a large number of pathological conditions including auto-immune disorders, cancer and cancer metastasis, pulmonary disease, vascular disease and Graft-versus-Host Disease (GvHD) in the setting of haematopoietic stem cell transplantation (HSCT)[3]. Some of these conditions are discussed in the following paragraphs.

Acute leukaemia

Acute leukaemia is the most common cancer in children[11] and can be divided into two forms: acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML). It is caused by a malignant transformation of haematopoietic progenitor cells (either lymphoid or myeloid) which results in indefinite expansion of this otherwise infrequently proliferating cell[12]. Due to this expansion, normal haematopoiesis is disturbed which either can lead to diminished counts of unaffected cells or an abnormal distribution of blood cells. Such bone marrow failure leads to the following clinical symptoms: anaemia (manifesting as fatigue and paleness), chronic infections and haemorrhages.
Due to improved treatment strategies, current overall survival of paediatric ALL is approximately 75%[13;14]. In the Netherlands, children diagnosed with ALL are nowadays treated according to the ALL-10 protocol. Over the years, several risk factors for a poor prognosis have been identified including: cytogenetic abnormalities, poor response to initial therapy or induction failure after 4-6 weeks of chemotherapy. Recently, a prospective study has shown that PCR-determined levels of Minimal Residual Disease (MRD) were also clinically relevant. Based on these MRD levels detected on two time points after induction therapy (day 33 and 79), patients could be divided into three groups: standard risk (SR), intermediate risk (MR) and high risk (HR)[14]. The aim of the ALL-10 protocol is to investigate whether therapy can be tapered for SR patients to reduce side effects while keeping the high cure rate, and whether intensification of therapy can improve the outcome for the MR and HR patients. For the HR patients, this intensive chemotherapy is very often followed by an allogeneic HSCT, providing the availability of a suitable stem cell donor[15].
Although treatment innovations have significantly improved the overall survival of ALL in the past decades, the expected cure rate for AML in children is still only 60%[16]. Whereas treatment strategies for ALL patients are quite clear, randomised trials are currently undertaken to define the best treatment protocol for each risk group in AML. In the AML-15 protocol, 3 risk groups are defined based on genetic analysis performed at diagnosis and proportion of malignant cells in the bone marrow after the first course of chemotherapy: good risk (GR), standard risk (SR) and poor risk (PR). About 20% of the patients belong to the GR group. They have favourable genetic abnormalities, irrespective of bone marrow status after the first course of chemotherapy. The SR group comprises about 50% of the patients, who have neither favourable nor adverse genetic abnormalities, and not more than 15% leukaemic blasts in the bone marrow after the first course of chemotherapy. Finally,

the PR group consists of about 20% of the AML patients, who have either more than 15% blasts in the bone marrow after the first course of chemotherapy, or adverse genetic abnormalities in the absence of favourable ones. Only children in the PR group are eligible for allogeneic HSCT.
Both in ALL and AML, bone marrow relapse is the major cause of treatment failure; the majority of patients who show such a relapse eventually die[11;17;18]. Comparison of leukaemic cells detectable at diagnosis, remission or early relapse shows that the clone present in samples taken after relapse was often already present at diagnosis, albeit at very low levels. At diagnosis, several phenotypically different leukaemic clones exist to a various extent. Chemotherapy targets the most abundantly present clone(s) at diagnosis. The chemotherapy-resistant clones will survive, although in very low, almost undetectable, levels at the time of clinical remission. However, these clones will expand, eventually leading to an early relapse. Late relapses probably occur due to the *de novo* development of a second leukaemia from the same pre-malignant clone[17] (Figure 3). Several studies have shown that peripheral blood (PB)- and bone marrow (BM)-derived ALL cells harvested at diagnosis and cultured *in vitro*, are significantly less resistant to a large number of drugs than AML cells[17;19-21]. This could be an explanation for the difference in outcome between paediatric ALL and AML.
Besides residing in the bone marrow, extramedullary sites may also be affected at diagnosis or at relapse of the leukaemia. Extramedullary disease has been reported in 30-50% of the children with ALL[22] and in 10-40% of the paediatric AML patients at diagnosis[23-28], and is thought to correlate with poor prognosis. Extramedullary leukaemia (EML) is defined as leukaemic cell infiltration in soft tissues, such as

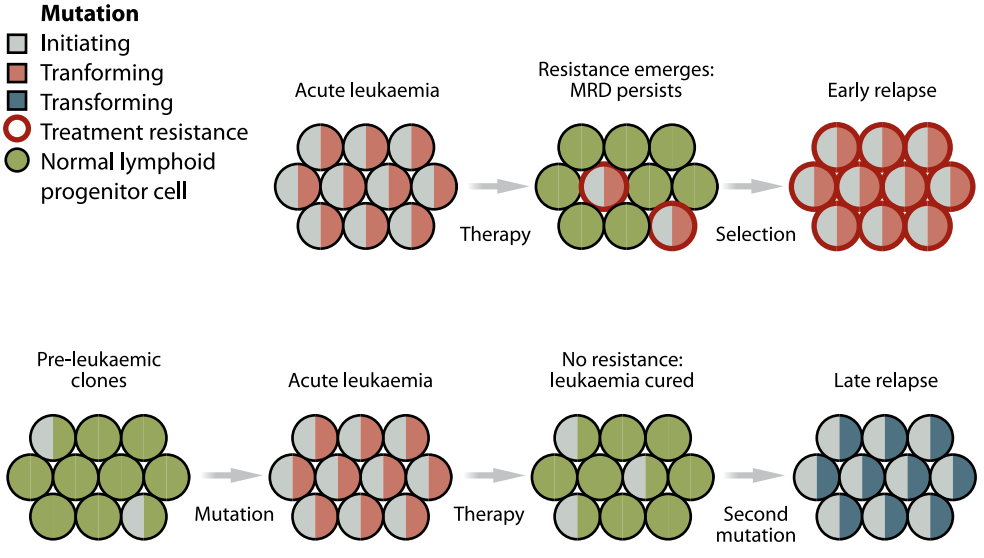

Figure 3. Potential mechanisms of relapse.
(adapted from: Bailey LC, Lange BJ, Rheingold SR, Bunin NJ. Bone-marrow relapse in paediatric acute lymphoblastic leukaemia. Lancet Oncol. 2008;9:873-883).

skin, muscles, bone, gingival tissue or brain. Whereas central nervous system (CNS) involvement is the most common location of EML in ALL, the skin is one of the main extramedullary sites in AML[23;27].

Chemokine-guided migration of leukaemic cells to extramedullary sites.

An increasing number of studies have provided evidence that the mechanisms of tumour cell migration resemble the mechanisms exploited by normal lymphocytes. Thus, chemokines and their receptors facilitate the distribution of leukaemic as well as non-malignant cells throughout the body. To date, only a few studies have focussed on the relation between specific homing characteristics and the occurrence of EML (either at diagnosis or at relapse); these studies mainly focussed on the interaction of CXCL12 and its receptor CXCR4. Variable expression of CXCR4 is seen on primary ALL blasts in the bone marrow, with significantly higher expression in the patients suffering from EML[29]. We set out to determine whether additional chemokines and chemokine receptors could play a role in determining the site of leukaemia relapse. We have a case in which expression of gut-homing molecules on PB-derived leukaemic blasts, at the time of diagnosis, was found to predict relapse of malignant disease in the gut later on (Figure 4)[30].

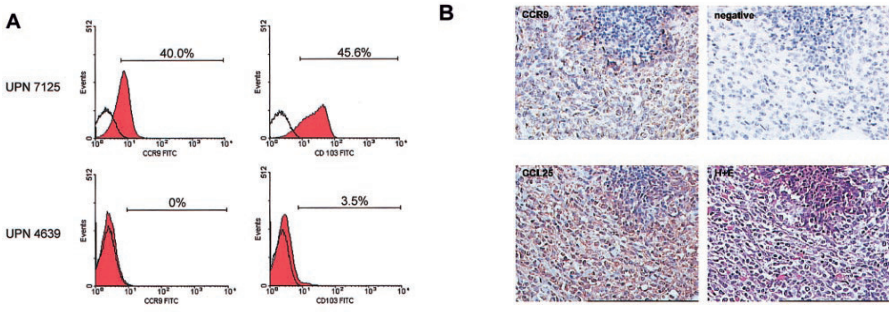

Figure 4. Unique expression of CCR9 and CD103 on the leukemic cells of patient 7125.
Multicolour flow cytometry was carried out on a peripheral blood sample obtained at diagnosis using a panel of chemokine receptor– and homing molecule–specific antibodies in combination with markers for T cells. (A) The unique expression of CCR9 and CD103 on the blast cells from patient 7125 is shown (top panel). The bottom panel shows a representative result for expression of these same receptors as found on T cells of 10 other T-ALL patients. Open histograms indicate level of control staining; red histograms, specific staining. Brackets and percentages denote the fraction of antibody-positive cells. (B) Immunohistochemistry was performed on the tumour cell infiltrate in the ileum of patient 7125. Using an anti-CCR9 polyclonal antibody and DAB detection (brown) there was clear positivity of the tumor cells for CCR9. The specificity of the CCR9 staining was confirmed by omitting the CCR9 antibody as a negative control. Furthermore, immunohistochemical staining using an anti-CCL25/TECK monoclonal antibody and NovaRed detection revealed a high expression of this CCR9 ligand in the tumour mass. The lower right picture shows haematoxylin and eosin (HE) staining of the same area of the affected ileum at relapse. Original magnification, 250X.

In AML, contradictive reports exist on the level of CXCR4 expression and extramedullary localisation of $CXCR4^+$ blast cells. It is thought that CXCR4/CXCL12 interactions play a role in the retention of AML blasts in the bone marrow, because bone marrow residing stromal cells are the main producers of CXCL12[31]. However, high expression of CXCR4 on AML blasts has been described to have a poor prognostic effect given the reported migration of $CXCR4^+$ blasts to extramedullary sites[32]. In order to shed more light on the role of chemokines and their receptors in paediatric AML patients suffering from EML, we investigated their expression patterns on PB- and BM-derived AML blasts as well as *in situ* on leukaemic blasts present in skin biopsies collected from patients with EML in the skin. The results of this study are described in **Chapter 2**.

Chemokine-guided T cell migration in immune disorders: Omenn Syndrome

Given that chemokines and their receptors play an important role in normal immunological processes, it is not surprising that they also appear to be involved in immune disorders. Omenn Syndrome (OS) is an inherited immunodeficiency characterised by lymphadenopathy, hepatosplenomegaly, chronic diarrhoea, exfoliative erythroderma and massively increased IgE levels[33;34]. Unlike severe combined immunodeficiency (SCID) patients, OS patients can have normal or even high lymphocyte counts. However, the *in vitro* proliferative capacity of T cells to respond to antigens is severely decreased.
In both OS and SCID patients, mutations in the RAG1, RAG2 and Artemis genes each affect T- or B cell development at an early stage by impairing recombination of V(D)J gene segments encoding the variable part of T cell receptor (TCR) and immunoglobulin molecules, respectively. Consequently, such patients display an abnormal B- and T cell development and a corresponding limited T cell repertoire (Figure 5). Although mature B cells are often completely absent, rearrangement of multiple TCR Vβ segments is, however, still possible, albeit that the resulting TCR repertoire of T cells is strongly reduced. Peripheral expansion of a limited number of T cell clones in response to infections[35] combined with an increased antigen exposure due to a defect in antigen clearance, may result in oligoclonal T cells in the circulation which appear to be chronically activated.
A hallmark of OS is the peculiar tissue distribution of T cells; the T cells typically accumulate in skin, gut and liver[36]. Although the underlying genetic abnormality of OS has been clarified[35], the underlying cause of skin, gut and liver homing of these T cells remains unclear. To provide some explanation of the remarkable clinical features of this disease, we investigated the chemokine receptor expression pattern by PB-derived T cells as well as local chemokine production in affected skin before and after application of the immunosuppressive drug Tacrolimus. This study is described in **Chapter 3**.

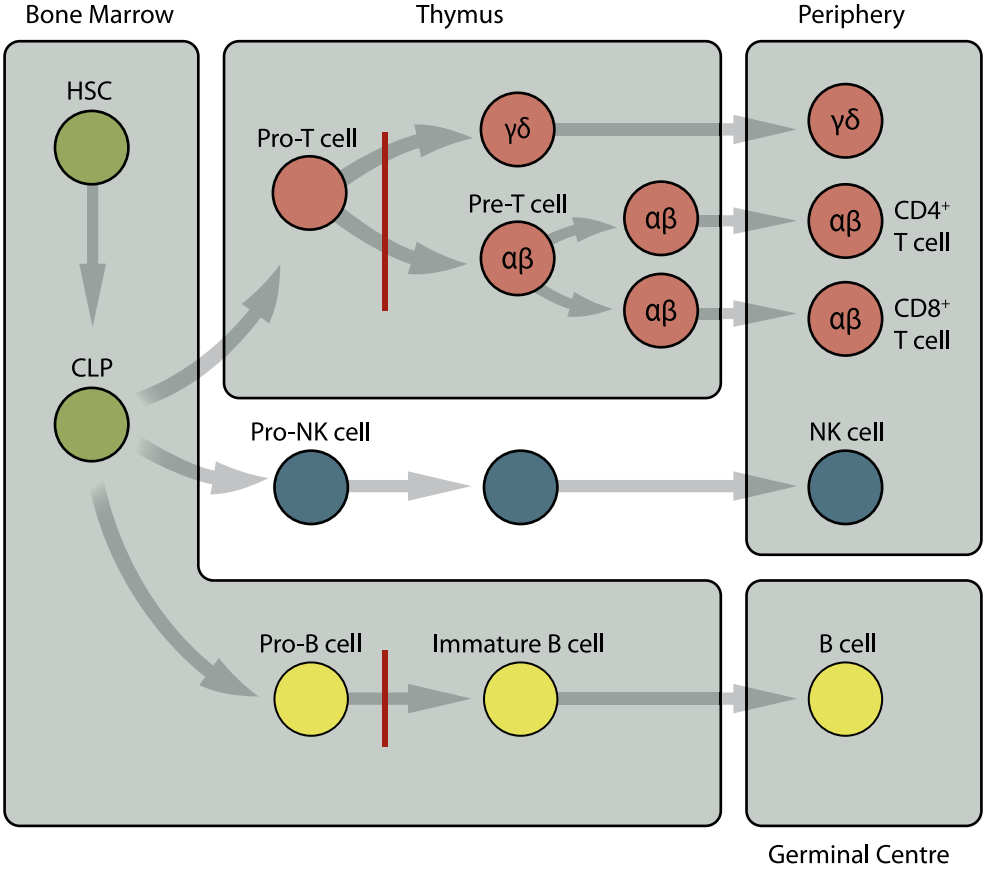

Figure 5. Schematic overview of lymphopoiesis. Red lines indicate the different stages where RAG and Artemis mutations block further differentiation. HSC = haematopoietic stem cell; CLP = common lymphocyte progenitor. Adapted from De Villartay JP, Fischer A, Durandy A. The mechanisms of immune diversification and their disorders. Nat.Rev.Immunol. 2003;3:962-972.

Stem cell transplantation

The only effective treatment option for OS patients is allogeneic HSCT, whereby the genetically aberrant precursor cells committed to the affected cell lineage(s) are replaced by healthy precursor cells. This will lead to normal B- and T cell maturation processes and, hence, diverse B- and T cell repertoires. Allogeneic HSCT is a well established, effective and commonly applied therapy for various haematological malignancies, benign haematological diseases, metabolic disorders and immunodeficiencies. After myeloablative conditioning and stem cell (SC) infusion, the patient's complete haematopoietic system, including cells repopulating the immune system, is replaced by that of the non-affected haematopoietic SC donor. A schematic overview of the general HSCT procedure in children, showing the possible conditioning variables and post HSCT complications, is given in Figure 6.

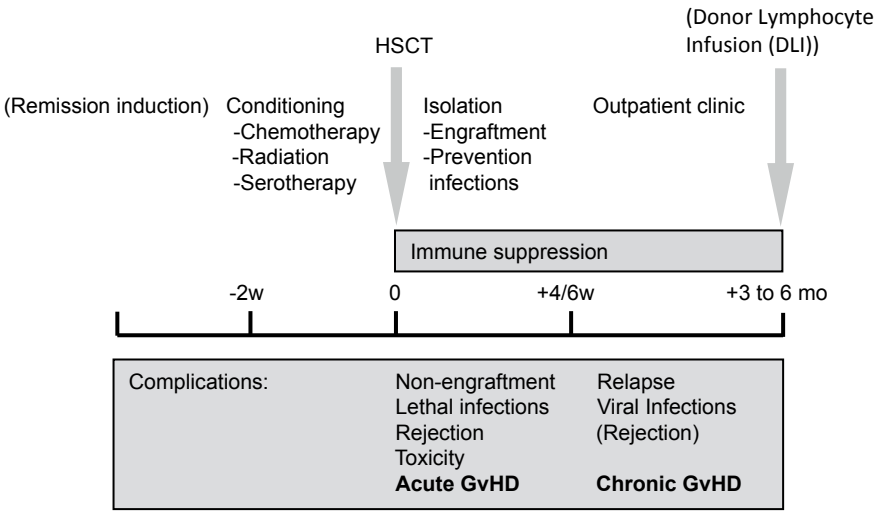

Figure 6. Schematic overview of the clinical HSCT protocol applied in children and possible post HSCT complications.

Complications associated with allogeneic haematopoietic stem cell transplantation

The two major, potentially life-threatening, complications seen shortly after allogeneic HSCT are graft rejection and Graft-versus-Host Disease (GvHD). Graft rejection is caused by remaining circulating T cells of host origin that have survived the pre-conditioning regimen. These patient-derived T cells recognise mismatched alloantigens expressed by the infused donor cells; this allorecognition leads to elimination of the infused graft. GvHD, on the other hand, is caused by T cells of donor origin which are transferred, along with the haematopoietic stem cells, into the immuno-compromised recipient. In this situation, donor T cells will be activated by mismatched human leukocyte antigens (HLA) and/or minor histocompatibility antigens (mHags) expressed by the recipient and not by the donor.
To minimise the risk of graft rejection or GvHD, the patient and donor should be , in the ideal situation, completely HLA matched. However, even when an HLA identical sibling is used as donor, rejection and GvHD can still occur; immune reactions in the latter setting are evoked by mHags. These antigens may differ between related donor and patient pairs. mHags are immunogenic peptides derived from intracellular

Next page: Table 2. Known minor antigens with their HLA restriction and distribution.
Adapted from: Spierings E, Goulmy E. Minor Histocompatibility antigens in biology and medicine. In: Mehra N, editor. HLA in medicine and biology, 2010. Tissue distribution from: http://www.lumc.nl/dbminor. Broad tissue distribution is considered expression by haematopoietic cells and non-haematopoietic cells such as fibroblasts and keratinocytes.

Autosomal

Minor H antigen	HLA-restriction	Tissue distribution	Cell types
LB-ADIR-1	HLA-A2	Restricted	Haematological malignancies, solid tumours
SP110	HLA-A3	Restricted	Haematopoietic cells, IFN-γ inducible
LB-PI4K2B-1	HLA-DQ6	Broad	
UGT2B17	HLA-A29	Restricted	DC, B cells, EBV-BLCLs, liver, intestine
UGT2B17	HLA-B44	Restricted	DC, B cells, EBV-BLCLs, liver, intestine
UGT2B17	HLA-A2	Restricted	DC, B cells, EBV-BLCLs, liver, intestine
HB-1	HLA-B44	Restricted	B-ALL, EBV-LCLs
HA-2	HLA-A2	Restricted	Haematopoietic cells
HA-8	HLA-A2	Broad	
ACC-1	HLA-A24	Restricted	Haematopoietic cells
ACC-2	HLA-B44	Restricted	Haematopoietic cells
CTSH/A31	HLA-A31	Restricted	EBV-BLCLs, AML
CTSH/A33	HLA-A33	Restricted	EBV-BLCLs, AML
HA-3	HLA-A1	Broad	
CD19	HLA-A2	Restricted	B cell lineage specific, including B cell malignancies
LRH-1	HLA-B7	Restricted	T cells, B cells, EBV-BLCLs, PHA blasts, NK cells, AML, CML
ACC-6	HLA-B44	Restricted	Haematopoietic cells
HA-1/A2	HLA-A2	Restricted	Haematopoietic cells, solid tumours
HA-1/B60	HLA-B60	Restricted	Haematopoietic cells
C19orf48	HLA-B7	Restricted	Solid tumours
SLC1A5	HLA-B61	Unknown	
LB-ECGF-1	HLA-B7	Broad	
PANE1	HLA-A3	Restricted	Lymphoid cells

Y-chromosome encoded

Minor H antigen	HLA-restriction	Tissue distribution	Cell types
A1/HY	HLA-A1	Broad	
A2/HY	HLA-A2	Broad	
A33/HY	HLA-A33	Broad	
B27/HY	HLA-B27	Restricted	Unknown
B52/HY	HLA-B52	Restricted	B and T lymphoblasts, Leukocytes, PHA blasts, EBVBLCLs, B cells, solid tumours, ALL, AML, Multiple Myeloma
B60/HY	HLA-B60	Broad	
B7/HY	HLA-B7	Broad	
B8/HY	HLA-B8	Restricted	Haematopoietic cells
DQ5/HY	DQB105	Broad	
DR15/HY	DR15	Broad	
DRB1*1501/HY	DRB11501	Broad	
DRB3*0301/HY	DRB30301	Broad	

proteins, which are encoded by polymorphic genes on autosomes and allosomes (sex chromosomes). These peptides are expressed at the cell membrane in the context of HLA molecules. These HLA/mHag peptide complexes are recognised by allo-reactive $CD4^+$ and $CD8^+$ T cells. Amino acid polymorphisms in these genes may result in 2 allelic counterparts. Generally, only the immunogenic peptide variant is capable of eliciting an allo-immune T cell response. Mismatches in mHags between patient and donor may result in immune reactions in Graft-versus-Host as well as Host-versus-Graft direction as discussed below[37].

The impact of mHags on the development of GvHD depends on their population frequencies[38] and tissue distribution[39]. With respect to the latter, two distinct patterns of expression have been described, i.e.: haematopoietic system-restricted expression or ubiquitous expression (Table 2). mHags with an ubiquitous or 'broad' expression pattern, i.e. the male chromosome-encoded mHag HY, are particularly relevant for the induction of GvHD. In contrast, mHags such as the autosomally encoded mHag HA-1, which are solely expressed by normal haematopoietic cells or leukaemic cells, are unlikely to cause GvHD. Evidence in support of this assumption has come from *in vitro* studies, in which HLA and mHag genotyped skin biopsies were incubated *in vitro* with HY or HA-1 specific $CD8^+$ Cytotoxic T Lymphocytes (CTL)[40]. While incubation with HY-specific CTL induced severe GvHD-like damage to skin cells, HA-1 CTLs induced no or only very mild skin destruction.

Various clinical studies have addressed the impact of mHag mismatching on the incidence of GvHD. Indeed, gender mismatching has been identified as a significant risk factor for the development of acute GvHD and transplant related mortality in male recipients of female stem cells[41]. In support of these epidemiological findings, elevated numbers of HY-specific T cells have been demonstrated in peripheral blood samples collected from male SCT patients who developed GvHD after receiving a bone marrow graft from a female sibling donor[42]. Whether these HY-specific T cells also infiltrate GvHD target tissues such as skin, gut or liver has not yet been investigated in human transplant patients. In **Chapter 4**, we describe how a newly available *in situ* staining technique enabled us to address this question in gender mismatched paediatric patient/donor combinations.

Acute GvHD

Acute GvHD is defined as a moderate to severe inflammatory response leading to tissue destruction mediated by alloreactive T cells post HSCT. This potentially lethal complication of allogeneic HSCT can be graded following the Glucksberg criteria listed in Tables 3 and 4[43].

The pathophysiology of acute GvHD can be divided into three different phases as depicted in Figure 6. The main events are: 1) activation of resident host antigen presenting cells (APCs), 2) activation and migration of donor T cells to target tissues and 3) tissue destruction by activated donor T cells. In the first phase, the conditioning regimen (chemotherapy and/or total body irradiation) leads to damage of host tissues. This tissue damage stimulates the secretion of pro-inflammatory cytokines, like IL-1 and TNF-α. These macrophage-derived cytokines induce or up regulate the expression of HLA and a variety of adhesion molecules on blood

Stage	Skin	GI tract	Liver
0	No rash due to GvHD	None	Bilirubin <35 µmol/l
1	Maculopapular rash <25% of body surface area	Diarrhea 500–1000 ml/day; nausea and/or vomiting	Bilirubin 35–50 µmol/l
2	Maculopapular rash 25-50% body surface area	Diarrhea 1000–1500 ml/ day	Bilirubin 51–102 µmol/l
3	Generalised erythroderma	Diarrhea >1500 ml/day Or cramps, or blood, or ileus	Bilirubin 103–225 µmol/l
4	Generalised erythroderma with bullous formation and/ or desquamation	Simultaneous presence of any two of the four criteria for stage 3 severity	Bilirubin >225 µmol/l

Table 3. Organ staging of aGvHD.

Grade	Skin (stage)	GI tract (stage)	Liver (stage)
0	0	0	0
I	1-2	0	0
II	1-3	1	1
III	2-3	2-3	2-3
IV	2-4	2-4	2-4

Table 4. Overall clinical grading of aGvHD.
Both tables are adapted from: Przepiorka D, Weisdorf D, Martin P *et al*. 1994 Consensus conference on acute GvHD grading. Bone Marrow Transplant 1995. 15: 825-828.

vessels present in host tissues. This, in turn, enhances the recognition of HLA and mHags expressed on host APC by infiltrating donor T cells. In phase 2, donor T cells become activated and start to proliferate, thereby secreting IL-2 and IFN-γ. These cytokines further induce T cell expansion, CTL and natural killer (NK) cell responses, and activate mononuclear phagocytes. During the third and last phase, more tissue is damaged by inflammatory mediators released by infiltrating leukocytes, such as perforin, granzyme B, FasL, and TNF-α. This ultimately results in an amplification of the tissue damage and clinical manifestation of GvHD symptoms[44].

In order to exert their damaging effect, activated alloreactive T cells must first migrate to particular tissue site(s). It is likely that these T cells are attracted by the same molecules as immune cells that fight pathogens invading peripheral tissues, i.e. locally produced chemokines interacting with their specific receptors on these T cells. To date, most of the work investigating the involvement of chemokines in GvHD has been carried out in experimental murine models. The production of various pro-inflammatory chemokines (CCL2, CCL3, CCL4, CCL5, CXCL9 and CXCL10) has been demonstrated in GvHD target organs, but true organ specificity of GVHD tissue-infiltrating T cells has not clearly been demonstrated.

During the onset of GvHD, manifestations of skin rash usually precedes the clinical manifestation of intestinal or liver GVHD, although acute GvHD also may remain limited to the skin. T cell migration pathways to the skin have been reasonably well characterised. Skin-homing T cells express cutaneous lymphocyte-associated

antigen (CLA) that, together with E-selectin, causes tethering of the cells along the endothelial wall (Figure 1). Subsequent activation and diapedesis of the T cells is driven by CCL17/CCR4 and CCL27/CCR10 interactions. Further recruitment of lymphocytes into the dermis appears to be mediated via CCR4/CCL17 interactions, whereas the CCL27/CCR10 pathway ultimately guides the cells towards the epidermal/dermal junction. In **Chapter 5**, we investigated the potential role of CCR10 and its ligand CCL27 in the migration of $CD4^+$ T cells to the skin. To this end, we analysed peripheral blood and skin tissues obtained from transplanted children who suffered from acute GvHD.

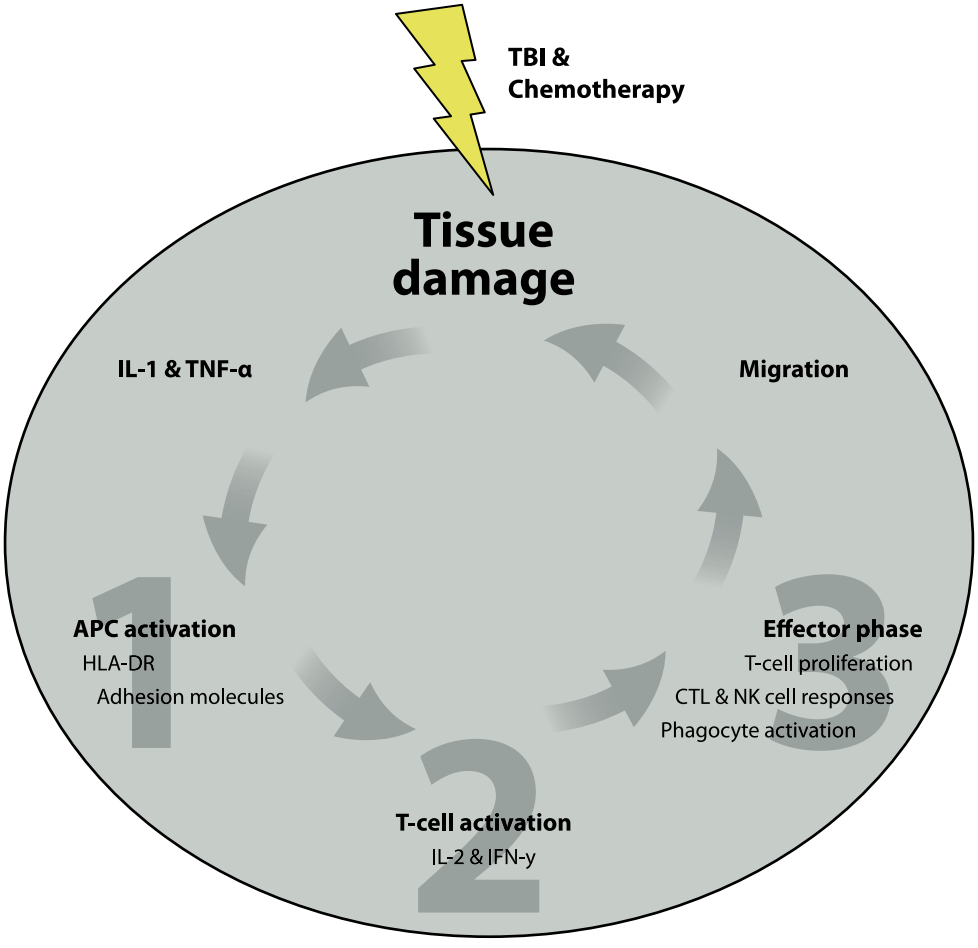

Figure 6. Pathophysiology of acute GvHD.

Chronic GvHD

Chronic GvHD (cGvHD) is a major long-term complication of allogeneic HSCT and a common cause of late death. It has a median time to onset of 4-6 months post HSCT, but can be diagnosed as late as one year after HSCT. The most common anatomical locations involved in the initial diagnosis of cGvHD are: skin, mouth, liver and eye[45,46]. Until a few years ago, the distinguishing factor between acute and chronic GvHD was the time point of clinical manifestation, i.e. whether tissue inflammation occurred before or after the first 100 days post-HSCT. However, consensus has been reached about a more explicit clinical definition of cGvHD; these criteria include the following clinical features: sclerosis, lichen-planus-like lesions, poikiloderma, oesophageal webs, bronchiolitis obliterans and fasciitis[47]. Fasciitis is characterised by a symmetrical inflammatory swelling of the extremities[48]. When accompanied by myalgia, this can cause severe functional impairment of the extremities.
Compared to aGvHD, the pathophysiology of cGvHD, and fasciitis in particular, is still poorly understood. It has been hypothesised that cGvHD results from a loss of peripheral immune tolerance towards self-antigens as reflected by the autoimmune-like clinical symptoms[46]. Histopathological findings include a diffuse lymphocyte infiltration of the oedematous fascia, which often extends to the muscle interstitium, and an increase of collagen fibres[49,50]. $CD8^+$ T cells are predominantly seen in these lymphocytic infiltrates.
$CD8^+$ cytotoxic T cells are, therefore, thought to be the main effector cells, which are likely activated locally by donor-derived helper T cells. This immune response may occur in situations where regulatory T cell numbers are low, i.e. after myeloabblative HSCT. Confirmatively, experimental GvHD models showed that the absence of T_{regs} resulted in uncontrolled expansion of T_H1 and T_H17 cells, leading to cytokine release and subsequent tissue damage[51]. In human cGvHD patients a significant decrease of the number of T_{regs} in peripheral blood was seen; their levels returned to normal after resolution of the disease[52-54]. It remains, however, unclear whether the T_{reg} number in peripheral blood is representative of their numbers and corresponding suppressive activity in secondary lymphoid organs and cGvHD target tissues.
Besides the involvement of different T cell subsets, the observation of a wide variety of (auto) antibodies in the serum of cGvHD patients also suggest a role for (donor) B cells in the induction or perpetuation of cGvHD[55-59]. Several studies have shown a significantly higher occurrence of auto antibodies in patients with cGvHD as compared to those without[50-52]. Additionally, antibodies to Y chromosome-encoded minor Histocompatibilty antigens have been found in male recipients of female haematopoietic stem cell grafts, correlating with the occurrence of cGvHD[53].
In order to exert their local damaging effect, lymphocytes have to migrate from the circulation into the fascia. Chemokines and their receptors generally play an important role in cellular trafficking to inflamed tissues. In contrast to our findings in aGVHD of the skin (Chapter 6), information on the role of chemokines and their receptors in cGvHD is currently unavailable. In a first attempt to address this complex issue, we have studied tissue biopsies derived from three cGvHD patients in whom fasciitis manifested as the main clinical feature of late HSCT-related complications. **Chapter 6** describes which type of immune cells have infiltrated the fascia and which

chemokine/receptor combination(s) might have facilitated the migration of the cells to the fascia.

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Chapter

2

Chemokine/chemokine receptor interactions in extramedullary leukaemia of the skin in childhood AML: differential roles for CCR2, CCR5, CXCR4 and CXCR7

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Abstract

Chemokine receptor/ligand interactions orchestrate the migration of cells to peripheral tissues such as the skin. We analysed chemokine receptor expression by acute myeloid leukemic (AML) cells present in peripheral blood (n=7), bone marrow (n=6) or skin (n=11) obtained from 15 paediatric AML patients with skin involvement and in 10 AML patients without skin involvement. High percentages of circulating $CCR2^+$ AML cells were only detected in patients with extramedullary disease. Skin-residing AML cells displayed a different set of receptors *in situ*, namely: CCR5, CXCR4, CXCR7 and CX3CR1. These results suggest the involvement of different chemokine/chemokine receptor interactions in homing and retention of AML blasts in the skin.

Introduction

AML is characterised by uncontrolled proliferation of bone-marrow (BM)-residing myeloid progenitor cells, which are arrested in their maturation process[1]. The prognosis of childhood AML has significantly improved, given that approximately 60% of the patients experiences long-term survival to date[2]. Extramedullary disease (EML), defined as the presence of leukaemic blasts in skin, muscle, bone, gingival tissue or brain, may manifest in 10-40% of paediatric AML patients at diagnosis. EML seems to correlate with poor prognosis in some, but not all, studies[3,4].

Chemokines play an important role in tumour cell migration and infiltration of distant organ sites. This multi-step process requires the sequential engagement of adhesion molecules and activation through chemokine receptors[5,6]. To date, most studies addressing the involvement of chemokines and their receptors in the tropism of leukaemic cells have concentrated on the interaction of CXCL12 and its receptor CXCR4. Given that BM stromal cells are major producers of CXCL12[7] and CXCR4 expression is thought to be higher on BM-residing blasts than on circulating blasts, CXCR4/CXCL12 interactions likely facilitate the retention of AML blasts in the BM[8]. Consequently, high CXCR4 expression by AML cells is considered as an independent risk factor for relapse and poor overall survival[9]. Contradictory, this seems to be associated with extramedullary involvement[10,11]. CXCR4 has long been considered the sole receptor for CXCL12. Recently, CXCL12 was also reported to be the ligand of a novel chemokine receptor, CXCR7[12]. Unlike other chemokine receptors, CXCR7 lacks the ability to mediate chemotaxis and calcium mobilisation after ligand binding. Instead, CXCR7 is thought to regulate tumour cell survival, clustering and growth[13]. Given the paucity of data regarding the role of specific chemokine receptors and their ligands in the migration of myeloid blasts to extramedullary sites, we investigated their expression on leukaemic blasts derived from peripheral blood (PB) and BM as well as their *in situ* expression profile in AML affected skin biopsies.

UPN	Sex	Age	Presentation	% Blasts in BM	% Blasts in PB	Extramedullary leukaemia site (if present) besides skin	Included in
Skin involvement at diagnosis							
1	F	15	M2	77	99		FC, IHC
2	F	14,7	M2	67	51		FC
3	M	0	M4	ND	82		FC
4	F	0	M4	55	16		IHC
5	F	1	M5	30	3		IHC
6	M	1	M5	ND	ND		IHC
7	F	0	M0	85	95	CNS	FC, IHC
8	M	11,9	M0	39	6	CNS	IHC
9	F	6	M2	65	65	CNS	FC, IHC
10	M	14,4	M2	86	80	CNS	FC, IHC
11	M	12,7	M5	87	48	CNS	FC, IHC
12	F	1	M4/M5a	35	0	Bone	IHC
13	F	3	M7	41	67	Eye	FC
14	M	15,8	M1	92	ND	CNS, lung, eye	FC, IHC
15	M	1	M5	72	12	Eye, extradural chloroma, LN	FC
No extramedullary manifestations of disease at diagnosis							
16	M	12,1	M0	89	82		FC
17	F	5	M1	85	78		FC
18	M	14	M2	45	73		FC
19	F	2	M4	71	22		FC
20	F	15,7	M4	57	15		FC
21	F	10	M4	54	8		FC
22	M	13,3	M4	88	68		FC
23	F	10,3	M5	97	ND		FC
24	M	9	M5	89	91		FC
25	M	2	M5a	95	95		FC

Table 1: Patient characteristics at diagnosis.
Abbreviations: UPN: Unique Patient Number; BM: Bone Marrow; PB: Peripheral Blood; CNS: Central Nervous System; LN: Lymph Node; Chloroma: granulocytic sarcoma mostly beneath the periosteum of the skull, spine or ribs; FC: Flow Cytometry; IHC: Immunohistochemistry.

Materials & Methods

Patients

Fifteen paediatric AML patients with confirmed skin involvement at diagnosis and 10 control patients without EML were enrolled in the study. Patient characteristics are described in Table 1. Formalin fixed, paraffin embedded and tumour cells containing skin biopsies were obtained from 11 out of 15 AML patients with skin involvement. Cryopreserved peripheral blood mononuclear cells (PBMC) and/or BM mononuclear cells (BMMC) were available for flow cytometric analysis (n=20). Patient materials, collected at diagnosis, were obtained from several Dutch paediatric oncology centres via the Dutch Childhood Oncology Group (DCOG, The Hague, the Netherlands;

protocol: OC-2001-013) or from the Women's and Children's Hospital (Adelaide, Australia). In line with the Helsinki guidelines, approval was obtained from the Institutional Review Board to use leftover biological material routinely obtained for diagnostic purposes for related research.

Flow Cytometric analysis

Chemokine receptor expression was analysed by flow cytometric analysis on a FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Selection of AML cells was based on their FSC/SCC pattern combined with specific markers such as CD7, CD13, CD14, CD33, CD34, CD56 and HLA-DR). The percentage of positive cells was analysed using Cellquest software.

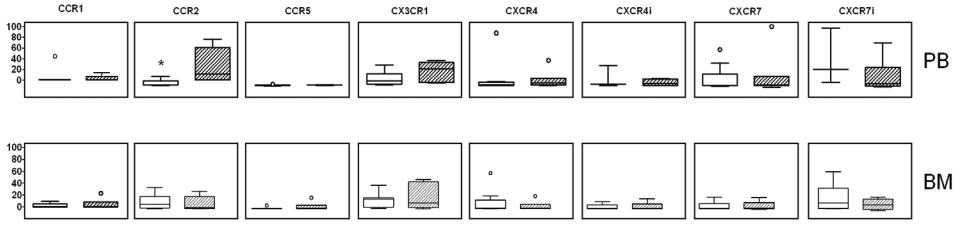
Immunohistochemical analyses

Combined immunofluorescent (IF) staining of tumour cell-specific markers and chemokine receptors was performed in cases where antibodies to the tumour specific markers were available. Enzymatic immunohistochemical (IHC) staining was performed in all other cases, as well as for analysing chemokine ligand expression, 4 µm paraffin sections were pre-treated as described before[14]. Subsequently, the slides were incubated overnight with the primary unconjugated antibodies followed by the relevant isotype-specific, Alexa Fluor 488 or 594 labelled, secondary antibodies (Invitrogen). Results were analysed by confocal microscopy (LSM 510 confocal microscope, Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). For IHC staining, positive cell were visualised using Envision or LSAB+ (Dako, Heverlee, Belgium) and DAB detection. Replacement of the primary antibodies by PBS/BSA 1% was used as a negative control for both staining methods. Enzymatic staining was scored as previously described[15]. In short, the intensity was graded as: 0, absent; 1, weak; 2, moderate and 3, intense and the percentage of positive tumour cells as: 0 = absent; 1 = 1%-10%; 2 = 10%-25%; 3= 25%-50%; 4 = 50%-75% and 5 = >75% positive tumour cells. Sections were considered positive when a combined score was higher than three. Immunofluorescent slides were scored positive when >50% of the tumour cells expressed the relevant chemokine receptor.

Statistical Analysis

Flow cytometric data are presented as median and interquartile range (Tukey) of the percentage of positive blasts. Differences in expression between AML patients with and without skin involvement were assessed using a Mann–Whitney test.

A

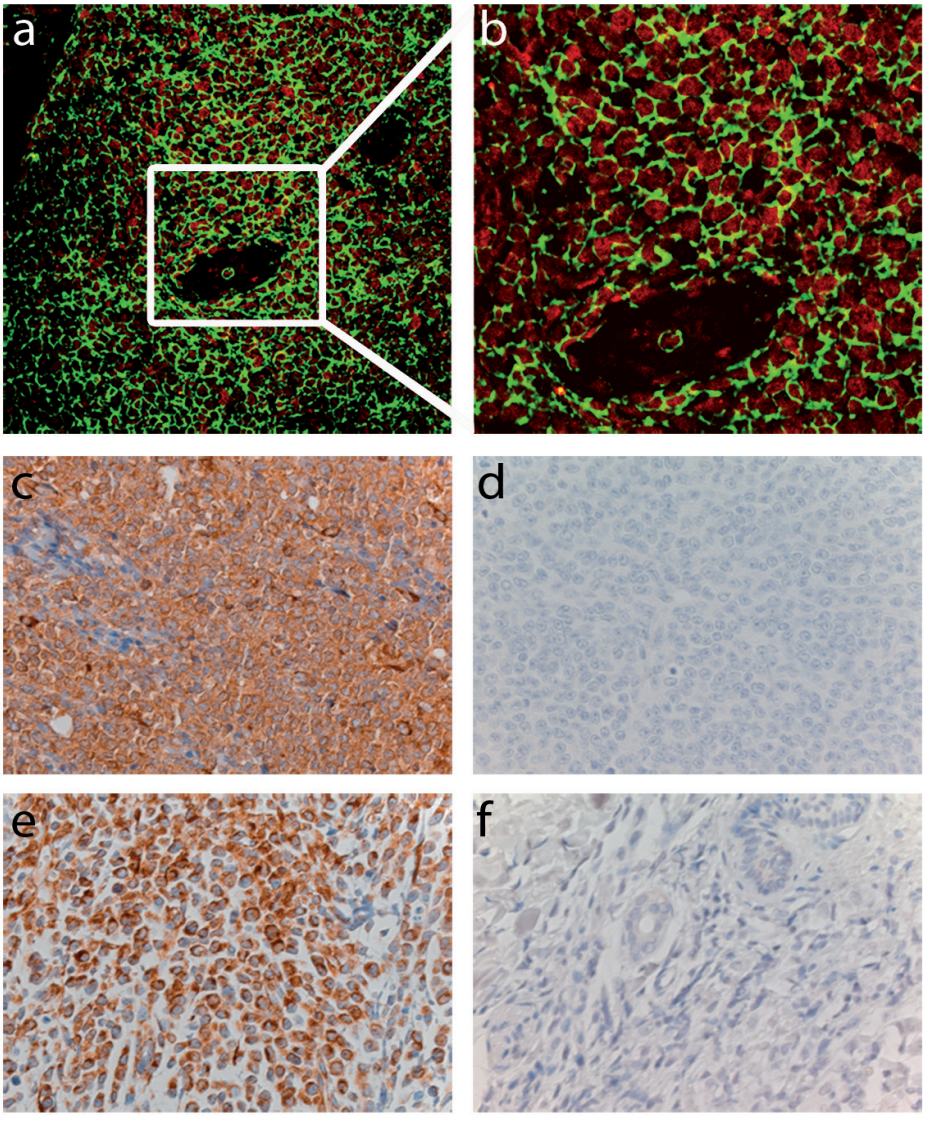


B

Patient	CCR1	CCR2	CCL2	CCR5	CCL3	CCL5	CXCR4	CXCL12	CXCR7	CXCL11	CX3CR1	CX3CL1
1	- (2)	- (3)	- (0)	8	8	- (0)	8	- (3)	8	- (1)	8	- (3)
4	-	6	7	8	8	- (0)	+	7	5	- (2)	-	- (1)
5	-	6	- (3)	8	- (1)	- (0)	+	8	+	- (0)	-	- (3)
6	-	- (1)	- (3)	8	8	- (0)	+	8	+	- (0)	+	7
7	-	7	- (2)	8	nd	nd	-	6	-	- (0)	-	- (2)
8	-	- (1)	nd	8	nd	nd	+	7	+	- (0)	-	nd
9	-	4	- (3)	8	5	- (1)	+	6	+	- (0)	+	6
10	-	6	- (3)	8	nd	nd	+	5	+	- (0)	-	- (2)
11	-	8	4	8	4	- (1)	+	6	+	- (1)	-	- (1)
12	- (1)	5	- (0)	8	8	- (3)	8	- (3)	7	- (0)	8	7
14	nd	- (2)	nd	8	nd	- (0)	+	- (2)	+	- (2)	5	- (1)
Total	0/10	7/11	2/9	11/11	6/7	0/8	10/11	8/11	10/11	0/11	5/11	3/10

Figure 1: *Ex vivo* and *in situ* chemokine and chemokine receptor expression by AML cells.
(A) Flow cytometric results are given as median percentage of positive cells within the blast population with their interquartile range. Outliers are indicated with an open circle. The upper row shows chemokine receptor expression on AML blasts from PB; the lower graphs display the expression by BM-derived AML blasts. CXCR4i and CXCR7i indicate intracellular staining. Chemokine receptor expression by blasts of AML patients without extramedullary involvement is represented by the open boxes (PB: n=3-9, BM: n=7-9). Hatched boxes represent chemokine receptor expression by blasts of AML patients with skin involvement (PB: n=6-7, BM: n=6). Significant differences between the groups are indicated with an asterisk.
(B) The table shows immunohistochemical staining results. Numbers in brackets represent the combined score for intensity and percentage positive tumour cells as described in the Materials & Methods section. The numbers in the lower row represent the total number of positive cases out of the number of evaluable biopsies.
(C, next page) Representative pictures of immunohistochemical stainings. (a) Double immunofluorescent staining on a skin biopsy of a representative patient identified a large infiltrate of $CD43^{pos}$ tumour cells (green) with, in this case, intracellular localisation of CXCR4 (red). (b) A cropped image of this picture. Single enzymatic stainings (visualised by the red/brown colour) showing CXCR7 (c) and CXCL12 (e). Omission of the primary antibodies was used as negative control (d and f). (a-f) Magnification: 250x.

c



Results and Discussion

Given that extramedullary leukaemia occurs in about 25% of paediatric AML patients, understanding the migration process of AML blasts to peripheral tissues is necessary for further improvement of currently available treatment options. We performed the first comprehensive analysis of chemokine receptor/ligand expression patterns expressed by AML cells in blood, BM and affected skin of paediatric AML patients. Due to scarcity of tissue, we could, however, not test the complete set of available antibodies on each biopsy.

Traditional skin homing receptors and adhesion molecules like CCR4, CCR10[16] and CLA were expressed at very low levels on PB- and BM-derived AML blasts (data not shown). No statistically significant differences between patients with or without skin involvement were observed for CCR1, CCR5, CX3CR1, CXCR4(i) and CXCR7(i) expressed by blasts cells (Fig. 1A). In contrast, significantly (P=0.009) increased percentages of $CCR2^+$ AML blasts were observed in AML patients with EML. The percentages of blast cells in the blood of these patients ranged from 8.8 to 75.0% (median 18.6%); in patients without EML these percentages ranged from 0.3 to 15.1% (median 0.9%) (Fig. 1). These results are in line with a study of Cignetti *et al.* reporting a correlation between co-expression of CCR2/CCL2 and extramedullary involvement in adult AML patients[17]. Furthermore, $CCR2^+$ tumour cells could also be visualised in the majority (63.4%) of skin biopsies (Figure 1B). Surprisingly, and in contrast to the Cignetti study[17], one of the ligands for CCR2, CCL2, was only observed in two of these biopsies. This observation implies that CCR2 expressing AML cells exploit another ligand (i.e. CCL7, CCL8, CCL13 or CCL16[18]) for skin-homing.

Analysis of BM-derived blasts demonstrated substantial inter-patient variation (Figure 1). No statistically significant differences were observed in the expression of the chemokine receptors CCR2, CX3CR1 and CXCR7i between the 2 patient groups. In addition, CCR1, CCR5, CXCR4, CXCR4i and CXCR7 were only occasionally expressed on BM-derived AML blasts. Although age, gender and FAB classification were evenly distributed between the two groups, we cannot exclude that these data are affected by a certain degree of heterogeneity in both study groups. A major difference in chemokine receptor expression by PB- or BM-derived AML blasts between the different FAB classifications was, however, not observed.

In line with the low levels of $CCR1^+$ AML cells detected in PB and BM samples, skin-infiltrating leukaemic cells did not express CCR1 (Figure 1B). CX3CR1 and CX3CL1 were expressed by 45.5% and 30% of the biopsies, respectively. Additionally, no difference was seen in CX3CR1 expression by PB- or BM-derived blasts obtained from patients with or without EML. Thus, CX3CR1/CX3CL1 interactions probably do not play a major role in the migration of AML blasts to the skin.

A key observation in our study is the expression of CCR5 in all skin biopsies analysed (n=11). We additionally studied 2 of the 5 chemokines that bind to this receptor[18], i.e. CCL5 and CCL3. While CCL5 could not be detected in any of 8 biopsies studied, CCL3 could be visualised in 85.7% of the biopsies. Given that CCR5 was not expressed by leukaemic blasts in either PB or BM, we speculate that CCL3 might be involved in retention of AML blasts in the skin rather than in skin-homing.

The expression of CXCR4 was mainly intracellular (Figure 1C, picture a,b), and present in almost all evaluable cases (90.9%). Interestingly, CXCR7, associated with tumour cell growth and survival, was expressed in the skin of the same patients (Figure 1C, picture c). CXCL12, the ligand of both CXCR4 and CXCR7, was expressed in 72.7% of the biopsies (Figure 1C, picture e). Skin expression of CXCL11, the alternative ligand for CXCR7, could not be demonstrated (0/11). FACS analysis did not show a significant difference between patients with and without EML regarding CXCR4 or CXCR7 expression by either PB- or BM-derived AML blasts (Figure 1A). Although the AML blasts in the skin clearly expressed CXCR4, CXCR7 and CXCL12, the relatively low expression in PB and BM renders the role of these receptors in skin-homing of leukaemic blasts unlikely. A role for both CXCR4 and CXCR7 in tumour cell survival has been described[13,19]. In a pilot experiment, we observed that the viability of primary AML cells was more reduced when cultured in the presence of blocking anti-CXCR7 antibody prior to CXCL12 exposure than after CXCR4 blockade with AMD3100 (data not shown). Together with earlier reported data[20], these preliminary observations point to a role for CXCR7/CXCL12 in the survival of AML blasts.

Based on our *ex vivo* and *in situ* observations, we hypothesise that CCR2 expression by circulating AML blasts facilitates homing to the skin in response to an as yet unidentified locally produced chemokine. Subsequently, CCR5/CCL3 and CXCR4/CXCL12 interactions facilitate the retention of AML cells in the skin, where CXCR7/CXCL12 interactions subsequently prolong their survival.

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Conflict of interest

The authors declare no competing financial interests.

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Chapter

3

Decrease of skin infiltrating and circulating CCR10^{+} T cells coincides with clinical improvement after topical tacrolimus in Omenn syndrome

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Abstract

Omenn syndrome (OS) is a rare form of severe combined immunodeficiency, often characterised by an early onset of generalised erythrodermia caused by a massive infiltrate of the skin by auto-reactive T cells. We describe the case of an OS patient in which we observed a high percentage of skin-homing, circulating $CD4^+$ and $CD8^+CCR10^+$ T cells. Treatment of the erythrodermia with topical tacrolimus resulted in a significant clinical improvement of the skin, which was associated with normalisation of intradermal CCL27 expression. This coincided with both a reduced cutaneous T cell infiltrate as well as a specific decrease in circulating $CCR10^+$ T cells. Thus, topical tacrolimus treatment appears to reduce CCL27 expression, thereby specifically inhibiting infiltration of skin-specific T cells.

Introduction

Omenn syndrome (OS) is an inherited immunodeficiency disorder with autoimmune-like manifestations. No or very few circulating mature B cells are found in these patients in contrast to normal or even elevated numbers of poorly functional, activated, T cells in the blood. These T cells infiltrate the skin, gut, liver and spleen resembling graft-versus-host disease[1].

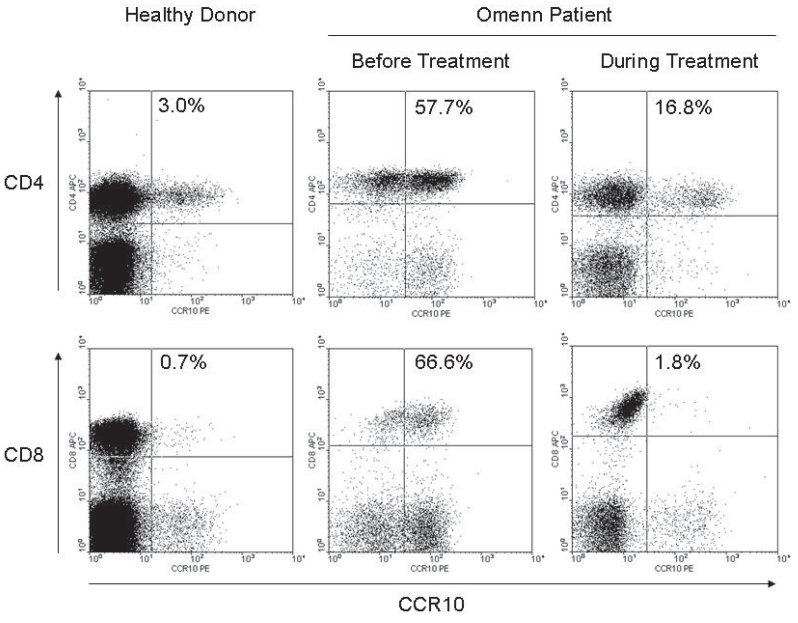
Lymphocyte expression of surface adhesion and chemokine receptors is required for appropriate tissue and microenvironmental localisation. This tissue-specific homing is particularly well illustrated by the distinct mechanisms used by lymphocytes in homing to the skin[2]. Effector/memory T cells with skin-tropism are easily identified by their expression of the cutaneous lymphocyte antigen (CLA). In addition, CLA^+ T cells selectively express two chemokine receptors, CCR4 and CCR10, whose ligands CCL17 and CCL27 are expressed on the luminal surface of cutaneous post-capillary venules. These skin-homing T cells have clearly been shown to play a role in many inflammatory skin diseases[3].

One way to down-regulate or modulate T cell activity is by the use of immunosuppressive drugs belonging to the group of calcineurin inhibitors. Besides their primary effect, the inhibition of the synthesis of T cell growth factors, there is now also some evidence that these drugs down-modulate chemokine receptor expression, thus interfering with migration of T cells to the inflammatory site[4]. In the present study we describe a unique skin homing profile on the peripheral blood $CD4^+$ and $CD8^+$ T cells of an OS patient and the effect of topical tacrolimus treatment on this T cell population.

Clinical Course and Results

The patient is the second child of consanguineous parents born at term after an uncomplicated pregnancy. His five year old sister is healthy and the family history is negative for immunodeficiencies but positive for atopy. In the first week he presented with erythematous skin lesions, initially diagnosed as congenital eczema and treated

A



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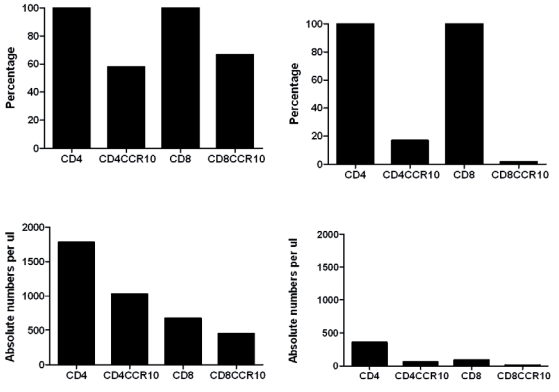
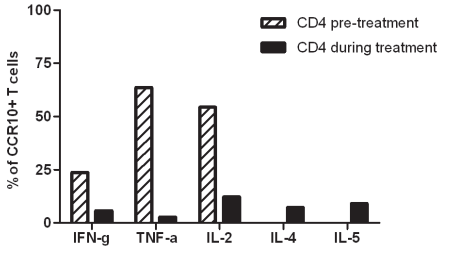


Figure 1. Increased percentages of both CD4⁺ and CD8⁺ T cells expressing the skin-homing receptor CCR10 in the peripheral blood of Omenn syndrome patient (OS1).
(A) Flow cytometric analysis showing an increased percentage of both CD4⁺ and CD8⁺ CCR10⁺ T cells amongst peripheral blood lymphocytes of a patient with Omenn syndrome (OS1) compared to a representative healthy paediatric control donor. Tacrolimus treatment resulted in a substantial decrease in the percentage of both circulating CD4⁺ and CD8⁺ CCR10⁺ T cells. The numbers in the FACS plots represent the percentage of CCR10⁺ cells within the CD4⁺ or CD8⁺ T cells.
This decrease is also true for the absolute numbers as depicted in (B).

with topical steroids. During the first two months he developed food intolerance and low-grade enteritis. Hypo allergic formula only resulted in limited and unstable improvement. At 2.5 months he presented with rhinitis, fever (38-39ºC) and dyspnoea which improved after treatment with amoxicillin but the erythrodermia persisted and he developed generalised lymphadenopathy, conjunctivitis and otitis externa. Blood examination demonstrated a leukocytosis (27x 109/L) with a marked eosinophilia of 20-30%. An immunodeficiency was suspected and at three months the child was referred to our hospital for further evaluation.

Lymphocyte subset analysis showed that virtually all $CD3^+$ cells were T cell receptor α/β and $CD45RO^+$ and there was a lack of circulating B cells. Chimerism analysis revealed that all lymphocytes were of patient origin, excluding transferred maternal T-lymphocytes. Therefore the patient was diagnosed as severe combined immunodeficiency with an OS presentation. Genetic analysis revealed a homozygous deletion (c.591delT) in the RAG1 gene, resulting in a frame shift and a premature termination at amino acid position 20

Due to the fact that this patient, indicated as OS1, presented with a generalised skin rash, skin-homing T cells were investigated in the peripheral blood. Flow cytometric analysis revealed high percentages of both $CD4^+$ (57.7%) and $CD8^+$ (66.6%) T cells that expressed the skin-homing receptor CCR10, compared to the paediatric healthy donor (Figure 1). These T cells all had a memory phenotype ($CD45RO^+$) and co-expressed the activation marker HLA-DR (51.5% $CD4^+CCR10^+$ and 61.1% $CD8^+CCR10^+$) and the skin-homing adhesion molecule CLA (88.4% $CD4^+CCR10^+$ and 93.2% $CD8^+CCR10^+$) (data not shown). Furthermore, a significant percentage of the $CCR10^+$ T cells also expressed homing receptors for secondary lymphoid organs (CD62L: 44.8% $CD4^+CCR10^+$ and 59.9% $CD8^+CCR10^+$; and CCR7: 31.7% $CD4^+CCR10^+$ and 42.8% $CD8^+CCR10^+$; data not shown). High percentages of $CD4^+CCR10^+$ and $CD8^+CCR10^+$ T cells were also observed in the blood of an unrelated other OS patient (OS2) with similar cutaneous manifestations, with 62.7% of the $CD4^+$ T cells and 73.9% of the $CD8^+$ T cells expressing CCR10 (data



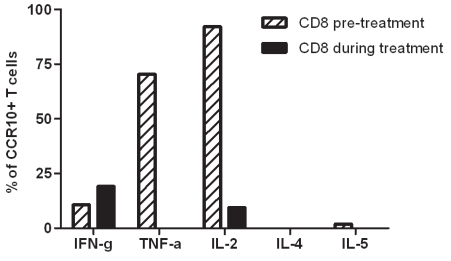
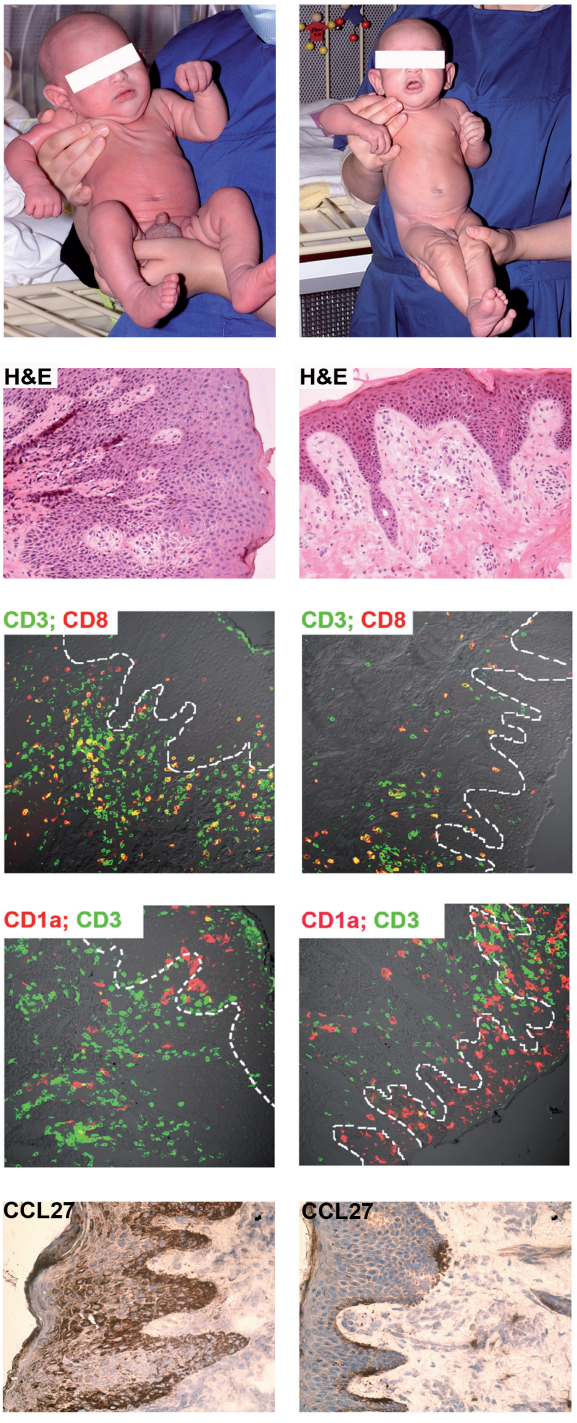


Figure 2. Intracellular cytokine staining reveals a pro-inflammatory profile of the circulating $CCR10^+$ T cells from an Omenn syndrome patient (OS1), which is reduced upon topical Tacrolimus treatment.

Intracellular staining of cytokines (IFN-γ, TNF-α, IL-2, IL-4 and IL-5) produced by $CD4^+$ and $CD8^+$ $CCR10^+$ peripheral blood T cells upon PMA/Ionomycin stimulation in vitro of PBMC from a patient with Omenn syndrome (OS1) before and during treatment with topical tacrolimus.


H&E
H&E
CD3; CD8
CD3; CD8
CD1a; CD3
CD1a; CD3
CCL27
CCL27

not shown). Heteroduplex PCR analysis of the T cell receptor V_β profile of sorted $CCR10^+$ T cells (both $CD4^+$ and $CD8^+$) showed a polyclonal profile (data not shown). This indicates that there is no specific clonal expansion within the $CD4^+$ $CCR10^+$ and $CD8^+CCR10^+$ T cells.

Topical tacrolimus (Astellas Pharma Europe Ltd, Staines, UK) resulted in significant cutaneous improvement within 48h. During 7 weeks of treatment, tacrolimus blood levels between 1.9 and 12.8 ng/ml were observed, accompanied by a further reduction in erythrodermia even in non-treated skin areas. We investigated whether this treatment had an effect on peripheral blood skin-homing T cells. Figure 1 depicts a substantial decrease in the percentage of both circulating $CD4^+$ and $CD8^+CCR10^+$ T cells 49 days after the start of treatment, coinciding with a decrease in CLA^+ peripheral blood T cells (data not shown). A considerable reduction is also seen in the absolute numbers of $CD4^+CCR10^+$ (17.4 times lower) and $CD8^+CCR10^+$ (224.5 times lower) T cells. This decrease is much greater than that of the total $CD4^+$ (5.1 times lower) and $CD8^+$ (7.7 times lower) T cell populations, indicating the specificity of the decline (data not shown).

Similarly, Figure 3 shows that during tacrolimus treatment, the pre-existing dense infiltrate of both $CD4^+$ and $CD8^+$ T cells was significantly reduced whereas the number of $CD1a^+$ Langerhans cells increased in the epidermis after treatment, in keeping with a previous report[5]. Immunohistochemical analysis also revealed that elevated expression of the CCR10 ligand, CCL27, in the epidermis of the affected skin (Figure 3) was reduced to that of healthy control skin following tacrolimus (data not shown). Previous reports on the T_H1/T_H2 profile of T cells from OS patients have given conflicting results[6;7]. Here, pre-treatment, the $CCR10^+$ T cells from OS1 clearly produced the pro-inflammatory cytokines TNF-α ($CD4^+$: 63.5% and $CD8^+$: 70.4%) and IL-2 ($CD4^+$: 54.4% and $CD8^+$: 92.2%) but only marginally produced the anti-inflammatory cytokines IL-4, and IL-5 upon *in vitro* stimulation with PMA/Ionomycin (Figure 2). However, during treatment the TNF-α and IL-2 production by the $CCR10^+CD4^+$ and $CCR10^+CD8^+$ T cells was significantly diminished (Figure 2). This holds also true for the $CCR10^-$ T cells. When we determined the potential inhibitory effect of tacrolimus on cytokine production by the $CCR10^+$ T cells *in vitro*, tacrolimus addition to cultures of PBMC resulted in clear suppression of TNF-α and IL-2 production in keeping with the clinical response (data not shown).

Figure 3 (previous page). Photographs and immunohistochemical analysis showing the modification of histological features during treatment with tacrolimus.

The patient presented with erythematous skin lesions, which upon topical tacrolimus treatment resulted in significant cutaneous improvement. Informed consent from the parents was given for photograph usage. Before treatment a skin biopsy showed a markedly active lesion with prominent epidermal hyperplasia (as displayed by the haematoxylin and eosin, H&E, stain), a large infiltrate of both $CD4^+$(green colour depicting absence of CD8 on CD3 T cells) and $CD8^+$ T cells (yellow due to co-localisation of CD3, green and CD8, red), a reduced epidermal expression of CD1a cells (red) and a diffuse and strong expression of CCL27 (single enzymatic staining detected by the brown colour) in the epidermis. During treatment, normal skin histology was restored with an increased number of epidermal CD1a cells, only small groups of infiltrating T cells and basal expression of CCL27. The dotted white line represents the epidermal/dermal border.

Discussion

We have described the case of an OS patient, OS1, displaying typical OS immunological features and presenting with early onset erythrodermia. One striking feature of the peripheral blood T cells of OS1 and OS2, was the high percentage of both CD4 and CD8 T cells expressing CCR10. Although CCR10 has been clearly associated with the homing of $CD4^+$ T cells to the skin[8;9], few reports show any involvement on $CD8^+$ T cells[10]. To date, studies analysing the T cell infiltrates in other skin diseases such as psoriasis and atopic dermatitis indicated that CCR10 is selectively expressed on $CD4^+$ T cells[11;12]. Thus, this is the first report showing the presence of such a large percentage of $CD8^+CCR10^+$ T cells in an inflammatory skin disorder. Further geno- and phenotyping of both the $CD4^+$ and $CD8^+CCR10^+$ T cells showed that these polyclonal T cells were all $CD45RO^+$, mainly HLA-DR^+ and co-expressed CLA, confirming their activated state and skin-tropism.

Tacrolimus is an immunosuppressive drug that is effective in the treatment of various skin diseases such as psoriasis and atopic dermatitis[13]. Although its inhibition of intracellular signalling pathways resulting in a lack of cytokine gene expression and T cell activation is well documented[14], emerging evidence indicates that immunosuppressive drugs can down-modulate chemokine or chemokine receptor expression, preventing migration of T cells to the inflammatory site[15-17]. Treatment of OS1 with topical tacrolimus not only resulted in clear clinical improvement of his skin but also in a large decrease in the percentage and absolute numbers of circulating $CD4^+CCR10^+$ and $CD8^+CCR10^+$ T cells. This seems to contrast with a previous report where administration of oral tacrolimus failed to control the erythrodermia in a child with OS[11]. Whilst achieving similar blood levels of tacrolimus (between 1.8 and 4.4 ng/ml in this reported case compared to between 1.9 and 12.8 ng/ml in OS1) the main difference seems to be the route of administration of tacrolimus leading to the highest concentration in the skin when topically applied. The clinical improvement in the skin and substantial decrease in skin-homing T cells in the peripheral blood of OS1 following treatment, was reflected by a clear reduction of activated T cells in the affected skin as well as a marked decrease in the expression of the CCR10 ligand, CCL27. As would be expected, treatment with tacrolimus was also associated with a reduced production of the inflammatory cytokines TNF-α and IL-2 by peripheral blood T cells. Therefore, reduction in dermal T cell numbers could be explained by several observations from OS1. Firstly, TNF-α is known to stimulate the production of CCL27 by keratinocytes[15,16]. Thus, the reduction of TNF-α expression may have caused the decline of CCL27 expression in the skin leading to a subsequently diminished recruitment of T cells. It is also possible, that tacrolimus has a direct effect on CCR10 expression by T cells, as has been shown for other immunosuppressive drugs. However due to limited patient material we were unable to test this.

In summary, our findings illustrate that topical treatment with tacrolimus has the potential to reduce a CD4/CD8 T cell mediated skin inflammatory process by a mechanism which involves down-regulation of skin specific chemokines and chemokine receptors.

Material and Methods

Flow Cytometry and intracellular cytokine staining

PBMC were stained for flow cytometry using an unlabeled anti-CCR10 primary antibody (clone 36; DNAX Research Inc, Palo Alto, CA) visualised with either a goat anti-mouse IgG1-PE or -FITC conjugated secondary antibody (Southern Biotechnology Associates Inc. Birmingham, Alabama). The cells were also stained with peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5)-conjugated anti-CD4 (BD Biosciences, San Diego, CA, USA), allophycocyanin (APC)-conjugated anti-CD8 (Immunotech, Marseille, France) and FITC-conjugated CD45RO (DAKO, Glostrup, Denmark), FITC-conjugated HLA-DR and CLA (BD Pharmingen, San Diego, CA). Staining for intracellular cytokines was performed as described previously[3]. After using the Fix and Perm permeabilisation kit (Caltag, Burlingame, CA) cells were stained with PE-conjugated cytokines: anti-IFN-γ, anti-TNF-α, anti-IL-2, anti-IL-4, or anti-IL-5 (BD Biosciences). Flow cytometry was performed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) and data analysis using CellQuest software.

Immunohistochemistry

Immunohistochemical analysis of tissues sections was performed as described previously[3]. Double stainings with cell-specific markers (CD1a; mouse IgG1, CD4; mouse IgG2a; Neomarkers (Fremont, CA, USA), CD8; mouse IgG2b; Novocastra (Newcastle upon Tyne, UK), CD3; rabbit IgG; DAKO) were detected by fluorescence using the relevant secondary goat anti-mouse or goat anti-rabbit isotype specific Alexa Fluor 488, Alexa Fluor 647 or Alexa Fluor 546 secondary antibodies (Invitrogen, Carlsbad, CA, USA). Results were analysed by confocal microscopy using a Carl Zeiss MicroImaging, Inc. LSM 510 confocal microscope. In the case of the single enzymatic stains, CCL27 (mouse IgG2a; R&D Systems, Abingdon, UK) was detected using a goat anti-mouse-biotin labeled secondary antibody (DAKO) followed by StreptABComplex/horseradish peroxidase (DAKO) and finally diaminobenzidine (DAB) detection. For all stainings replacement of the primary antibodies by PBS/BSA 1% was used as a negative control. Under these conditions no specific staining was identified.

Conflict of Interest

The authors state no conflict of interest.
Informed consent was obtained from the parents regarding the use of photographs. Institutional approval is provided to use left over biological material obtained for diagnostic purposes for related research in line with the Helsinki guidelines.

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Chapter

4

A possible role for CCL27/CTACK-CCR10 interaction in recruiting $CD4^+$ T cells to skin in human graft-versus-host disease

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Abstract

Graft-versus-host disease (GvHD) is a serious complication of allogeneic stem cell transplantation (SCT) affecting the skin, gut and liver. The involvement of distinct organs suggests a role for tissue-specific chemokines and their receptors in directing activated donor T cells to these sites. In this study the potential involvement of the skin-specific CCL27/CTACK-CCR10 interaction was investigated in 15 paediatric SCT patients with skin GvHD. During the course of skin GvHD, peripheral blood T cells from these patients contained a high proportion of $CD4^+CCR10^+$ T cells that disappeared after the GvHD was resolved. These cells were $CD45RO^+$, expressed additional skin homing markers (cutaneous lymphocyte-associated antigen and CCR4), and produced the T cell helper type 1-cytokines tumour necrosis factor-α and interleukin-2. The increase in $CD4^+CCR10^+$ T cells was absent in SCT patients without GvHD. Immunohistochemical investigations showed $CD4^+CCR10^+$ T cells in the GvHD skin biopsies of the same patients, but not in the gut biopsies of patients also suffering from gut GvHD. The infiltration of $CD4^+CCR10^+$ T cells in the GvHD-affected skin correlated with an enhanced epidermal expression of CCL27/CTACK, the ligand for CCR10. These findings support the involvement of CCL27/CTACK-CCR10 interaction in recruiting $CD4^+$ T cells to the skin, thus contributing to the pathogenesis of acute GvHD.

Introduction

Allogeneic stem cell transplantation (SCT) is a well established and effective therapy for various haematological malignancies and inherited disorders[1]. However, the success of SCT is hampered by the occurrence of lifethreatening graft-versus-host disease (GvHD), which manifests as progressive immune destruction of skin, intestines and liver[2]. Matching patient and donor for human leucocyte antigens (HLA) significantly reduces the risk for GvHD. Nonetheless, despite T cell depletion and pharmacological GvHD prophylaxis, GvHD remains a frequently occurring complication[3].

Acute GvHD is due to the recognition by alloreactive donor T cells of major histocompatibility complex (MHC) disparities or of minor histocompatibility antigens presented by host MHC proteins[4]. In order to induce GvHD, alloreactive donor T cells must first migrate to a particular tissue site where they exert their effector function. As migration of immune cells is regulated by chemokines and their receptors[5], it is likely that these molecules also control the selective migration of activated alloreactive T cells to distinct organs in GvHD. To date, all the work investigating the involvement of chemokines in GvHD has been carried out in experimental murine models. Although the elevated expression of various proinflammatory chemokines, such as CCL2/MCP-1, CCL3/MIP-1-a, CCL4/MIP-1-b, CCL5/RANTES, CXCL9/MIG and CXCL10/IP-10 has been demonstrated in the target organs of GvHD[6-8], further investigations into the exact relevance of these chemokines during GvHD are limited. In one study, blockade of T cell migration into the liver, using an anti-CCR5 antibody, downmodulated GvHD activity at this tissue site, thus illustrating that the specific

expression of particular chemokine receptors and production of their corresponding ligands in GvHD target organs does indeed appear to be important in the recruitment of alloreactive T cells[9,10].

Despite the fact that the skin is an early target of GvHD and usually precedes intestine and liver involvement, until now there has been little data to show the involvement of chemokines and their receptors in the pathogenesis of skin GvHD. The mechanisms mediating memory T cell recruitment to the skin have now been fairly well characterised[3]. T cells homing to the skin express the cutaneous lymphocyte-associated antigen (CLA) that allows them to interact with E-selectin on endothelial cells[11,12]. Although CLA mediates specific tethering of the T cells, the activation and subsequent diapedesis is due to specific chemokines[13]. Several chemokines and their receptors are associated with skin-homing $CD4^+$ T cells, namely CCR4 and its ligands CCL17/TARC and CCL22/MDC, and CCR10 together with its ligand CCL27/CTACK. CCL17/TARC has been shown to be constitutively expressed and hyperinducible on cutaneous venules[14], while CCL27/CTACK is produced by keratinocytes[15]. It is thought that the CCL17/TARC-CCR4 pathway recruits lymphocytes into the dermis, whereas the CCL27/CTACK-CCR10 pathway may guide them all the way up to the epidermis, thus suggesting that at least one of these chemokine-mediated pathways must be functional to effectively recruit T cells to inflammatory skin[14-17]. There is now considerable evidence showing the involvement of these chemokines and their receptors in various inflammatory skin diseases, including atopic dermatitis, psoriasis and atopic eczema[16,18-20].

To date, no definitive roles have yet been identified for specific chemokine/receptor interactions in the recruitment of activated donor T cells to the skin during acute GvHD. Thus, in the present study, the potential role of CCR10 and its ligand CCL27/CTACK was investigated in paediatric patients suffering from skin GvHD. The finding of a significant population of $CD4^+CCR10^+$ T cells not only in the peripheral blood of these patients but also within the skin GvHD sites, along with an enhanced expression of CCL27/CTACK, supports the involvement of CCL27/CTACK-CCR10 interactions in the development and pathogenesis of skin GvHD.

Materials and methods

Patients

After obtaining informed consent, sequential blood samples were obtained from 23 paediatric patients who had received an allogeneic SCT for the treatment of a variety of haematological malignancies. Fifteen of these patients suffered an acute GvHD involving the skin. Eight of these patients also suffered from gut GvHD involvement. Another eight patients did not experience acute GvHD at all. A combination of ciclosporin A (2 mg/kg/d intravenously) and a short course of methotrexate (10 mg/m2, at days +1, +3 and +6 after SCT) was used as GvHD prophylaxis in all patients. Acute GvHD was diagnosed and graded in all patients according to the standard Glucksberg criteria[21]. Systemic treatment of acute GvHD consisted of continuation of ciclosporin A (2 mg/kg/d intravenously or 6 mg/kg/d orally) and methylprednisolone (2

mg/kg/d initial dose). Medication was tapered following clinical improvement. Table I outlines the transplantation-related details of the patients involved in this study. Skin and gut biopsies were taken from the affected sites when GvHD was suspected based on clinical criteria[21], just before treatment was started. The biopsies were then frozen in TissueTeck and stored at -80°C or paraffin embedded.
For chimaerism analyses, mononuclear cells and granulocytes were isolated from the bone marrow and peripheral blood at regular intervals post-transplant. Percentages of donor-host chimaerism for recipients of sex-mismatched SCT were evaluated by fluorescent *in situ* hybridisation for X and Y chromosomes, while for recipients of sex-matched SCT, fluorescent-based multiplex polymerase chain reaction amplification of short-tandem repeat sequences discriminative of donors and hosts was used. All patients investigated in this study displayed full donor chimaerism post-transplant. This study was approved by the Review Board of the LUMC for medical ethics.

Flow cytometry

Primary antibodies used for flow cytometry were as follows: anti-CCR1, CCR2, phycoerythrin (PE)-conjugated anti-CCR3, CCR5, CCR6, CCR9, CXCR4, CXCR5 and CXCR6 (R&D Systems, Minneapolis, MN, USA); anti-CCR4, CCR7, CXCR3 and fluorescein isothiocyanate (FITC)-conjugated anti-CLA (BD Pharmingen, San Diego, CA, USA); CD103-FITC, CD62L-FITC and CD45RO-FITC (Dako, Glostrup, Denmark); CCR8 (Alexis Biochemicals, San Diego, CA, USA); CCR10 (clone 37; DNAX Research Institute, Palo Alto, CA, USA); CD25-FITC, HLA-DR-FITC, CD69-FITC and CD57-FITC (BD Biosciences, San Jose, CA, USA). For detection of the unlabelled primary antibodies, the cells were stained with the relevant PE- or FITC-conjugated isotype-specific secondary antibody (Southern Biotechnology Associates Inc., Birmingham, AL, USA). For phenotypic determination, the cells were then stained with peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5)-conjugated anti-CD4 (BD Biosciences) and allophycocyanin (APC)-conjugated anti-CD8 (Immunotech, Marseille, France). Intracellular detection of perforin expression was analysed on $CCR10^{+}$ cells by first staining for CCR10 followed by a PE-conjugated mouse IgG1. The cells were then permeabilised using the Fix and Perm permeabilisation kit (Caltag, Burlingame, CA, USA) and stained with the following directly conjugated antibodies: perforin-FITC (Holzel Diagnostika, Koln, Germany), CD4-PerCP-Cy5.5 (BD Biosciences) and CD8-APC (Immunotech). Four-colour flow cytometry was performed on a fluorescence-activated cell sorting (FACS) Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) using cellquest software.

Immunohistochemistry

Four micrometer cryosections of skin or gut biopsies were fixed in cold acetone, dried at room temperature for 5 min, and then rehydrated for 5 min in phosphate-buffered saline (PBS). Tissues were then blocked with 10% normal goat serum (Dako) for 30 min before incubation with primary unconjugated antibodies for 2–3 h at 4°C. Double stains with primary anti-chemokine receptors (CCR10; clone 1908;

Patients	Age (years)	Donor	Diagnosis	Conditioning	TCD	GvHD prophylaxis	Onset GvHD (wks post HSCT)	max. grade	Organ involved	Treatment	Max. % $CD4^+CCR10^+$	wks after SCT	Absolute total CD4 numbers	Absolute total $CD4^+CCR10^+$ numbers
with GvHD														
1	10	IRD	ALL	Cy, VP16, TBI	-	MTX, CsA	2	1	skin	MP	19,2	25	9	1,6
2	16	MUD	ALL	VP16, ATG Cy, TBI	-	MTX	3	1	skin	MP	29,5	11	27,1	8
3	4	ORD	ALL	Cy, VP16, TBI	-	MTX, CsA	5	1	skin	MP	25,2	14	168	42,3
4	17	IRD	AML	Cy, TBI	-	MTX, CsA	2	2	skin	MP	17,8	7	110,6	17,8
5	2	CB	ALL	VP16, ATG, TBI	-	CsA	5	2	skin	CsA, local steroids	46,3	8	-	-
6	9	IRD	ALL	VP16, TBI	-	CsA	10	1	skin	local steroids	14,4	25	-	-
7	1	MUD	OP	Bu, Cy	-	MTX, CsA	12	3	skin & gut	MP	19,7	12	75,3	10,1
8	4	MUD	ALL	Cy, VP16, TBI	-	MTX, CsA	5	2	skin & gut	MP	43,5	13	100,4	43,7
9	15	MUD	ALL	Cy, VP16, TBI	-	MTX, CsA	6	2	skin & gut	MP	33,8	33	94,5	28,3
10	10	MUD	SAA	OKT3, Cy, TBI	-	MTX, CsA	5	3	skin & gut	MP, CsA, MMF	21,7	32	-	-
11	13	MUD	ALL	Cy, VP16, TBI	-	MTX, CsA	10	2	skin & gut	MP	24,1	14	90	12
12	<1	ORD	OS	Bu	-	MTX, CsA	25	3	skin & gut	MP	26,9	25	-	-
13	4	MUD	aCML	Cy, ATG, TBI	$CD34^+$ T cell addback	MTX, CsA	2	3	skin & gut	MP	14,4	6	129	18,6
14	8	MUD	ALL	VP16, ATG, TBI	-	MTX, CsA	2	2	skin & gut	MP, CsA	19,5	9	-	-
15	13	IRD	MDS RAEBt	Bu, Cy, Mel	-	CsA	5	4	skin, gut & liver	MP, MSC	14,7	7	75	11
without GvHD														
16	<1	MUD	CHS	Flu, Bu, Cy	T/B E-rossetting	CsA					7,8	20	1026,2	80
17	1	MUD	ALL	VP16, Bu, ATG, Cy	T/B E-rossetting	CsA					6,4	23	853,9	49,5
18	<1	MUD	ALL	VP16, Bu, Cy	$CD34^+$ T cell addback	CsA					11,3	28	527	59,6
19	9	MUD	ALL	Cy, VP16, TBI	-	MTX, CsA					7,6	5	19	1,4
20	4	IRD	WAS	Bu, Cy	-	MTX, CsA					3,3	11	223	7,4
21	13	MUD	Thal	Bu, Cy, Mel	-	MTX, CsA					5,6	30	269	15,1
22	7	IRD	aCML	Cy, TBI	-	MTX, CsA					5,5	11	435,6	8,3
23	14	MUD	ANLL	Bu, Cy, Mel	-	MTX, CsA					14	5	181	16,7

DNAX), in combination with cell-specific markers, CD4 (mouse IgG2a; Neomarkers, Fremont, CA, USA), CD8 (mouse IgG2b; Novocastra, Newcastle upon Tyne, UK), CD3 (rabbit IgG; DAKO), CD45RO (mouse IgG2a; Dako), Ki67 (mouse IgG1; Dako) were detected fluorescently using the relevant secondary goat anti-mouse or goat anti-rabbit isotype-specific Alexa Fluor 488 or Alexa Fluor 546 secondary antibodies (Molecular Probes, Leiden, the Netherlands). Replacement of the primary antibodies by PBS/bovine serum albumin 1% was used as a negative control. Results were analysed by confocal microscopy using LSM 510 confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA).
In the case of single enzymatic stains, CCL27/CTACK (goat IgG; R&D Systems) was incubated overnight at room temperature on paraffin sections that had been subjected to heat-mediated antigen retrieval in a microwave using citrate buffer (10 mmol/l, pH 6.0). CCL17/TARC (rabbit IgG; Peprotech, London, UK) was incubated overnight on cryosections at 4°C. The bound primary antibodies were detected using a rabbit anti-goat-biotin or a swine anti-rabbit-biotin labelled secondary antibody (Dako), respectively, followed by StreptABComplex/horseradish peroxidase (Dako) and finally VECTOR NovaRed (Vector Laboratories, Burlingame, CA, USA) detection. To test the specificity of immunostaining, the primary antibody was omitted. Under this condition no staining was identified.

Intracellular cytokine staining

Flow cytometry analysis for intracellular cytokines was performed by stimulating peripheral blood mononuclear cells (PBMC) with a combination of 200 ng/ml phorbol myristate acetate (PMA) and 500 ng/ml of Ionomycin (Sigma-Aldrich, St Louis, MO, USA) for 1 h. Control PBMC were left unstimulated. Stimulated and non-stimulated cells were then cultured for 16 h at 37°C and 5% CO_2 in the presence of 5 lμ/ml Brefeldin A (Sigma-Aldrich). Cells were then aliquoted and stained with anti-CCR10 (DNAX), followed by mIgG-APC (BD Pharmingen) and then directly labelled with anti-CD3-PerCP-Cy5.5 and CD4-FITC. After washing, the cells were permeabilised using the Fix and Perm permeabilisation kit (Caltag) and stained with the following PE-conjugated cytokines: anti-interferon (IFN)-γ, anti-tumour necrosis factor (TNF)-α, anti-interleukin (IL)-2, anti-IL4, anti-IL-5, anti-IL- 10, anti-IL-12 (BD Biosciences) and anti-transforming growth factor (TGF)-β (IQ Products, Groningen, the Netherlands). Four-colour flow cytometry was performed on a FACS Calibur using cellquest™ software (Becton Dickinson).

Table I (previous page). Overview of all SCT patients and their transplantation-related details.
IRD, Identical Related Donor; MUD, Matched Unrelated Donor; ORD, Other Related Donor; CB, Cord Blood; MSC, Mesenchymal Stem Cells; ALL, Acute Lymphoblastic Leukaemia; AML, Acute Myeloid Leukaemia; OP, Osteopetrosis; SAA, Severe Aplastic Anaemia; OS, Omenn Syndrome; aCML, atypical Chronic Myeloid Leukaemia; MDS RAEBt, Myelodysplastic Syndrome; CHS, Chediak-Higashi; WAS, Wiskott-Aldrich Syndrome; Thal, Homozygote B Thalassaemia; ANLL, Acute Non Lymphocytic Leukaemia; TCD, T cell depletion; Cy, cyclophosphamide; VP16, Etoposide; TBI, Total Body Irradiation; ATG, Antithymocyte Globulin; OKT3, muromonoab-CD3; MMF, Mycophenolate Mofetil; Bu, Busulphan; Mel, Melphalan; Flu, Fludarabin; MTX, Methotrexate; CsA, Cyclosporine A; MP, Methylprednisolone. Absolute numbers are in counts per μl.

Statistical analysis

Flow cytometric data on CCR10 expression are expressed as median and range. Differences in CCR10 expression between healthy controls, SCT patients without GvHD and GvHD patients were first assessed using the Kruskal-Wallis test. When significant ($P < 0.05$), pair-wise comparisons were performed using the Mann-Whitney test. The Mann-Whitney test was also performed to analyse the absolute CD4 and CCR10 numbers in the peripheral blood of SCT patients with and without GvHD.

Results

Identification of peripheral blood $CD4^+$ $CCR10^+$ T cells in skin GvHD patients

In order to identify the tissue-homing capability of T cells involved in acute GvHD, the expression of chemokine receptors and adhesion molecules was studied on T cells in peripheral blood taken from 23 paediatric patients at regular intervals following allogeneic SCT. Due to the fact that all the 15 GvHD patients included in this study suffered from skin GvHD, particular attention was paid to the previously described skin-associated homing markers, namely CLA and CCR10. Interestingly, there was no significant increase of any of these skin-homing markers on the peripheral blood $CD8^+$ T cells of all the GvHD patients studied. In contrast, however, this analysis revealed a significant increase in the percentage of $CD4^+CCR10^+$ T cells in the peripheral blood of all patients (15/15) who had been diagnosed with GvHD of the skin. At the peak of this response, i.e. 0-27 weeks after GvHD onset, the percentage of $CD4^+$ T cells that expressed CCR10 ranged from 14.4% to 46.3% (median: 21.7%) in the 15 skin GvHD patients studied (Table I). Figure 1A shows the increase in $CD4^+CCR10^+$ T cells in a representative skin GvHD patient (patient 3 in Table I). This increase in CCR10 expression on CD4+ T cells was statistically significant (Kruskal-Wallis: $P < 0.05$, Mann-Whitney: $P \leq 0.001$) compared with that seen on the $CD4^+$ T cells of SCT patients without GvHD during the same period after transplant (median: 7%; range from 3.3% to 14%; n = 8 studied) and healthy paediatric donors (median: 2.8%; range from 0.6% to 6.5%; n = 13 studied, Figures 1A and D).

The relative increase in $CD4^+CCR10^+$ T cells in skin GvHD patients also coincided with an increase in the percentage of $CD4^+CLA^+$ T cells (Figure 1B), although the kinetics of CCR10 and CLA disappearance were not completely similar in all patients. Indeed, multicolour flow cytometry showed that >50% of the $CD4^+CCR10^+$ T cells co-expressed CLA (Figure 1C).

The absolute numbers at or near the peak of CCR10 expression are given in Table I for those patients for whom this data were available. The median of the absolute CD4 numbers in the SCT patients without GvHD was significantly higher (Mann-Whitney: $P < 0.05$) than in the GvHD patients. However, there was no significant difference between the absolute $CD4^+CCR10^+$ numbers of these two patient groups. The expression of CCR10 by $CD4^+$ peripheral blood T cells appeared to correlate with the duration of skin activity in the patients with skin GvHD. For the majority of GvHD

patients studied (10/15), skin GvHD activity resolved very rapidly following systemic steroid treatment and this was reflected by increased percentages of peripheral blood $CD4^+CCR10^+$ T cells for only a short period of time (mean: 14 weeks). Figure 2A shows an example of the typical kinetics of the $CD4^+CCR10^+$T cell population from a representative patient (patient 4 in Table I). In contrast, in the case of a skin GvHD

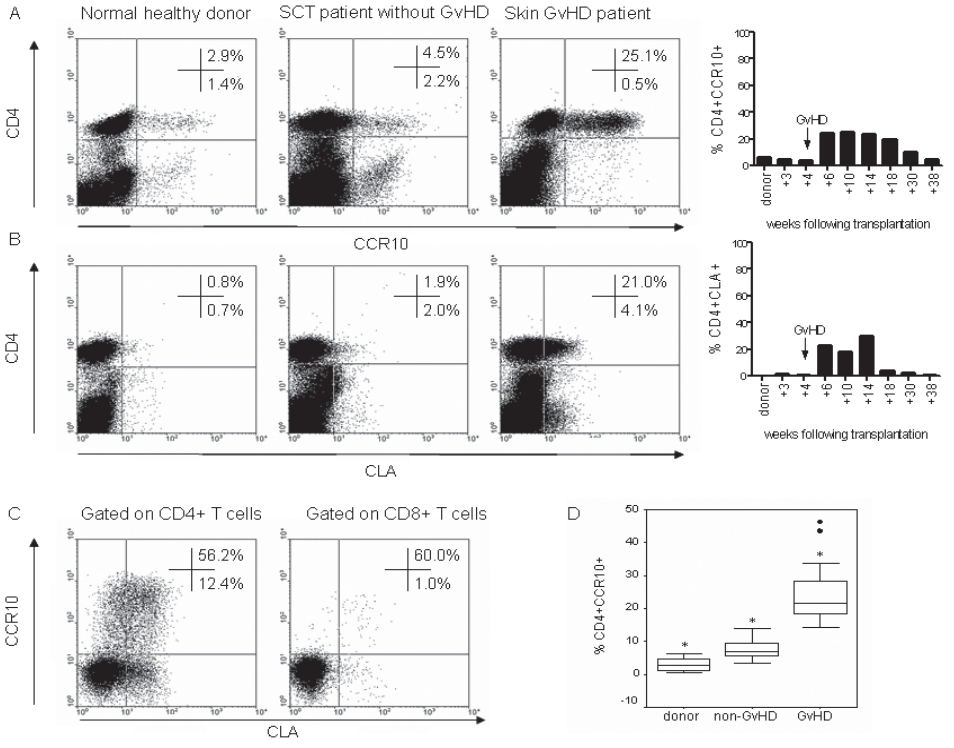

Figure 1. Increased percentages of $CD4^+$ T cells expressing the skin-homing markers CCR10 and cutaneous lymphocyte-associated antigen (CLA) in a representative patient with skin graft-versus-host disease (GvHD).

(A) Fluorescence-activated cell sorting (FACS) analysis showing an increased percentage of $CD4^+CCR10^+$ T cells amongst peripheral blood lymphocytes of a representative patient with skin GvHD (patient 3 in Table I, 6 weeks post-stem cell transplantation (SCT)])compared with a SCT patient without GvHD (patient 17 in Table I, 7 weeks post-SCT) and a healthy control (normal healthy donor). The percentage of $CD4^+CCR10^+$ T cells remained high in the peripheral blood of the skin GvHD patient for several months following the initial diagnosis of skin GvHD. (B) The increased percentage of $CD4^+CCR10^+$ T cells also coincided with an increase in $CD4^+CLA^+$ T cells in the same patient. (C) Multicolour FACS analysis showed that the $CD4^+CCR10^+$ T cells highly expressed CLA. In contrast, there was negligible expression of CCR10 and CLA on the $CD8^+$ T cells of the same skin GvHD patient. The numbers in the FACS plots represent the percentage of CCR10 (A) or CLA (B) positive cells within the $CD4^+$ T cells (top right quadrant) and CD4) T cells (bottom right quadrant) respectively; (C) percentage of CLA positive cells in $CCR10^+$ (top right quadrant) and CCR10) T cells (bottom right quadrant). (D) Median and range of $CD4^+CCR10^+$ T cells at the peak of expression of all healthy paediatric donors (n = 12), SCT patients without GvHD (n = 8) and GvHD patients (n = 15). Two outliers from the GvHD group are shown as closed circles. Differences between the groups were statistically analysed using the Kruskall-Wallis and Mann-Whitney test and were significantly different ($P < 0.001$) as indicated by the asterisk.

patient who did not respond immediately to systemic steroid treatment and showed protracted skin activity (patient 8 in Table I), increased percentages of peripheral blood $CD4^+CCR10^+$ T cells were observed for a much longer period of time (26 weeks, Figure 2B). The specificity of the increased percentages of $CD4^+CCR10^+$ T cells to the disease activity was further demonstrated by the fact that SCT patients without skin GvHD showed no increase in peripheral blood $CD4^+CCR10^+$ T cells

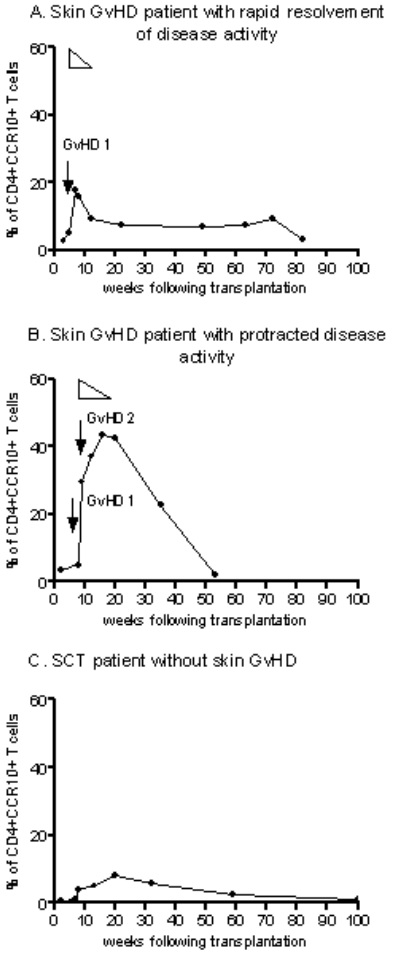

Figure 2. Increased percentages of $CD4^+CCR10^+$ peripheral blood T cells correlate with duration of disease activity in the skin.
(A) Fluorescence-activated cell sorting (FACS) analysis showing a rapid increase and decrease in the percentage of $CD4^+CCR10^+$ peripheral blood T cells in a stem cell transplantation (SCT) patient whose skin graft-versus-host disease (GvHD) responded rapidly to treatment (patient 4 in Table I). (B) In contrast, another skin GvHD patient who did not respond immediately to steroid treatment and showed protracted skin activity (patient 8 in Table I), had increased percentages of peripheral blood $CD4^+CCR10^+$ T cells for a prolonged period of time. (C) Representative FACS analysis of a SCT patient without any skin GvHD activity (patient 16 in Table I) shows no increase in the percentage of peripheral blood $CD4^+CCR10^+$ T cells following transplantation.

following transplantation. A representative patient is shown in Figure 2C (patient 16 in Table I).

Additional phenotypical analysis of the $CD4^+CCR10^+$ T cells (Figure 3) showed that this population stained positive for the memory T cell marker, $CD45RO^+$ (mean: 96 ± 2.4%). These $CD4^+CCR10^+$ T cells clearly displayed a profile of surface markers indicating preferential homing to the skin, as evidenced by their low expression of the lymphoid homing marker CCR7 and gut-associated homing integrin CD103, and high expression of the skin homing receptors CLA (mean: 74.4 ± 23.8%) and CCR4 (mean: 88.9 ± 6.6%). Indeed, the $CD4^+CCR10^+$ T cells expressed significantly higher levels of these skin homing molecules than the $CD4^+CCR10^-$ population. Analysis of other chemokine receptors whose ligands have previously been found in inflamed skin showed that the $CD4^+CCR10^+$ T cells displayed fairly high levels of CXCR3 (mean: 25.5 ± 17.0%), although still lower than the $CD4^+CCR10^-$ T cells (mean: 48 ± 17.0%), and very low levels of CCR6 (mean: 1.4 ± 1.3%). Stains were also performed to elucidate the activation state and possible function of the $CD4^+CCR10^+$ T cells. Analysis of the expression of the activation markers CD25 and HLA-DR showed that the $CD4^+CCR10^+$ T cells displayed a higher expression of these markers when compared with the $CD4^+CCR10^-$ T cell population.

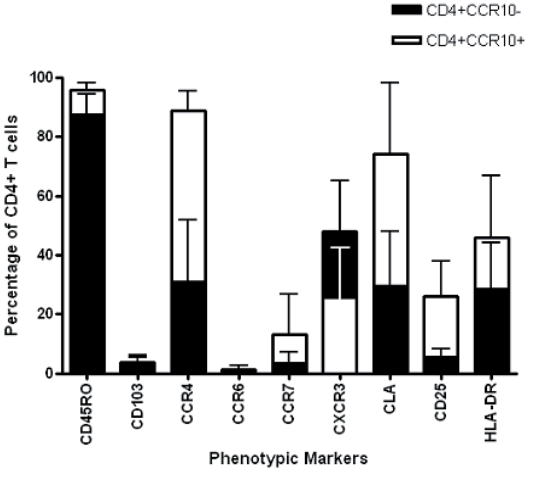

Figure 3. Comparison of the phenotype of $CD4^+CCR10^+$ and $CD4^+CCR10^-$ T cells in patients with skin graft-versus-host disease (GvHD).

Multicolour fluorescence-activated cell sorting analysis of the $CD4^+CCR10^+$ and $CD4^+CCR10^-$ T cell populations at the peak of the CCR10 expression showed a significantly increased expression of the skin-homing markers cutaneous lymphocyte-associated antigen and CCR4 and the activation markers CD25 and human leucocyte antigen-DR on the $CD4^+CCR10^+$ T cell population compared with the $CD4^+CCR10^-$ T cell population. The data are expressed as mean ± SD of results obtained from n = 8 patients with skin GvHD.

CD4⁺CCR10⁺ T cells infiltrate skin but not gut GvHD sites

To determine whether the CD4⁺CCR10⁺ T cells were also present in the skin of patients with acute GvHD, biopsies were taken from the affected skin sites of these patients early after the first clinical signs of GvHD. Multicolour immunofluorescent staining was performed on cryosections from four GvHD patients using antibodies specific for T cell markers in combination with either CCR10 or other phenotypic markers (Figure 4A-E). These results showed that there was a mixture of CD4⁺ and CD8⁺ T cells infiltrating GvHD skin, but only the CD4⁺ T cells expressed CCR10 whereas the CD8⁺ T cells did not, reflecting what was seen in the peripheral blood. Analysis of normal skin and skin from a transplant patient that did not suffer from GvHD showed that there was no significant T cell infiltrate in either biopsy (data

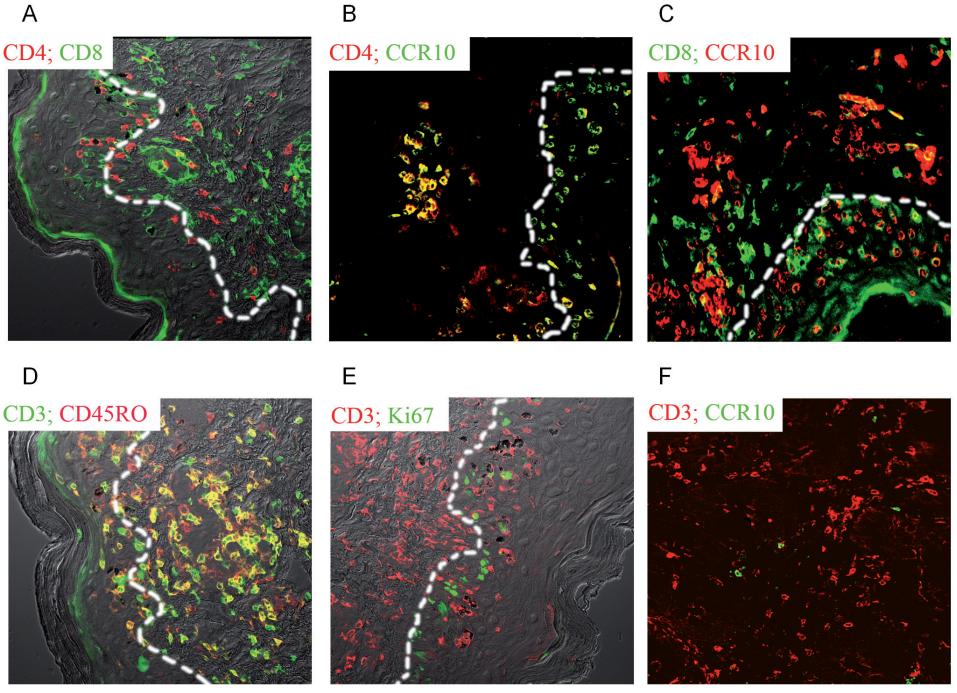

Figure 4. Immunohistochemical analysis of T cells infiltrating sites affected by graft-versus-host disease (GvHD).
(A) Double immunofluorescent staining on a skin GvHD biopsy of a representative patient affected by skin and gut GvHD, identified a mixture of both CD4⁺ (red) and CD8⁺ (green) T cells infiltrating skin GvHD sites. (B) The CD4⁺ T cells expressed CCR10 as evidenced by the yellow colour produced due to co-localisation of CD4 in red and CCR10 in green. (C) In contrast, the CD8⁺ T cells (green) did not express CCR10 (red). (D) The majority of the T cells in the skin GvHD site were CD45RO⁺ (yellow) but none of them expressed the proliferative marker Ki67 (green) (E). There was, however, Ki67 expression by the epidermal cells in the basal layer of the skin. The dotted white line denotes the epidermal–dermal junction. (F) Analysis of CD3⁺ T cells (red) and CCR10 (green) in the gut GvHD biopsy from the same patient showed no co-localisation of these markers. Magnification, 250x.

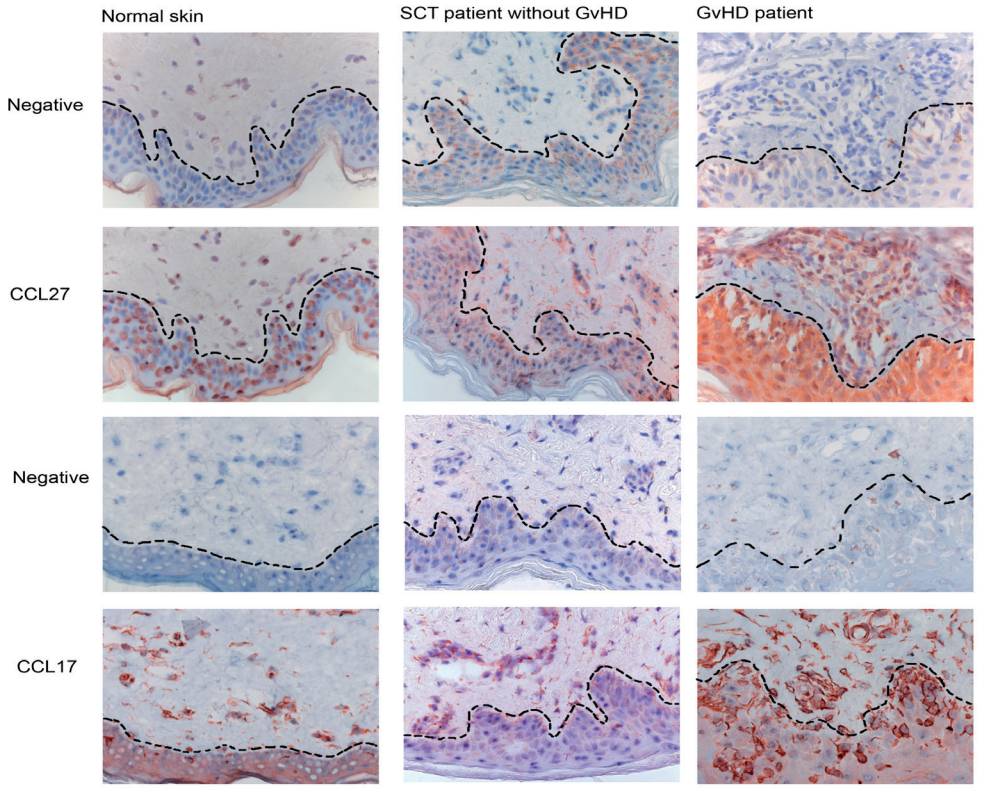

Figure 5. Expression of the skin-specific chemokine CCL27/CTACK and CCL17/TARC in normal versus graft-versus-host disease (GvHD) affected skin biopsies.
Single enzymatic staining for CCL27/CTACK and CCL17/TARC (detected by the red/brown colour) showed an enhanced expression of these chemokines in representative GvHD skin compared with skin of a healthy control and of a stem cell transplantation patient who did not suffer from GvHD. The CCL27/CTACK was clearly enhanced in the epidermis of the GvHD skin and both CCL27/CTACK and CCL17/TARC were expressed by the cellular infiltrate in the dermis and epidermal junction of GvHD skin. Omission of the primary antibody was used to show the specificity of the staining (negative controls). The dotted line represents the epidermal–dermal junction. Magnification, 600x.

not shown). Further analysis of the T cells infiltrating GvHD skin confirmed that the majority were indeed $CD45RO^+$ and none of the T cells expressed the proliferative marker Ki67. The only expression of Ki67 was due to epidermal cells within the basal layer. To further demonstrate the specificity of the $CD4^+CCR10^+$ T cells for the skin GvHD sites, gut biopsies from four of the skin GvHD patients who also suffered from a gut GvHD were investigated for the presence of $CD3^+CCR10^+$ T cells. All four gut GvHD biopsies consistently showed no co-localisation of CD3 and CCR10, thus confirming that CCR10 is only involved in the homing of T cells to skin and not gut GvHD sites (Figure 4F).
The selective presence of $CD4^+CCR10^+$ T cells in the skin GvHD biopsies of patients with skin and gut GvHD strongly supported preferential migration of these T cells to the skin. Therefore, expression of both the CCR10 ligand, CCL27/CTACK, and the

CCR4 skin-associated ligand, CCL17/TARC, was also investigated in skin GvHD biopsies. Single enzymatic staining for these chemokines was also performed on normal skin from healthy donors and from SCT patients without GvHD (Figure 5). As previously reported[15,16], CCL27/CTACK was only weakly expressed by keratinocytes in the epidermis of unaffected skin whereas CCL17/TARC was expressed by a subset of dermal vessels and cells just below the epidermal-dermal junction[22]. In the skin of SCT patients who did not suffer from GvHD, the expression of both CCL27/CTACK and CCL17/TARC was only slightly increased, whereas in the skin of GvHD patients, the expression of both these ligands was clearly enhanced. CCL27/CTACK expression was greatly upregulated within the epidermis and there was also expression of both CCL27/CTACK and CCL17/TARC by infiltrating lymphocytes.

Intracellular cytokine staining reveals the production of TNF-α and IL-2 but not IFN-γ by CD4$^+$CCR10$^+$ T cells in GvHD patients

To try and elucidate the possible function of the CD4$^+$CCR10$^+$ T cells, the production of cytokines with or without stimulation of the cells with PMA/ionomycin was investigated at the peak of CCR10 expression (mean: 16.5 weeks post-SCT) in the GvHD patients for whom enough material was available (n = 4, patients 1, 3, 8 and 10 in Table I). In these patients, the CD4$^+$CCR10$^+$ T cells were shown to consistently produce TNF-α (range, 5.1-35.2%; mean, 18.1 ± 13.1%) and IL-2 (range, 4.6-27.2%; mean 13.7 ± 10.8%) upon stimulation (Figures 6A and C). In the SCT patients without GvHD (n = 5, patients 16, 17, 19, 21 and 23 in Table I; mean: 23 weeks post-SCT), the CD4$^+$CCR10$^+$ T cells produced TNF-α (range 39.6-71.2%; mean 55.8% ± 14.9%), IL-2 (range, 37.7-58.5%; mean 49.7 ± 9.9%) and interestingly, IFN-γ (range, 6.3-36.4%; mean 25.1 ± 12.4%, Figures 6B and D). In contrast, in the GvHD patients IFN-γ was only produced by the CD4$^+$CCR10$^-$ T cells, as shown in the FACS plots of a representative patient in Figure 6C. The overall production of cytokines by CD4$^+$ T cells (both CCR10$^+$ and CCR10$^-$) of SCT patients without GvHD was higher than in the GvHD patients (Figures 6A and B). This may be explained by the prednisolone treatment received by all GvHD patients (n = 4). In one patient (patient 8 in Table I), sufficient cells were available to study cytokine expression at the time of GvHD before prednisolone treatment was started. In this case, the percentages were 2.5 times higher than those during treatment but the same expression pattern was seen, i.e. only TNF-α and IL-2 were expressed and not IFN-γ (data not shown). Neither CD4$^+$CCR10$^+$ nor CD4$^+$CCR10$^-$ T cell populations of both SCT patients with and without GvHD produced any of the T cell helper type 2 (Th2) cytokines IL-4 and IL-5 or the suppressive cytokines IL-10 and TGF-β upon stimulation (data not shown).

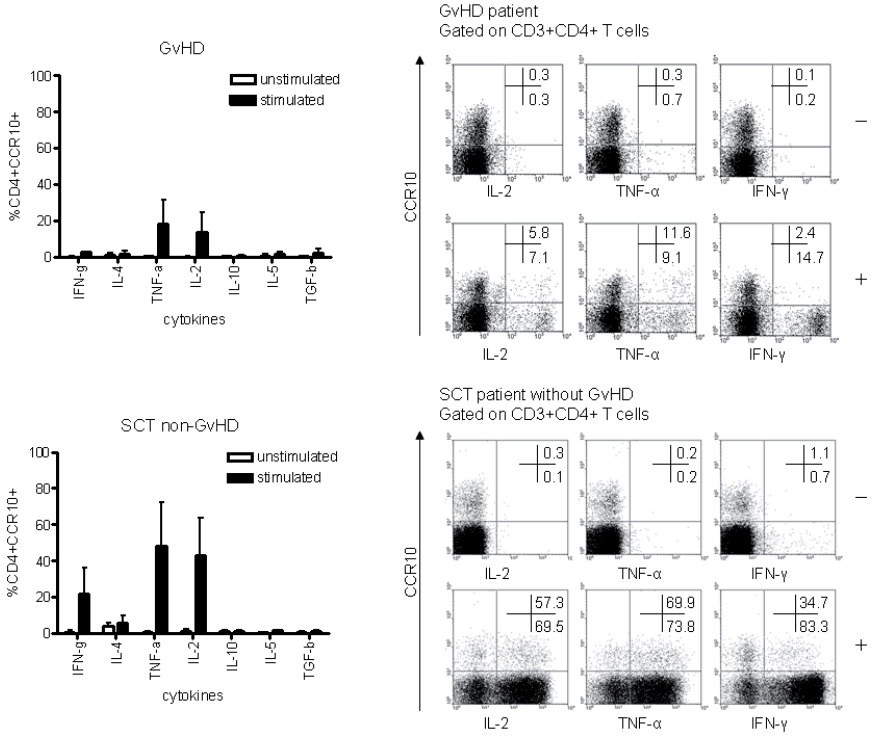

Fig 6. Intracellular cytokine staining reveals production of TNF-α and IL-2 but not IFN-γ by the CD4$^+$CCR10$^+$ T cells.

(A) Mean intracellular staining of cytokines by CD4$^+$CCR10$^+$ T cells at the peak of the CCR10 expression [mean: 16.5 weeks post-stem cell transplantation (SCT)] of n = 4 graft-versus-host disease (GvHD) patients (patients number 1, 3, 8 and 10 in Table I) and (B) of n = 5 SCT patients without GvHD (patients numbers 16, 17, 19, 21 and 23 in Table I, mean: 23 weeks post-SCT), with (+) and without (-) PMA/ionomycin stimulation in vitro. (C) Representative FACS analysis of the cytokine production by CD4$^+$CCR10$^+$ peripheral blood T cells from a patient with skin GvHD (patient number 1 in Table I) and (D) a SCT patient who did not suffer from GvHD (patient number 17 in Table I). The numbers represent the percentage of CD4$^+$CCR10$^+$ (top right quadrant) or CD4$^+$CCR10$^-$ (bottom right quadrant) T cells that express a particular cytokine.

Discussion

Acute GvHD is a major complication of allogeneic SCT resulting in morbidity and mortality. Thus, investigating the mechanisms behind the migration of alloreactive T cells to the sites of GvHD is fundamental in understanding the pathogenesis and course of GvHD. While chemokines and their receptors have been studied extensively in murine GvHD models, this is the first study to report on their involvement in human GvHD. As skin is an early target of GvHD, we focused on the skin-homing chemokine receptor CCR10 and its ligand CCL27/CTACK.

In this study, analysis of chemokine receptor expression by the peripheral blood T

cells of paediatric skin GvHD patients showed a clear increase in the percentage of $CD4^+$ T cells expressing CCR10. This relative increase in $CD4^+CCR10^+$ T cells was clearly specific for the skin GvHD patients, as the SCT patients without GvHD did not show such an increase in this population of T cells. This was independent of any transplantation-related factors as both groups with and without GvHD were similarly heterogeneous in their treatment protocol. Furthermore, the appearance of $CD4^+$ $CCR10^+$ T cells in the circulation was independent of the presence or absence of gut GvHD and the duration of the increased percentages of this population in the peripheral blood of skin GvHD patients appeared to correlate with the disease activity in the skin. Analysis of the absolute numbers of lymphocytes revealed that the $CD4^+$ T cell population was dramatically affected by the prednisolone treatment, causing lymphopenia in all the GvHD patients compared with the non-GvHD patients. In this manner the proportion of $CD4^+CCR10^+$ T cells in the circulation of GvHD patients increased although the absolute numbers did not change significantly. This greater proportion of $CD4^+CCR10^+$ T cells in the circulation of the GvHD patients, together with the upregulated expression of the ligand for CCR10, CCL27, in the skin of these patients, results in the infiltration of this population in the skin. Further characterisation of the $CD4^+CCR10^+$ T cell population in the peripheral blood of skin GvHD patients showed that they also highly expressed other skin-homing associated markers, such as CLA and CCR4. Interestingly, $CD4^+CCR10^+$ T cells lack expression of CCR6, which was recently reported to be involved in the development of GvHD across an MHC class II barrier in mice[23]. Thus, this phenotype of $CD4^+CCR10^+$ T cells clearly indicated a preferential migration of these cells to the skin. This was confirmed by immunohistochemical investigations showing the presence of $CD4^+CCR10^+$ T cells in the skin but not in gut GvHD biopsies of the same patients. Furthermore, there was clearly an enhanced expression of the ligand for CCR10, CCL27/CTACK in the epidermis of GvHD-affected skin compared to skin of SCT patients without GvHD and of healthy controls. Interestingly, the lymphocytic infiltrate in the skin biopsies also stained positive for CCL27/CTACK, raising the possibility that the infiltrating cells themselves may contribute to the further recruitment and retention of $CCR10^+$ T cells.

Direct evidence for the role of CCR10-CCL27/CTACK in T cell recruitment to the skin has already been shown in lesional skin biopsies taken from patients suffering from skin disorders, such as atopic dermatitis, psoriasis and nickel contact allergy. A strong CCR10 expression was observed on skin-infiltrating dermal leucocytes and intra-epidermal lymphocytes, providing evidence for a role of CCR10 expressing T cells in these diseases[11-14,19]. In all these diseases, the CCR10-expressing T cells were $CD4^+$ and no $CD8^+CCR10^+$ T cells could be detected. This is in keeping with a study by Hudak *et al*[24], who showed that bloodderived $CCR10^+$ T cells are predominantly within the $CD4^+$ T cell subset whereas $CD8^+$ T cells only have negligible expression of CCR10. We also found the same in patients with skin GvHD after allogeneic SCT, with CCR10 only being expressed at significant levels by the $CD4^+$ T cells in both the peripheral blood and GvHD skin. Thus, the $CD8^+$ T cells must use other chemokine receptor/ligand interactions to home to and enter the skin. Indeed, unpublished work from our laboratory showed that the majority of T cells infiltrating GvHD skin, both $CD4^+$ and $CD8^+$, also expressed CXCR3, the ligands of which (CXCL9/MIG,

CXCL10/IP-10 and CXCL11/I-TAC) have previously been shown to play a role in attracting activated T cells to inflamed skin[25]. The expression of CCL27/CTACK has previously been reported to be increased in inflammatory conditions, such as atopic dermatitis, psoriasis and contact dermatitis[16,18]. Our finding - that this is also true for GvHD skin lesions, is in keeping with the fact that CCL27/CTACK has been shown to be induced by IL-1 and TNF-α and, to a lesser extent, by IFN-γ[15], all cytokines associated with the initial inflammatory response in acute GvHD. In addition to the presence of inflammatory cytokines, CCL17/TARC (the ligand for CCR4), was reported to be significantly upregulated during the first week post-transplant in a murine model of GvHD[26]. The present study in human skin GvHD also showed a significant increase in the expression of this ligand in affected skin. This chemokine was recently shown to augment the CCL27/CTACK production in keratinocytes that have been prestimulated with TNF-α[27]. This in turn would lead to an enhanced skin-specific attraction of $CCR10^+$ T cells through CCL27/CTACK.

The T cells infiltrating skin GvHD sites were all confirmed to be memory T cells, as evidenced by their expression of CD45RO. However, none of these T cells expressed the proliferative marker Ki67 in the skin GvHD site. This finding strongly suggests that the T cells involved in GvHD do not arise from donor cell expansion in target organs, such as the skin, but are in fact activated and expanded within the draining lymphoid tissues before migrating to the skin. This is supported by experiments that have used green fluorescent protein transgenic donor cells to track their migration during the first week post-transplant in a fully MHC-mismatched murine allo-bone marrow transplantation model[28]. This study showed that, within hours of transplantation, donor T cells partitioned to lymphoid tissues where the allogeneic T cells expanded within 2-3 days. Following this period, allogeneic T cell numbers increased in GvHD target organs[28].

In GvHD, the presentation of alloantigens of host origin induces the activation of donor T cells and the subsequent production of cytokines. The T_H1 cytokines are preferentially produced and have been implicated in the pathophysiology of acute GvHD[29]. In the present study, *in vitro* stimulation of the $CD4^+CCR10^+$ T cell population found in the peripheral blood of skin GvHD patients indeed resulted in production of the T_H1-cytokines TNF-α and IL-2 but not IFN-γ. The production of TNF-α and IL-2 was reduced when compared with the $CD4^+CCR10^+$ T cells of the SCT patients who did not suffer from GvHD, probably due to the prednisolone treatment received by the GvHD patients. In one patient, for whom material was available at the time of GvHD before prednisolone treatment, the same expression pattern was seen; however, the percentages were higher than during treatment. This suggests that prednisolone had an effect on the level of cytokine production by these T cells but the lack of IFN-γ production was not due to the treatment. Considerable production of IFN-γ was, however, observed for the $CD4^+CCR10^-$ T cells in SCT patients, irrespective of the occurrence of GvHD, and for the $CD4^+CCR10^+$ T cells of SCT patients without GvHD. This is in keeping with the fact that high levels of IFN-γ can prevent the occurrence of GvHD[30]. There was no expression by either the $CD4^+CCR10^+$ or $CD4^+CCR10^-$ T cell population of IL-4, IL-5, IL-10 or TGF-β. Although the $CD4^+CCR10^+$ T cells were found to be perforin negative (data not shown), the fact that they produce TNF-α and IL-2 upon activation could still suggest a potentially detrimental role for these cells

in the pathophysiology of GvHD. Both TNF-α and IL-2 have been shown to have a pivotal role in controlling and amplifying the immune response against alloantigens. However, further *in vitro* work is needed to elucidate any, e.g. effector or regulatory, function of the $CD4^+CCR10^+$ T cell population during skin GvHD. The appearance of this population after the onset of clinically apparent skin GvHD makes the possibility of a regulatory role of these T cells attractive. In addition, more evidence for this role lies in the fact that the CCR10 percentage remains high after clinical resolvement of GvHD.

In summary, the present study has shown a role for the CCL27/CTACK-CCR10 interaction in the recruitment of activated donor $CD4^+$ T cells to sites of human skin GvHD. Although this is probably not the only chemokine/chemokine receptor pair to be involved in skin GvHD, as evidenced by the high expression of CCL17/TARC in skin GvHD biopsies, identification of such specific chemokine/receptor interactions involved in tissue-specific targeting of T cells to GvHD organs may become potential targets for the development of novel strategies to prevent the occurrence of GvHD.

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Chapter

5

In situ detection of HY-specific T cells in acute Graft-versus-Host Disease-affected male skin after sex-mismatched stem cell transplantation

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Abstract

HY-specific T cells are presumed to play a role in acute graft-versus-host disease (aGvHD) after female-to-male stem cell transplantation (SCT). However, infiltrates of these T cells in aGvHD-affected tissues have not yet been reported. We evaluated the application of HLA-A2/HY dextramers for the *in situ* detection of HY-specific T cells in cryopreserved skin biopsy specimens. We applied the HLA-A2/HY dextramers on cryopreserved skin biopsy specimens from seven male HLA-A2$^+$ paediatric patients who underwent stem cell transplantation with confirmed aGvHD involving the skin. The dextramers demonstrated the presence of HY-specific T cells. In skin biopsy specimens of three male recipients of female grafts, 68% to 78% of all skin-infiltrating CD8$^+$ T cells were HY-specific, whereas these cells were absent in biopsy specimens collected from sex-matched patient-donor pairs. Although this study involved a small and heterogeneous patient group, our results strongly support the hypothesis that HY-specific T cells are actively involved in the pathophysiology of aGvHD after sex-mismatched stem cell transplantation.

Introduction

Acute graft-versus-host disease (aGvHD) is a life-threatening complication of allogeneic stem cell transplantation (SCT) mainly affecting the stem cell recipient's skin, liver, and/or gastrointestinal tract[1]. Matching the stem cell donor and recipient for HLA significantly reduces the risk for aGvHD and chronic GvHD[2]. In the HLA-matched SCT setting, the development of GvHD is caused by donor T cells specific for ubiquitously expressed minor histocompatibility antigens[3], such as the Y chromosome-encoded HY antigens. Clinical results indicate that male recipients of female stem cells are at the greatest risk for GvHD[4].

Previous reports on the presence of HY-specific CD8$^+$ T cells in peripheral blood samples of adult male patients who developed aGvHD after sex-mismatched SCT[5,6] suggest the involvement of these cells in the pathophysiology of GvHD. This assumption is supported by results from *in vitro* studies using an *ex vivo in situ* skin explant model in which HY-specific cytotoxic T lymphocytes (T_{CTL}) cause GvHD-like tissue destruction when added to male skin tissues expressing the relevant HY-presenting HLA class I molecules[7]. However, whether the presence of circulating HY-specific CD8$^+$ T cells detected before and after the onset of aGvHD reflects an active contribution of these cells to tissue destruction has remained unclear. We applied a dextramer-based staining technique to retrospectively analyse the *in situ* presence of HLA-A2/HY–specific CD8$^+$ T cells in archived cryopreserved skin biopsy specimens from 7 HLA-A2$^+$ male paediatric patients who developed aGvHD of the skin after undergoing allogeneic HLA-matched unrelated SCT with a male or a female donor.

Methods

Study participants

All skin biopsy samples analysed in this study were derived from HLA-A2$^+$ male individuals and were collected after written informed consent was obtained in accordance with the Declaration of Helsinki. Skin samples were collected from five healthy adult volunteer donors and from seven paediatric patients who underwent allogeneic SCT for the treatment of various hematologic and nonhematologic disorders (Table 1). Patient selection was based on the expression of HLA-A2 by patient and donor, the development of histologically confirmed aGvHD[8] involving the skin and the availability of a cryopreserved skin biopsy specimen collected before the initiation of first-line treatment with corticosteroids. All but two patients (UPN 567 and UPN 577) received a 10/10-matched (HLA-A, -B, -C, -DQ, and -DR) stem cell graft from an unrelated donor. Three of the seven male patients (UPN 567, UPN 473, and UPN 463) received a stem cell graft from a female donor. The use of peripheral blood mononuclear cells (PBMCs) and skin samples for the underlying study was approved by Leiden University Medical Centre's Medical Ethics Committee.

Multimeric HLA class I /peptide complexes

Conventional allophycocyanin (APC)-conjugated HLA-A2 tetramers and phycoerythrin (PE)-conjugated HLA-A2 dextramers (Immudex, Copenhagen, Denmark), both containing the HLA-A2-restricted HY peptide FIDSYICQV (designated as HLA-A2/HY tetramers or HLA-A2/HY dextramers), as well as control PE-conjugated HLA-A2 dextramers containing the influenza peptide GILGFVFTL (HLA-A2/flu dextramers) were prepared as previously described[6,9,10]. The specificity and sensitivity of these HLA-A2/peptide multimers were routinely determined by fluorescent activation cell-sorting analysis (Supplementary Figure S1), as described previously[6,9].

Immunofluorescent staining and confocal laser scanning microscopy analysis of cryopreserved skin explant tissue and aGvHD-affected skin tissue

Before cryopreservation, skin explant assays were performed with freshly obtained healthy male skin biopsy specimens that were coincubated *in vitro* for 72 hours at 37°C and 5% CO2 with 1x10^6 HY-specific T_{CTL}, as described in detail elsewhere[7,11]. Control skin biopsy specimens, prepared from the same healthy donors, were incubated with HLA-A2/HA-1–specific T_{CTL}. Of note, the latter T cells infiltrate human skin in the presence of HA-1$^+$ dendritic cells[12], but unlike the HY-specific T_{CTL}, HA-1-specific T_{CTL} do not cause GvHD-like tissue destruction[7,12]. Cryosections (6 mm) were prepared from both snapfrozen skin explant tissue and cryopreserved aGvHD-affected skin biopsy specimens. After acetone fixation, the sections were stained at 4°C with PE-conjugated HLA-A2/HY dextramers or control PE-conjugated HLA-A2/Flu dextramers, followed by appropriately diluted rabbit anti-PE antibody (Biogenesis, Poole, United Kingdom) and cyanin 3-labeled goat anti-rabbitF(ab)2 antibody (JacksonImmunoResearch Laboratories,West Grove, PA). All cryosections analysed

in this study (from volunteer donors and from SCT patients) were simultaneously stained with FITC-conjugated CD8 antibody (BeckmanCoulter, Woerden, the Netherlands)[11]. Combined immunofluorescent staining using antibodies specific for CD3 (Dako, Glostrup, Denmark), CD4 (Neomarkers via Immunologic, Duiven, the Netherlands), CD8 (Novocastra via Leica Microsystems B.V., Rijswijk, the Netherlands) or CD8 (Beckman Coulter)/CD45RO (Dako)/granzyme B (Sanquin, Amsterdam, the Netherlands) was performed as described previously[11,13]. All sections were mounted with vectashield (Vector Laboratories, via Reactolab SA, Servion, Switserland) and analysed on a Leica TCS SP confocal laser scanning system (Leica Microsystems B.V).
Images were collected sequentially using a 40x numerical aperture 1.4 objective. Color photographs were generated as electronic overlays. For each patient except UPN 520, between four and 18 consecutive cryosections were scored for the presence of $CD8^+$ HLA-A2/HY dextramer-negative and $CD8^+$ HLA-A2/HY dextramer-positive T cells. A single section prepared from a biopsy specimen from UPN 520 was scored in a similar way. Each section consisted of five to seven confocal images of 512 mm x 512 mm x 6 mm (W x L x H) each, covering the complete section. All positively staining cells were individually analysed while scanning in the z-axis direction to obtain a threedimensional confocal image window, to exclude inclusion of noncellular structures in the total number of positive cells counted in nearly the complete biopsy.

Statistical analysis

In each patient, the mean number of HLA-A2/HY dextramer-positive $CD8^+$ T cells per cryosection was calculated. Differences between the mean values observed in the biopsy specimens of sex-matched and sex-mismatched recipients were analysed by a two-tailed unpaired t-test using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA).

Results

Onset of aGvHD coincides with an increase in peripheral blood lymphocytes

All patients exhibited the first clinical symptoms of aGvHD in the skin between 12 and 35 days after SCT (Table 1). Results on post-SCT peripheral blood lymphocyte recovery were available in six of the seven patients (Supplementary Figure S2). The missing data for UPN 577 were due to early death, at day +15 after SCT. Although total peripheral blood lymphocyte counts were not in the range of those obtained before SCT (2745 ± 809/mL), all six patients displayed an increase in absolute numbers of lymphocytes at or shortly after the day of skin biopsy collection (range, 80-1073/mL). $CD4^+$ and $CD8^+$ T cell subset analysis was performed on blood samples collected shortly before and after onset of aGvHD from UPN 567, UPN 574, UPN 501, and UPN 520. In line with data on total lymphocyte counts measured in parallel, both $CD4^+$ and $CD8^+$ T cell counts were increased after the onset of aGvHD.

Patient ID	Age at SCT, years	Diagnosis	Conditioning Regimen	Donor Sex	Transplantation Type[a]	Donor Chimerism, %[b]	Clinical Onset of Acute Skin GvHD[c]	Time of Skin Biopsy (Grade)[c]	Minor H Antigen Mismatch in Graft-versus-Host Direction
UPN 567	4	CML	TBI, Cy, ATG	F	PBSC	ND	12	16 (I)	A2/HY, B60/HY, HA-1, HA-8, ACG-2
UPN 473	7	SAA	TAI, Cy, ATG	F	BM	100%	22	24 (I-II)	A2/HY
UPN 463	6	X-ALD	Bu, Cy, ATG	F	BM	ND	13	14 (I-II)	A2/HY, B8/HY
UPN 574	4	SAA	Cy, Treo, ATG	M	BM	100%	14	16 (II)	-
UPN 577	7	MDS RAEBII	Bu, Cy, Mel, Cam1H	M	PBSC	ND	12	14 (II)	HLA-2/LB-ADIR-1
UPN 501	7	X-LPD	Bu, Cy, ATG	M	BM	100%	14	18 (I)	HLA-2/LB-ADIR-1
UPN 520	15	ALL	TBI, Cy, VP16, ATG	M	BM	100%	35	39 (II)	HLA-A2/HA-8

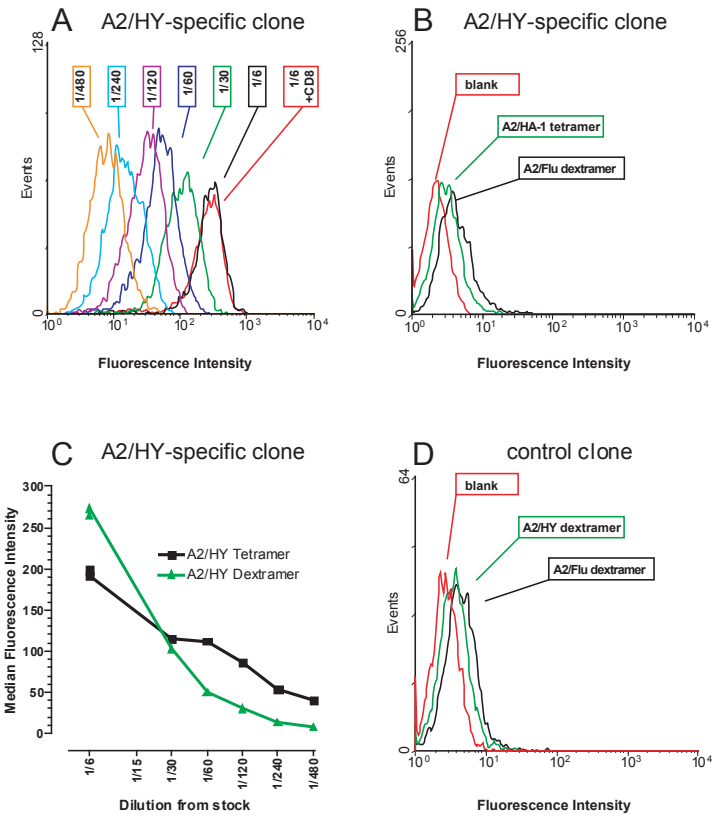
Table 1. Patient Characteristics.

CML indicates chronic myelogenous leukemia; SAA, severe aplastic anemia; X-ALD, X-linked adrenoleukodystrophy; MDS RAEBII, myelodysplastic syndrome (refractory anemia with excess number of blasts); X-LPD, X-linked lymphoproliferative disease; ALL, acute lymphoblastic leukemia; TBI, total body irradiation; TAI, thoracoabdominal irradiation; Cy, cyclophosphamide; ATG, anti thymocyte globulin; Bu, busulfex; Treo, treosulphan; Mel, melphalan; Cam1H, Campath (alemtuzumab); VP16, etoposide; PBSC, G-CSF mobilized peripheral blood stem cells; BM, bone marrow; ND, not determined.

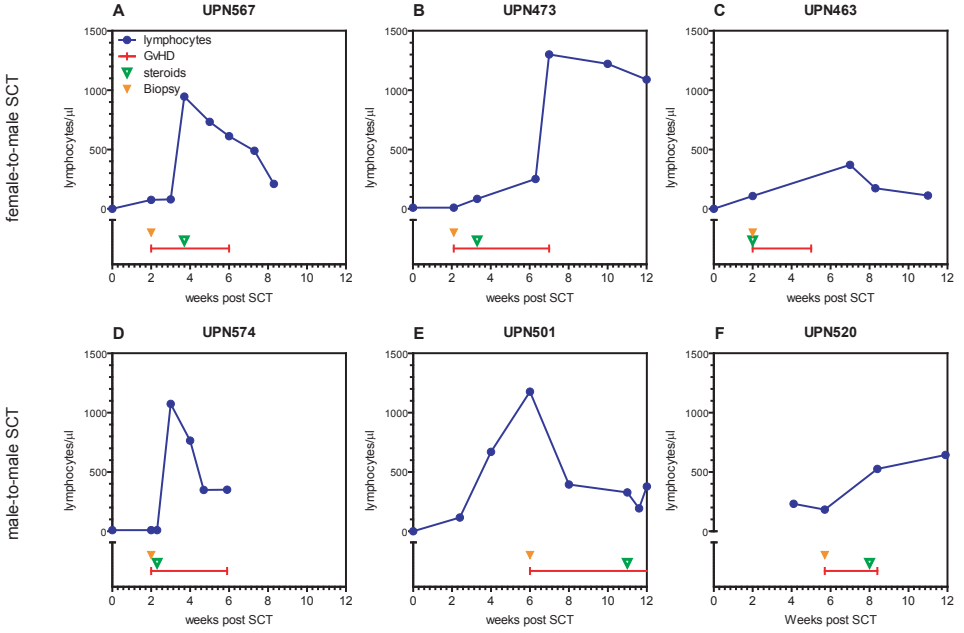
[a] T cell depletion of the graft through antibody-induced T cell rosetting (UPN 463), purified CD34 with T cell add-back (UPN 567), or Campath "in the bag" (UPN 577).

[b] Percentage donor chimerism was determined by VNTR analysis (PowerPlex 16) on PBMCs collected between 21 and 28 days post-SCT.

[c] Days after hematopoietic stem cell graft infusion, followed by the Lerner GvHD grading[11] in parentheses.

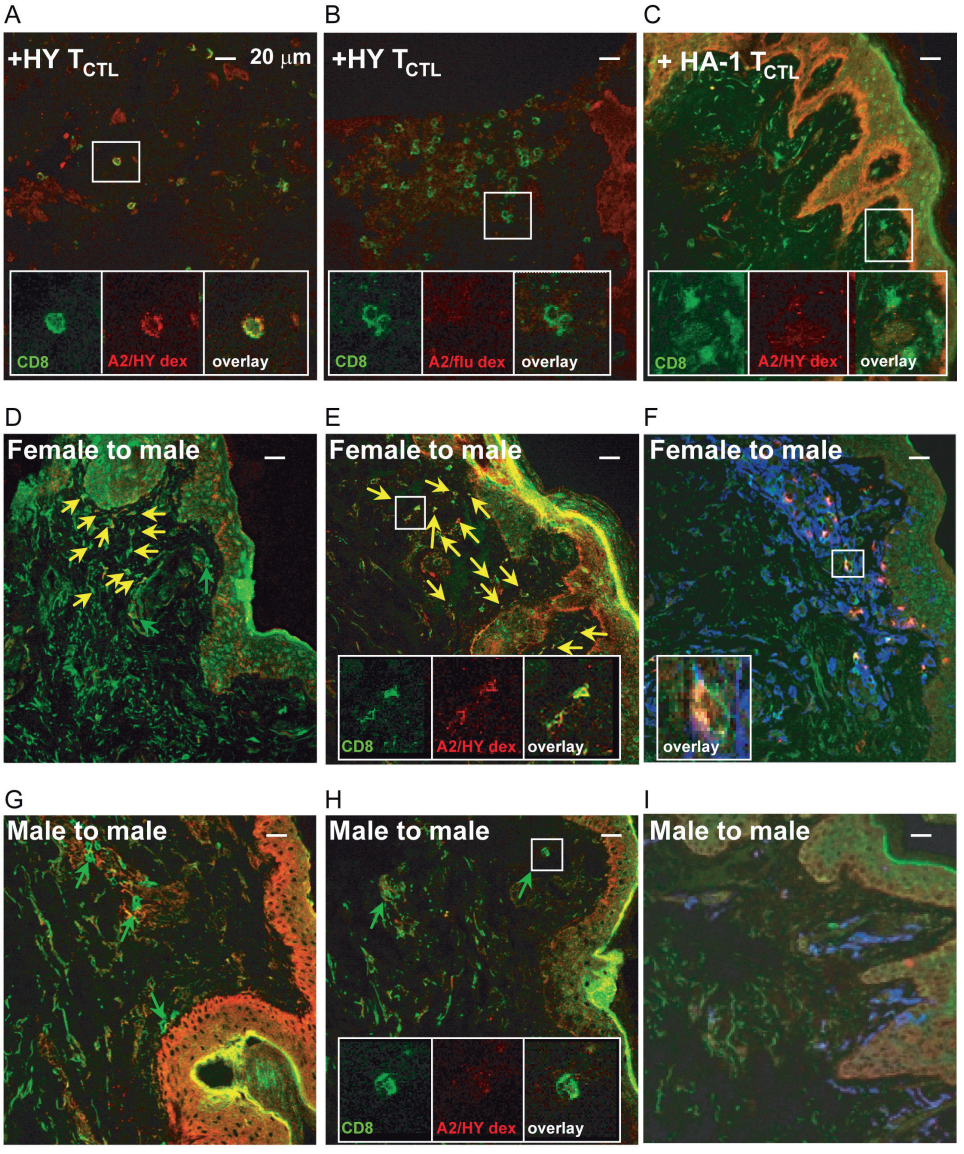


Supplementary figure S1. Validation of the tetrameric and dextrameric HLA/peptide complexes. Specificity and sensitivity of the HLA-A2/HY dextramers was evaluated by fluorescent activation cell-sorting (FACS) analysis. (A) A2/HY-specific T-cell clone 21-17 was incubated with A2/HY dextramer or tetramers in serial dilutions (ranging from 1/6 to 1/480) of the dextrameric and tetrameric stocks in combination with CD8 antibodies. Additionally, the 1/6 dilution (red) was used to stain in the absence of CD8 antibodies, in order to investigate the potential influence of CD8 staining on the dextramer avidity. (B) To address the specificity of the staining, control stainings were performed using the A2/HA-1 tetramer (green) and the A2/FLU dextramer (black). (C) To compare the results from the serial tetramer (black) and dextramer (green) dilutions, MFI were plotted per dilution. (D) Allo-specific HLA-A2-restricted T_{CTL} A24 was stained with A2/HY dextramer (green) and with A2/Flu dextramer (red), both in a 1/6 dilution. In all analyses, median fluorescence intensities were recorded.



Supplementary figure S2. The onset of aGVHD in the skin coincides with an increase in the number of circulating lymphocytes.
The absolute number per microliter (ml) blood of total lymphocytes, CD3 co-expressing $CD4^+$ T cells and $CD8^+$ T cells was evaluated in peripheral blood samples collected up to 6 weeks after gender-mismatched (UPN 567, left plot) or gender-matched (UPN 574, right plot) allogeneic SCT.

Figure 1. *In situ* staining of skin-infiltrating $CD8^+$ T cells reveals the presence of both granzyme B^+ and HLA-A2/HY dextramer$^+$ cells in aGvHD-affected skin tissue obtained after female-to-male SCT (next page).
Skin explant tissue derived from healthy male HLA-$A2^+$ volunteer donors was used to validate the sensitivity and specificity of HLA-A2/HY dextramers (red) for combined *in situ* labeling of skin-infiltrating $CD8^+$ T cells (green). These skin segments were incubated with either HY-specific T_{CTL} (A and B) or control HA-1-specific T_{CTL} (C) before cryopreservation and immunofluorescent labeling. Double-labeled cells, which are HY-specific $CD8^+$ T cells, become yellow in the overlay illustrations, as depicted by the representative cell in the lower right overlay insert of (A). Single-labeled cells (ie, $CD8^+$ cells) remain green in the overlay pictures, as depicted by the representative cells in the lower right insert of (B) and (C). (D and E) Combined immunofluorescent staining of cryosections prepared from aGvHD-affected skin biopsy specimens obtained from two male recipients of a female graft (D, UPN 473; E, UPN 567). The cryosections were incubated with HLA-A2/HY dextramers (red) and CD8 antibodies (green). Yellow arrows indicate co-localisation of HLA-A2/HY dextramers and CD8 antibodies on the same cell. (F) Combined CD8 (green), CD45RO (dark blue), and granzyme B (red) staining in a cryosection prepared from the same individual as shown in (E). (G and H) The identical staining procedure as described in (D) and (E) but applied to cryosections obtained from two male recipients of a male graft (G, UPN 501; H, UPN 574). Green arrows indicate $CD8^+$ cells not expressing the T cell receptor that specifically binds HLA-A2/HY dextramers. The higher magnification of a representative single-labeled cell in (E) depicts a $CD8^+$ T_{CTL} that is not HY-specific. Note that keratinocytes located at the dermal–epidermal junction may display some nonspecific red staining when incubated with dextramer preparations. (I) Combined CD8 (green), CD45RO (dark blue), and granzyme B (red) staining in a cryosection prepared from the same individual shown in (E).


A
+HY T_{CTL}
20 μm
CD8
A2/HY dex
overlay
B
+HY T_{CTL}
CD8
A2/flu dex
overlay
C
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CD8
A2/HY dex
overlay
D
Female to male
E
Female to male
CD8
A2/HY dex
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F
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Sufficient numbers of PBMCs from UPN 567 and UPN 473 (both male recipients of a female stem cell graft) were available for additional HLA-A2/HY tetramer analysis performed at 2 and 4 weeks (UPN 567) or 4 and 5 weeks (UPN 473) after SCT. The numbers of HY-specific T cells in $CD8^+$ cell fractions were below the detection limit in all four PBMC samples analysed (data not shown). Chimerism analyses performed on PBMC samples collected 21 to 28 days after SCT revealed complete donor origin in four (UPN 473, UPN 574, UPN 501, and UPN 520) of six patients (Table 1).

Validation of HLA-A2/HY Dextramers for in Situ Detection of HY-Specific T Cells in Cryopreserved Skin Explant Tissue

Failure to detect circulating HY-specific $CD8^+$ T cells after the onset of aGvHD in lymphopenic sex-mismatched recipients does not exclude their possible presence in aGvHD-affected tissues. Thus, we analysed archived frozen skin biopsy specimens from our patient cohort. Because the conventional HY tetramers do not stain frozen biopsy specimens[11], we first validated HLA-A2/HY dextramers on frozen sections prepared from *ex vivo in situ* skin explant tissue for their selective staining capacities[7,11]. To this end, HLA-A2/HY–specific T_{CTL} or control HLA-A2/HA-1-specific T_{CTL} were added exogenously to fresh skin tissue derived from HLA-$A2^+$ healthy male volunteer donors. Similar to conventional HLA-A2/HY tetramers[11], HLA-A2/HY dextramers stained skin-infiltrating HY-specific T_{CTL} when applied to viable skin explant tissue that was snap frozen after the addition of either staining reagent (data not shown). However, in the clinical setting of paediatric SCT, obtaining fresh tissue biopsy specimens for such experiments is difficult. Therefore, we analysed the capacity of HLA-A2/HY dextramers to stain HY-specific T_{CTL} when applied to already cryopreserved tissue. HLA-A2/HY dextramers, in contrast to HLA-A2/HY tetramers, were able to stain HY-specific T_{CTL} when applied to cryosections prepared from snap-frozen skin explant tissue (Figure 1A). Control HLA-A2/Flu dextramers did not label skin-infiltrating HY-specific T_{CTL} (Figure 1B). Furthermore, HLA-A2/HY dextramers did not stain cryosections preincubated with HA-1–specific T_{CTL} (Figure 1C). Collectively, these results show the specificity and applicability of HLA- A2/HY dextramers to visualise HY-specific $CD8^+$ T cells in cryopreserved skin tissue collected for clinical evaluation.

Presence of HY-specific $CD8^+$ T cells in aGvHD-affected skin collected from Male Recipients of Female Stem Cell Grafts

Historically collected cryopreserved skin biopsy specimens from seven paediatric patients with confirmed aGvHD of the skin were available for this analysis. The validated HLA-A2/HY dextramers were used to analyse these cryosections. The presence of HY-specific T_{CTL}, visualised by HLA-A2/HY dextramer and CD8 costaining cells, was found in two of the three male recipients of a female stem cell graft (Figures 1D and E). Higher-magnification images of two double-positive cells are shown in the insert at the bottom of Figure 1E. Of note, skin-infiltrating HY-specific T cells were observed in biopsy specimens collected as early as 12 to 13 days after sex-mismatched SCT (UPN 463 and UPN 567). These T cells accumulated at the

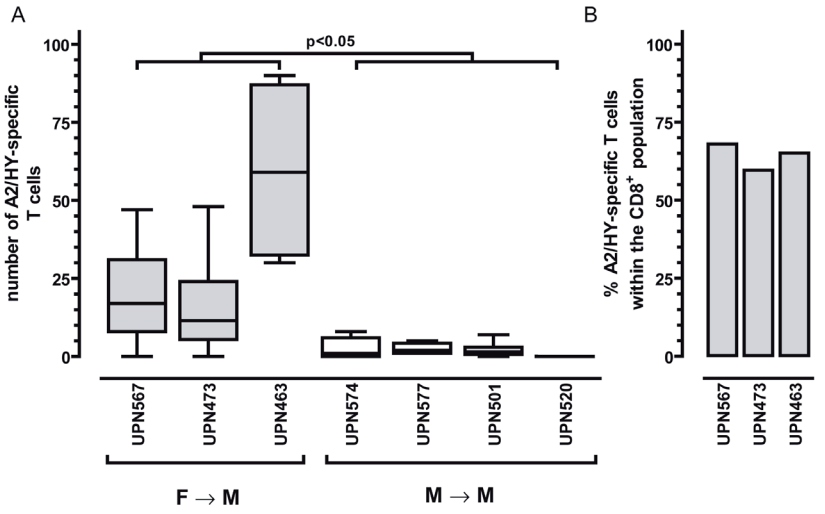

Figure 2. Acute GvHD after female-to-male SCT is associated with significantly higher numbers of skin-infiltrating CD8⁺ T cells containing a high percentage of cells specific for HY.
(A) Total numbers of CD8⁺ cells (left) and HLA-A2/HY dextramer⁺ CD8⁺ cells (right) were counted in 1-18 serial cryosections prepared from the same cryopreserved skin biopsy specimen collected shortly after clinical onset of aGvHD and before the start of first-line treatment. All positively staining cells were individually analysed while scanning in the Z-axis direction to obtain a 3-dimensional confocal image window, to avoid inclusion of noncellular structures in the total number of positive cells counted in nearly the complete biopsy specimen. For each individual patient, the box-and-whisker plot shows the mean number ± SD of cells counted per section as indicated on the Y-axis. The upper and lower ends of the boxes represent the upper and lower quartiles, respectively. Whiskers represent the lowest and highest observations. The solid line represents the median; *p<0.05. the y-axis. The solid line represents the median. (B) Percentage of HLA- A2/HY-specific T cells within the CD8⁺ population in the three female-to-male SCT recipients.

same location as HY-specific CTL clones applied in the skin explant assay[7], that is in the dermis as well as just below the dermal-epidermal junction (Figures 1D and E). At the same location, granzyme B-coexpressing CD8⁺ cells were visualised (Figure 1F), illustrating that aGvHD skin-infiltrating CD8⁺ T cells are "licensed to kill" and are not innocent bystanders. Unfortunately, the only available HLA-A2/HY dextramers and the granzyme B-specific antibody were both conjugated with PE, hindering the analysis of coexpression of these two markers by the same CD8⁺ T cell.
CD8⁺ HLA-A2/HY dextramer-positive T cells were not detected in any of the biopsy specimens obtained from male recipients of a male stem cell graft (Figures 1G and H). These cryosections contained only a few CD8⁺ T cells, as exemplified by the enlarged single positive green cell shown at the bottom of Figure 1H. But these CD8⁺ cells were not HY-specific and did not express granzyme B, in sharp contrast to the skin-infiltrating CD8⁺ cells shown in Figure 1F. Control staining of serial sections with HLA-A2/Flu dextramers revealed no positive signal in any of the biopsy tissues tested (data not shown).
Quantification of the CD8⁺ T cell infiltrates was performed on skin biopsy specimens from all seven patients (Figure 2). Significantly higher numbers of CD8⁺ HY-specific

T cells infiltrated the skin after sex-mismatched SCT than after sex-matched SCT (P<.05) (Figure 2A). In contrast to the biopsy specimens obtained after sex-matched SCT, high proportions (68%-78%) of aGvHD skin-infiltrating $CD8^+$ cells in the male recipients of a female graft were HY-specific (Figure 2B).

Discussion

HY-specific T cells may contribute to the development of GvHD in male recipients of female haematopoietic stem cell grafts[14]. Although numerous clinical studies have shown the influence of sex mismatching on SCT outcome, the *in situ* presence of HY-specific T cells in GvHD-affected tissues has not been shown until now. The available tetrameric HLA-HY peptide complexes do not stain infiltrated T cells in cryopreserved tissues.

In this study, we first validated the use of dextrameric HLA-A2/HY peptide complexes using an *ex vivo* skin explant model[7] with cryopreserved skin tissues of healthy male individuals. The newly developed dextrameric HLA-A2/HY peptide complexes allowed staining and analysis of infiltrating T cells in cryopreserved tissues. These results thus allowed us to perform a retrospective analysis of earlier stored clinical GvHD samples.

For the first time, using dextrameric HLA-A2/HY peptide complexes, we visualised HY-specific $CD8^+$ T cells in aGvHD-affected tissue obtained from male recipients of female stem cell grafts. Application of the PE-labeled dextrameric HLA-A2/HY complexes combined with CD8-specific antibodies showed skin-infiltrating HY-specific T cells in cryopreserved skin biopsy specimens obtained shortly after clinical manifestation of aGvHD in three male paediatric recipients of a female stem cell graft. Despite the first signs of lymphocyte recovery in the circulation, HY-specific T cells were not detected in peripheral blood samples collected from two of these three SCT recipients (UPN 567 and UPN 473) at 2 to 5 weeks after SCT. In previous work, we reported the presence of HY-specific T cells in the peripheral blood of adult male SCT recipients who developed aGvHD after receiving a non-T cell-depleted bone marrow graft from a female donor[6]. These seemingly contradictory results might be related to the significant differences in applied SCT protocols between paediatric and adult patients, such as composition of the applied stem cell grafts and the pre- and post-SCT applied immune suppression regimens. Of note, severe lymphopenia in the first few weeks after SCT, in combination with the limited volume of peripheral blood routinely collected from paediatric SCT patients, has hampered reliable HLA-A2/HY tetramer analyses in PBMCs. Thus, our analysis was limited, as two of the three paediatric male recipients of female grafts could be analysed. Note, however, that we found a relatively high number of HY-specific T cells migrating to the skin during the early stage of aGvHD. Whether or not this clarifies the absence of minor HY-specific T cells in the peripheral blood is a subject for extensive future analysis.

Given that TCR stimulation-induced cell cycle progression is required for the induction of granzyme B-containing cytolytic granules in both naïve and antigen-experienced T_{CTL}[15], the presence of highly granzyme $B^+/CD8^+$ in aGvHD-affected

skin suggests that these presumably cytolytic T cells are activated. As shown in Figure 1F, activated T_{CTL} ultimately release their cytolytic granules in the dermis or at the dermal–epidermal junction. At the same location, GvHD-like tissue destruction occurs *in vitro* when HY-specific T_{CTL} clones are applied in the skin explant assay[7]. We previously demonstrated that HLA-A2/HY-specific T_{CTL} clones lyse IFN-γ-activated HLA-$A2^+$ epidermal keratinocytes *in vitro*[16]. *In vivo*, IFN-γ may be derived from the activated T_{CTL} themselves and/or from activated, that is, CD45RO expressing $CD4^+$ T cells present in the same dermal infiltrates (Figure 1F).

The early appearance of HY-specific $CD8^+$ Tcells in aGvHD-affected skin raises the question whether these T cells arise from naïve or memory T cells, both of which are likely cotransferred along with the female haematopoietic stem cell graft into the male patient. Although we were not able to analyse pre-SCT collected female donor PBMCs in the present study, it should be noted that pregnancy can induce the generation and long-term persistence of minor histocompatibility antigen-specific T_{CTL}, including T cells specific for HY[17-19]. It is possible that such memory-type minor histocompatibility antigen-specific T cells are also present in haematopoietic stem cell products prepared from parous female donors. On re-encountering the relevant minor histocompatibility antigen in the stem cell recipient, these T cells may contribute to the development of aGvHD. Prospective analyses of the presence of cytolytic type T cells specific for ubiquitously expressed minor histocompatibility antigens in female stem cell products may identify SCT patient-donor pairs at risk for aGvHD.

In conclusion, the present study provides the first indication that HY-specific T cells are actively involved in the development of aGvHD after sex-mismatched SCT. The size of our study population was small and the patient population relatively heterogeneous, therefore, further studies on larger and more homogeneously treated patients are needed to evaluate the clinical significance of our observations.

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Authorship Statement:

Eric Spierings, Florry A. Vyth-Dreese, Maarten J. D. van Tol, and Els Goulmy designed the research; Jørgen Schøller, Stan Pavel, and R. Maarten Egeler provided crucial reagents and tissue specimens; Yeung-Hyen Kim, Claudia M. J. M. Faaij, Ellen Schrama, Trees A. M. Dellemijn, and Astrid G. S. van Halteren performed experiments and collected data; Yeung-Hyen Kim, Eric Spierings, Claudia M. J. M. Faaij, Florry A. Vyth-Dreese, and Astrid G. S. van Halteren analysed results and prepared figures; and Astrid G. S. van Halteren, Eric Spierings, Maarten J. D. van Tol, and Els Goulmy drafted the manuscript.

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Chapter

6

Fasciitis in chronic GvHD; Cross talk between host dermal macrophages and donor T cells

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In preparation

Abstract

The clinical and pathological features of chronic and acute Graft-versus-Host Disease (GvHD) differ considerably. Fasciitis is a classical and often severe morbidity-causing feature of cGvHD. In this study, we set out to determine which type of immune cells had infiltrated the inflamed fasciae of 3 paediatric patients who developed fasciitis after HLA identical hematopoietic stem cell transplantation (HSCT). Tissue sections prepared from Skin-Muscle-Fascia biopsies of these patients showed infiltrates of activated, mainly CD8 co expressing, $CD3^+$ T cells, which were found in close contact with $CD14^+CD163^+$ dermal macrophages. The infiltrates did not contain other additional professional antigen-presenting cell types. Chimerism analysis performed on blood samples collected shortly before the onset of fasciitis showed 100% donor chimerism in distinct myeloid cell types. In contrast, significant numbers of host cells could be detected *in situ* in areas enriched for $CD163^+$ macrophages. Although this study population is too small to draw any conclusion, these preliminary observations suggest that residual host-derived dermal macrophages and infiltrating donor-derived $CD8^+$ T cells are involved in the pathogenesis of cGvHD-associated fasciitis.

Introduction

Chronic Graft-versus-Host Disease (cGvHD) is a long-term complication of allogeneic haematopoietic stem cell transplantation (HSCT) and a common cause of late post-transplant morbidity and mortality. It occurs in approximately 25% of paediatric patients undergoing HSCT[1] and mainly involves the skin, mouth, liver and/or eyes. Traditionally, the distinction between acute (aGvHD) and chronic GvHD (cGvHD) used to be based on the observation whether tissue inflammation occurred before or after the first 100 days post-transplant. Since 2004, consensus has been reached about a more explicit clinical definition of cGvHD; these criteria include the following clinical features: sclerosis, lichen-planus-like lesions, poikiloderma, oesophageal webs, bronchiolitis obliterans and fasciitis[2]. The symmetrical inflammatory swelling typically associated with fasciitis, may cause severe functional impairment of the extremities[3]. Histopathological analysis of fasciitis-affected biopsies typically displays a diffuse $CD8^+$ dominated lymphocytic infiltration of the oedematous fascia, often extending to the muscle interstitium, and an increased deposit of collagen fibres[4;5]. This inflammatory reaction seems to be directed against local micro lesions in the fascia.

The pathogenesis of cGvHD in general, and fasciitis in particular, is still poorly understood. In contrast to aGvHD, in which apoptosis and necrosis of affected tissues is commonly seen, in cGvHD the affected tissues typically display inflammatory and fibrotic processes. It is thought that loss of peripheral tolerance to self-antigens form the basis of the auto-immune-like clinical symptoms of cGvHD[6]. Several cell types have been suggested to be involved in cGvHD. Donor $CD8^+$ cytotoxic T cells, the supposed main effector cells, are likely activated locally by donor-derived helper T cells. This immune response may occur in situations where regulatory T cell numbers

are low, i.e. after myeloabblative HSCT[7], although cGvHD also occurs in patients that received reduced intensity conditioning. A high variety of (auto) antibodies detectable in serum samples collected from cGvHD patients also suggest a role for donor B cells in the induction or perpetuation of chronic inflammation[8-13].
In order to exert their local damaging effect, activated donor type $CD8^+$ T cells have to migrate from the circulation into GvHD-affected sites; here they must reencounter their specific alloantigen in order to become 'licenced to kill'[14]. Chemokines and their receptors generally play an important role in lymphocyte trafficking to inflamed tissues. In contrast to data on aGVHD[15-17], information on the role of chemokines and their receptors in cGvHD is non-existing. In a first attempt to address the various types of immune cells putatively involved in the pathogenesis of fasciitis, we have studied tissue biopsies derived from three cGvHD patients in whom fasciitis manifested as the main clinical feature of late HSCT-related complications. We set out to determine which type of immune cells, besides $CD8^+$ T cells, had infiltrated the fascia and which chemokine/receptor combination(s) might be involved.

Patients & Methods

Patients

Three paediatric patients, all diagnosed with acute leukaemia (n=2 AML, n=1 ALL, Table 1, median age 15 years) received a bone marrow (BM) graft from an HLA-identical sibling donor. Ciclosporin A (CsA) was tapered 30 days after HSCT. One of the patients (UPN 581) suffered from aGvHD of the skin (grade 2/3) for which he was successfully treated with Prednisone (1 mg/kg, intermittently for 7 months). Starting at 22-39 weeks post HSCT, all three patients presented with fasciitis as the sole manifestation of cGvHD; their clinical symptoms were characterised by swollen limbs, muscle ache and severe moving impairment. A skin-muscle-fascia biopsy confirmed the presence of active chronic fasciitis, where after first line systemic treatment with steroids was initiated (except for UPN 581, who already started steroid treatment 4 weeks earlier). Two of the 3 patients are still in remission and off immunosuppressive therapy. UPN 612 was only treated with steroids; UPN 619 was treated with prednisone and CsA, which did not lead to sufficient improvement. CsA was tapered and sirolimus was started, resulting in a progressive cytopenia. Sirolimus treatment was stopped and Rituximab (RTX) was given. Fasciitis symptoms decreased and prednisone could be tapered. The third patient (UPN 581) received prednisone. Later Cellcept was added. This patient eventually died from steroid refractory cGvHD 2.4 years post HSCT. Control patients (n=13) were selected based on comparability of underlying disease (acute leukaemia), HSCT and donor type (HLA-identical sibling donor), and absence of acute (> grade 1) and/or chronic GvHD (n=10). The final 3 patients in the control group did have cGvHD but without fasciitis. For ethical reasons, it was not possible to obtain skin-muscle-fascia biopsies from any of these control patients. All patients are described in Table 1 and all but UPN 716 received TBI (2 x 6 Gy at days -1 and 0) as part of their conditioning regimen. All patients received CsA (2 mg/kg from day -1) and methotrexate (MTX, 10 mg/m2

UPN	Diagnosis	Age (years)	Conditioning	aGvHD grade	cGvHD grade	Onset cGvHD	Medication	Duration	Onset and Clear-ance Fasciitis	Skin Muscle Fascia biopsy	Timepoints		
											1	2	3
Patients													
581	AML rel	15	Cy	3	2	39	Prednison Cellcept	42-125 46-125	39/no	46	39	43	-
612	ALL	14	VP16	-	2	23	Prednison	17-52	23/37	26	10	16	28
919	AML rel	17	Cy	-	2	22	Prednison Ciclosporin Sirolimus Rituximab	39-73 39-58 58-62 61-64	22/73	39	29	47	52
Controls without cGvHD													
589	AML	11	Cy	-	-	-	-	-	NA	NA	23	57	-
592	ALL rel	11	Cy, VP16	-	-	-	-	-	NA	NA	23	-	-
595	ALL	7	Cy, VP16	-	-	-	-	-	NA	NA	24	39	51
630	ALL	16	VP16	1	-	-	-	-	NA	NA	24	41	49
632	ALL	10	VP16	-	-	-	-	-	NA	NA	27	38	50
645	ALL	6	VP16	-	-	-	-	-	NA	NA	24	38	58
649	ALL	9	VP16	-	-	-	-	-	NA	NA	26	36	51
677	ALL	15	VP16	-	-	-	-	-	NA	NA	23	38	51
711	ALL	4	VP16	-	-	-	-	-	NA	NA	41	61	68
713	ALL	17	VP16	-	-	-	-	-	NA	NA	22	36	47
Controls with cGvHD													
708	ALL	17	Cy, VP16	3	2	27			NA	NA	24	36	52
716	AML	16	Bu, Flu	3	2	14			NA	NA	26	39	-
722	ALL	17	VP16	1	1	14			NA	NA	27	39	54

Table 1. Characteristics of fasciitis patients and HSCT controls

AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; rel, relapse; Cy, cyclophosphamide; VP16, etoposide; Bu, Busulfex; Flu, Fludarabine. Dosing of conditioning regimen: Cyclophosphamide: 60 mg/kg, 2 days, 120 mg/kg in total; Etoposide (VP16) alone: 60 mg/kg, 2 days, 120 mg/kg in total; in combination with Cy: 350 mg/m2 , 2 days, 700 mg/m2 in total; Busulfex: 120 mg/m2, 4 days, 480 mg/m2 in total; Fludarabine: 40 mg/m2, 4 days, 160 mg/m2 in total. NA: not applicable; All time points are in weeks post HSCT unless mentioned otherwise.

Antibody	Clone	Manufacturer	Isotype	Technique
CCR1	53504	R&D Systems	mIgG2b	FACS
CCR2	48.607.121	R&D Systems	mIgG2b	FACS
CCR3	61828	R&D Systems	rat IgG2a	FACS/IHC
CCR4	1G1	BD Parmingen	mIgG1	FACS
CCR5	2D7	BD Parmingen	mIgG2a	FACS/IHC
CCR5	-	Abcam	polyclonal goat IgG	FACS
CCR6	53103	R&D Systems	mIgG2b	FACS
CCR7	150503	R&D Systems	mIgG2a	FACS
CCR8	-	Kordia/Alexis	polyclonal goat IgG	FACS
CCR9	112509	R&D Systems	mIgG2a	FACS
CCR10	-	Capralogics	polyclonal goat IgG	FACS
CD1a	O10	Neomarkers	mIgG1	IHC
CD3	-	Dako	polyclonal rabbit IgG	IHC
CD3	SK7	Becton Dickinson	mIgG1	FACS
CD4	1F6	NovoCastra	mIgG1	IHC
CD4	SK3	Becton Dickinson	mIgG1	FACS
CD8	4b11	NovoCastra	mIgG2b	IHC
CD8	B9.11	Beckman Coulter	mIgG1	FACS
CD20	L26	Dako	mIgG2a	IHC
CD25	2A3	Becton Dickinson	mIgG1	FACS
CD27	L128	Becton Dickinson	mIgG1	FACS
CD28	L293	Becton Dickinson	mIgG2bK	FACS
CD45RA	4KB5	Dako	mIgG1	IHC
CD45RA	HI100	BD Parmingen	mIgG2bK	FACS
CD68	514H12	AbD Serotec	mIgG2a	IHC
CD127	R34.34	Beckman Coulter	mIgG1	FACS
CD134	L106	Becton Dickinson	mIgG1	FACS
CD137	4B4-1	BD Pharmingen	mIgG1	FACS
CD161	191B8	Miltenyi	mIgG2a	FACS
CD163	10D6	NovoCastra	mIgG1	IHC
CLA	HECA-452	BD Parmingen	rat IgM	FACS
CXCR1	42705	R&D Systems	mIgG2a	FACS
CXCR2	48311	R&D Systems	mIgG2a	FACS
CXCR3	1C6/CXCR3	BD Parmingen	mIgG1	FACS
CXCR4	44716	R&D Systems	mIgG2b	FACS
CXCR5	51505	R&D Systems	mIgG2b	FACS
CXCR6	56811	R&D Systems	mIgG2b	FACS
CXCR7	-	Proteintech Europe	polyclonal rabbit IgG	FACS
FoxP3	PHC101	e-Biosciences	rat IgG2a	FACS
HLA DRα	TAL.1B5	Dako	mIgG1	IHC
Ki67	-	SantaCruz	polyclonal rabbit IgG	IHC

Table 2. Antibodies used for flow cytometry and immunohistochemistry.
All isotypes are mouse (m) unless indicated otherwise.

at days +1, +3 and +6) as GvHD prophylaxis. Diagnosis of acute and chronic GvHD was assessed according to consensus criteria[18;19]. Peripheral blood mononuclear cells (PBMC) of healthy donors (n=12), both children >12 years of age and adults between 24 and 46 years old, were used to analyse base-line chemokine receptor expression patterns by lymphocytes with flow cytometry.

Flow cytometry

Table 2 lists the primary antibodies that were used for flow cytometric analysis of peripheral blood-derived T cell subsets, B cells and the expression of chemokine receptors. For visualisation of bound unlabelled primary antibodies, PBMC were stained with the relevant FITC-conjugated isotype-specific secondary antibody (Southern Biotechnology Associates Inc., Birmingham, AL, USA). PBMC solely stained with secondary antibodies served as negative controls. Cells were fixed directly after staining using 4% paraformaldehyde. The T cells were further characterised using the following marker combinations: naïve (CD4/CD8, $CCR7^{+}CD45RA^{+}$), central memory (CD4/CD8, $CCR7^{+}CD45RA^{-}$), effector memory (CD4/CD8, $CCR7^{-}CD45RA^{-}$), CD45RA expressing effector memory (T_{EMRA}: CD4/CD8, $CCR7^{-}CD45RA^{+}$)[20], T_{regs} ($CD4^{+}CD25^{hi}CD127^{-}FoxP3^{+}$) and $T_{H}17$ ($CD4^{+}CD161^{+}CCR4^{+}CCR6^{+}$)[21]. The intracellular FoxP3 staining was performed according to the supplier's manual (eBiosciences, San Diego, CA, USA). The percentage of positive cells was measured on a fluorescence-activated cell sorter (FACS) Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and data were analysed using Cellquest software.
Monocyte numbers were obtained via standard cell count and differentiation methods using a haematology automated analyser (Sysmex, Etten-Leur, the Netherlands).

Immunofluorescent and enzymatic staining of biopsied tissues

Paraffin-embedded skin-muscle-fascia biopsies were obtained at disease onset and before initiation of first line immunosuppressive treatment (except UPN 581, who already started steroid treatment 4 weeks earlier). The antibodies that were used for immunohistochemistry are also listed in Table 2. Four µm paraffin sections were deparaffinised and subjected to heat-mediated antigen retrieval in a microwave using citrate buffer (10 mmol, pH 6.0) or EDTA buffer (10 mmol, pH 9.0). Non-specific staining was blocked by incubating the slides with normal goat and/or mouse serum for 30 min followed by overnight incubation at room temperature with primary non-conjugated antibodies. For multicolour stainings, primary antibodies were detected by fluorescence using the relevant isotype-specific, Alexa Fluor 488, Alexa Fluor 594 or Alexa Fluor 647 labelled, secondary antibodies (Molecular Probes, Leiden, the Netherlands). Replacement of the primary antibodies by PBS/1% bovine serum albumin (BSA) 1% served as a negative control. Staining results were analysed by confocal laser scanning microscopy using a LSM 510 confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA).
In case of enzymatic immunohistochemical staining, bound primary antibodies were detected using Envision (Dako, Heverlee, Belgium) followed by 3,3'-Diamino-benzidine-tetrahydrochloride (DAB) as earlier described[22].

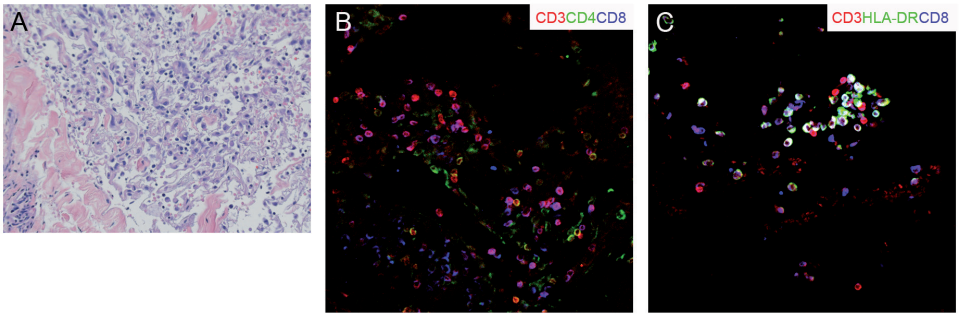

Figure 1. The fascia infiltrates in cGvHD patients are characterised by a gross amount of (activated) T cells
(A) Representative picture of H&E staining of a skin-muscle-fascia biopsy (UPN 619) showing a large infiltrate in the fascia around the muscle. (B) Combined immunofluorescent staining of CD3 (red), CD4 (green) and CD8 (blue) present in the same biopsy. Whereas rare CD4+ T cells are depicted in yellow, abundantly present CD8+ T cells are depicted in pink. (C) Combined immunofluorescent staining of the same biopsy of CD3 (red), HLA-DR (green) and CD8 (blue), indicating that the majority of fascia-infiltrating CD8+ T cells display an activated phenotype as depicted by white parts of the membrane.
Magnification: 250x

Detection of Y chromosome bearing cells by in situ hybridisation (ISH)

Y-chromosome ISH, performed as described previously[23], was used to investigate the number of residual male host cells in fasciitis-affected tissue obtained from the two gender mismatched HSCT pairs. In short, paraffin sections were deparaffinised and subjected to heat-mediated antigen retrieval using citrate buffer (10 mmol, pH 6.0). After digestion with 0.3% pepsin and dehydration, the sections were hybridised with digoxigenin (DIG) labelled Y-chromosome probe o/n at 37°C. In order to visualise bound probes, slides were subsequently incubated with mouse-α-DIG, rabbit-α-mouse and HRP-labelled swine-α-rabbit, respectively, followed by Novared.

Results & Discussion

In order to obtain more insight in the cellular events which take place around the onset of fasciitis as a classical symptom of cGvHD in allogeneic HSCT recipients, peripheral blood and diagnostic skin-muscle-fascia biopsies of 3 fasciitis patients were investigated in parallel. Enzymatic and fluorescent staining was performed to investigate the various cell types present in the fasciae of these patients. A representative picture of the localisation of an infiltrate is depicted in Figure 1A; the infiltrates were characterised by a large number of activated, i.e. HLA-DR$^+$, CD3 expressing T cells; the majority of these cells co-expressed CD8$^+$ (Figures 1C and 1B) which is in concordance with previous studies[4;5].
In order to investigate the distribution kinetics of immune cells in the peripheral blood, flow cytometric analysis was performed on PBMC of these patients collected before and during fasciitis. As shown in Figure 2, CD4$^+$ (A) or CD8$^+$ (B) T cell counts

in the 3 fasciitis patients were generally lower as compared to control HSCT patients without GvHD or to cGvHD patients who did not display fasciitis as their main clinical symptom. Further characterisation of these T cell subsets, as depicted in Figure 3, demonstrated low to normal percentages of $CD4^+$ naive and central memory (CM) type T cells (Figure 3A and 3B, respectively) in all cGvHD patients. $CD4^+$ effector memory (EM) T cell percentages were normal to high in all cGvHD patients (Figure 3C) and percentages of T_{EMRA} cells were increased in 2 out of 3 cGvHD patients without fasciitis (Figure 3D). For the $CD8^+$ naive T cell subset, normal to high percentages were seen in 2 out of 3 fasciitis patients, whereas decreased levels were seen in 3 cGvHD patients without fasciitis (Figure 3E). This observation held also true for the $CD8^+$ CM T cell subset (Figure 3F). Normal levels of $CD8^+$ EM T cells were seen in all cGvHD patients, except for the latest time points in 2 out of 3 fasciitis patients (Figure 3G). Finally, increased levels of $CD8^+$ T_{EMRA} cells were only seen in the 3 cGvHD patients without fasciitis. The increased percentages of naive $CD8^+$ T cells (Figure 3E) and decreased percentages of $CD8^+$ effector memory T cells (Figure 3G) in 2 of the fasciitis patients (UPN 581 and UPN 619) were likely the result of the extensive (steroid) treatment both patients received[24].

T_{regs} are thought to regulate the expansion of, amongst others, cytolytic type T cells and T_H17 cells[7]. Published data have reported a significant reduction of T_{regs} in the peripheral blood of cGvHD patients[7;25-28], resulting in an inverse correlation between Tregs and T_H17 numbers[7;29]. This is in contrast to our findings: compared to HSCT patients without cGvHD, HSCT patients with cGvHD (with and without fasciitis) showed similar percentages of T_{regs} (Figure 3I) and, irrespective of occurrence of fasciitis, the T_H17 percentages were increased in 3 out of 6 cGvHD patients (Figure 3J). Although the cGvHD patients in our study did not show decreased levels of Tregs, increasing this number could still have a beneficial effect on the clinical symptoms. Koreth *et al.* recently showed that daily subcutaneous administration of low-dose IL-2, which is critical for T_{reg} development, expansion, activity and survival, dramatically increased Treg numbers in steroid refractory cGvHD patients, resulting in reduced clinical symptoms[30].

Before any of the above described T cell subsets can exert their effect, they must first encounter professional antigen presenting cells (APC's). Several types of APC can reside in the skin, i.e., epidermal Langerhans cells (LC), $CD14^+CD1a^-CD163^+$ macrophages, $CD1a^+CD14^-$ and $CD1a^+CD14^+$ dermal dendritic cells (DC) in the deeper layers of the skin[31], but also $CD20^+$ B cells. Given that some studies suggest the involvement of B cells in cGvHD[8;10;11;32], their presence in the peripheral blood and fasciae was investigated. B cell numbers in the peripheral blood of all cGvHD patients (with and without fasciitis) appeared to be low to normal (Figure 2C). Despite their presence in peripheral blood, enzymatic staining of the biopsies showed complete absence of CD20 expressing B cells in inflamed fasciae (data not shown). Additional staining of the fasciitis-affected biopsies revealed that dermal macrophages were not only abundantly present in all biopsies, but also in close proximity of T cells (Figure 4A). Only a few single $CD14^+$ cells, either type 1 macrophages or infiltrating monocytes, were additionally observed. Monocyte numbers in the peripheral blood of cGvHD patients were normal to high (Figure 1D). Furthermore, $CD1a^+$ cells were clearly found in the unaffected epidermis and not in the cellular infiltrates

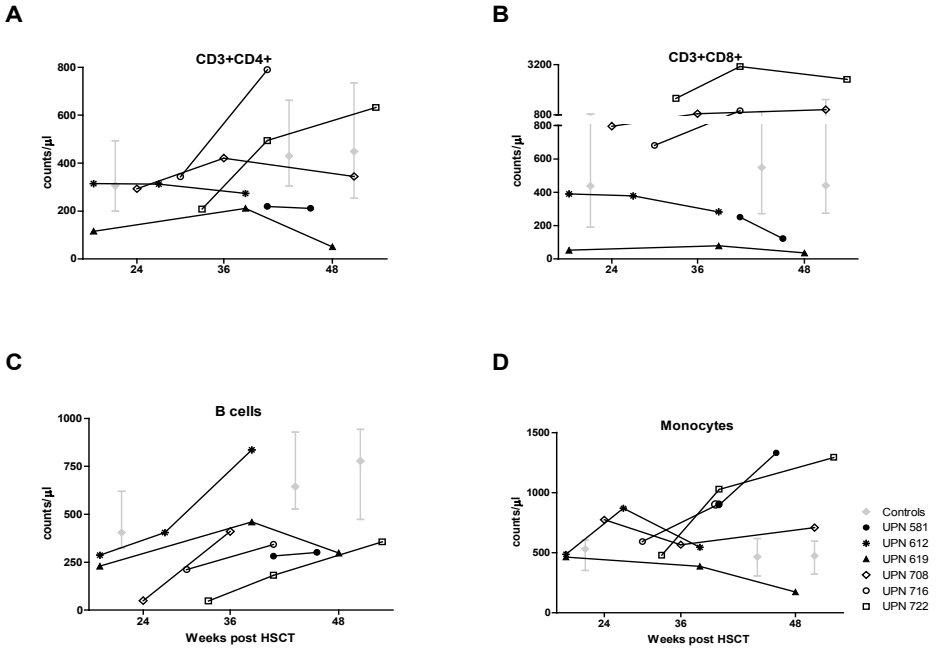

Figure 2. Post-HSCT reconstitution of immune cells in paediatric HSCT patients
Flow cytometric analysis was used to investigate the reconstitution of circulating immune cells within the peripheral blood mononuclear cells (PBMC). This analysis reveals decreased numbers of $CD4^+$ (A) and $CD8^+$ (B) T cells in the three fasciitis patients (closed black symbols) as compared to three cGvHD patients without fasciitis (open symbols) and ten HSCT patients without cGvHD (median: grey diamonds and 5-95% CI: bars), and low to normal levels of B cells (C) in cGvHD patients (with and without fasciitis) as compared to HSCT patients without cGvHD. (D) Monocyte numbers are comparable between the three groups with the exception of UPN 581, with intractable cGvHD and fasciitis, and UPN 722, with resolved cGvHD without fasciitis.

present in the deeper layers of the skin (data not shown). Additionally, when H&E staining (Figure 4B) was compared to single enzymatic CD163 staining the same macrophage-like cells could be visualised in the same area of the sections (Figure 4C). Haniffa *et al.* observed that residual $CD163^+$ dermal macrophages are able to promote donor-derived $CD8^+$ T cell proliferation and cytokine release when tested in an allogeneic set-up[31]. Additionally, evidence exists that tissue DC may reside from a distinct monocyte subset (expressing CD14, CD16 and CX3CR1)[33;34]. The single $CD14^+$ cells found in the fasciae of our patients could, therefore, be the precursors of donor-derived APC.
Two of the 3 fasciitis patients were males transplanted with a stem cell graft derived from a female sibling donor. Using in situ hybridisation (ISH) to specifically detect the presence of Y-chromosome bearing cells, male cells could be visualised in the fascia infiltrates of both patients (Figure 4F). While these infiltrates obviously comprised stromal cells of host origin (i.e. fibroblasts and endothelial cells), these cells may be

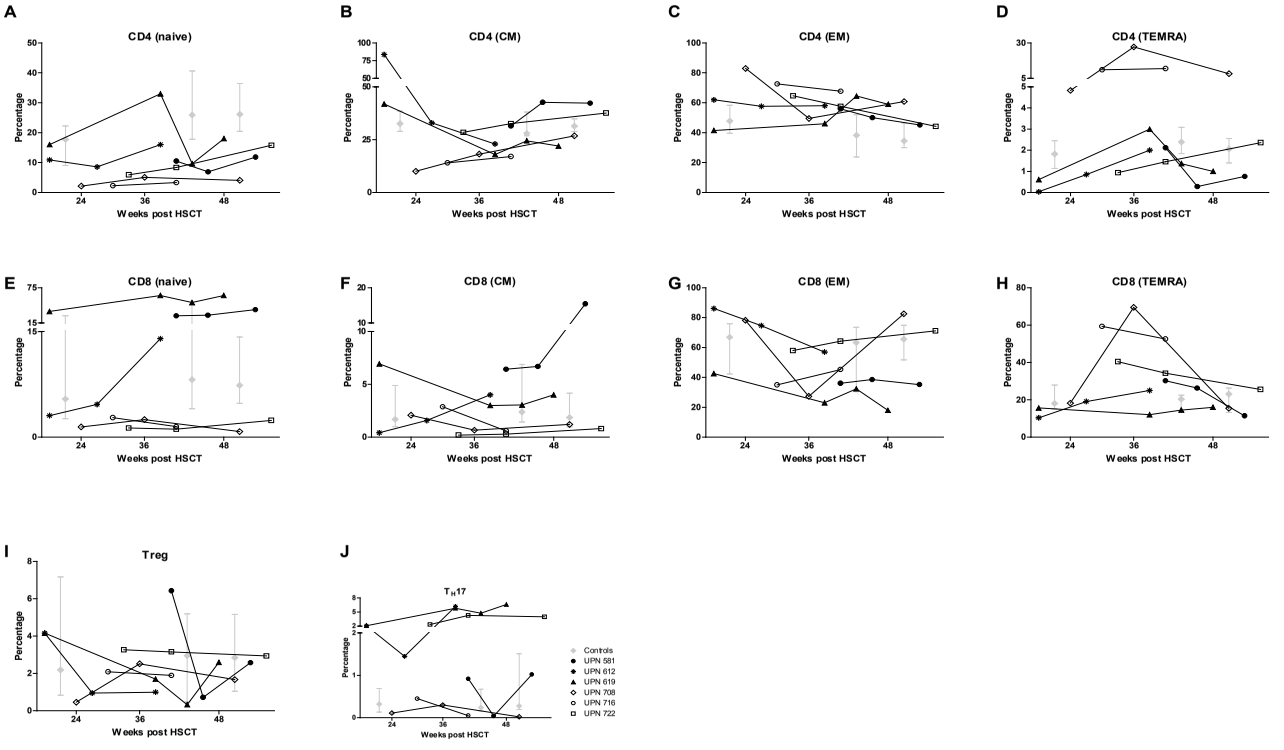

Figure 3. T cell subset characterisation

$CD4^+$ and $CD8^+$ T cells were further analysed for the combined expression level of several markers (CCR7, CD45RA, CD25, CD127, FoxP3, CD161, CCR4 and CCR6) known to be specifically expressed by distinct T cell subsets. The upper row (A to D) shows the percentages of Naïve, Central Memory (CM), Effector Memory (EM) and CD45 expressing effector memory (T_{EMRA}) T cells within the $CD4^+$ T cell compartment. The second row (E to H) shows the same subset analysis on circulating $CD8^+$ T cells. The bottom row shows T_{regs} (I) and T_H17 (J) subsets analysed within the $CD4^+$ T cell population. The closed black symbols represent the three HSCT patients with fasciitis, open symbols represent the three cGvHD patients without fasciitis. For comparison, the median (grey diamonds) and 5-95% CI (vertical bars) of ten HSCT patients without cGvHD are shown.

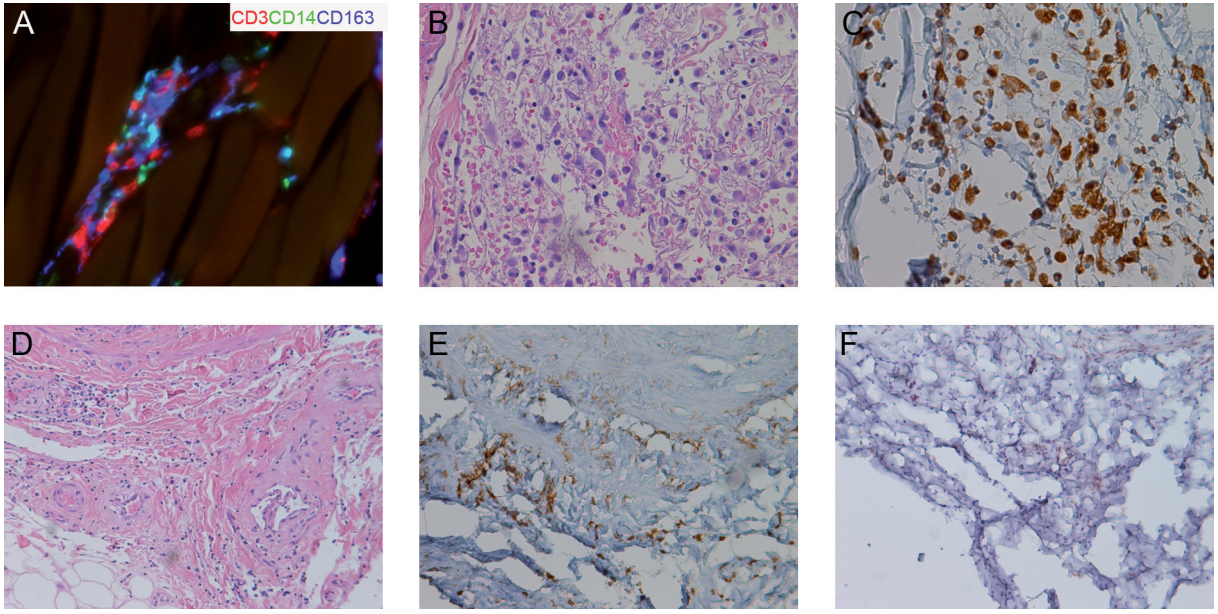

Figure 4. Dermal macrophages and host-derived cells are present in the fascia infiltrates.
(A) Triple immunofluorescent staining of the fascia infiltrate in a male fasciitis patient transplanted with a graft from a female donor (UPN 581), identified the occurrence of dermal macrophages ($CD14^{+}CD163^{+}$, turquoise) in close proximity of $CD3^{+}$ T cells (red). (B) H&E staining of fascia infiltrate in UPN 619, showing large macrophage-like cells. (C) Enzymatic CD163 staining of the same area of the same biopsy, indicating that the macrophage-like cells in the infiltrate are most likely dermal macrophages. (D) H&E staining of fascia infiltrate in UPN 612 (male patient transplanted with a female donor). (E) Enzymatic CD163 staining of the same area of the same biopsy. (F) *In situ* hybridisation of the Y-chromosome (red dots) in the same area of the same biopsy. The fascia infiltrate consists of both Y-chromosome positive cells (host origin) and Y-chromosome negative cells (donor origin).

accompanied by infiltrating female donor-derived monocytes and T cells. Routinely performed chimerism analysis (Powerplex 16) was used to analyse the level of donor chimerism in total PBMC collected before or at the same time when the patients were biopsied. Furthermore, chimerism analysis was performed on fractionated blood cells collected prior to the onset of fasciitis. To this end, two cell populations were separated by FACS sorting, i.e. $CD11c^+/CD14^+/CD20^-/CD3^-$ (monocytes) and $CD11c^+/CD14^-/CD20^-/CD3^-$ (myeloid DC). As also observed for unfractionated PBMC, these sorted populations showed full donor chimerism. However, when the CD163 staining results where compared to the Y-specific ISH data (Figure 4D-F), the Y-probe was clearly visible in the same region of the infiltrates as the abundantly present $CD163^+$ cells. Due to technical limitations, we were unfortunately not able to combine these two separate staining techniques. Nonetheless, as shown by Haniffa *et al*[31], dermal macrophages have a slow turnover after HSCT as indicated by a mean survival of host-derived cells of 100 days post HSCT. Given that we analysed our patients in between 70 and 364 days post-HSCT, we speculate that at least part of the $CD163^+$ cells present in the inflamed fascia must be of host origin. Additionally, mouse models of GvHD have shown that donor APC are required for maximal CD8-mediated GvHD. Donor APC were not necessary for the initiation but did intensify

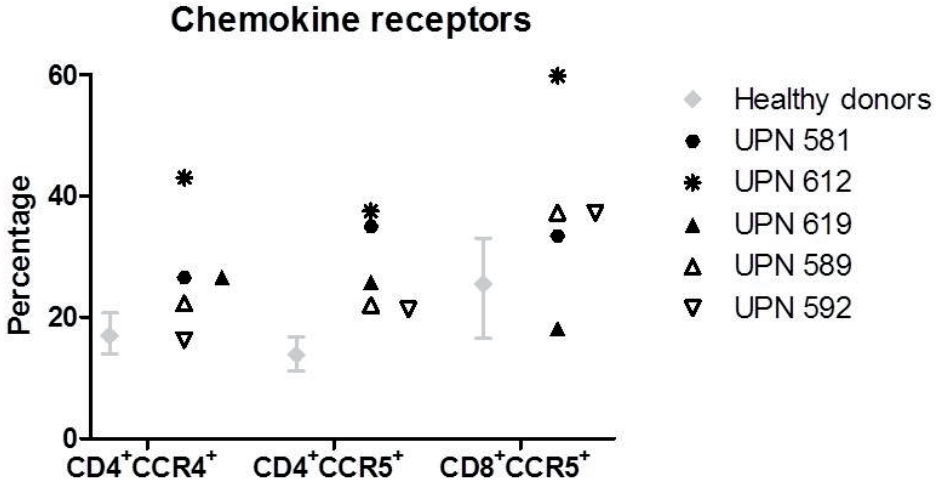

Figure 5. Chemokine receptor expression in peripheral blood T cells.
$CD4^+$ T cells in the peripheral blood of cGvHD patients at the time of fasciitis express higher levels of CCR4 and CCR5 as compared to healthy control HSCT donors (median: grey diamonds and 5-95% CI: bars) and two HSCT patients without acute or chronic GvHD (UPN 589 and 592, open triangles). The only difference in chemokine receptor expression on $CD8^+$ T cells between fasciitis patients and controls is an increased expression of CCR5 in UPN 612. The expression of other chemokine receptors, i.e., CCR1-3, CCR6-10, CXCR1-7) on both $CD4^+$ and $CD8^+$ T cells of the fasciitis patients is not different as compared to healthy control HSCT donors or the two HSCT patients without GvHD (data not shown).

the GvHD reaction[35]. It is, therefore, not surprising that we observed Y^+ cells in the fascia infiltrates.

The infiltration of peripheral tissues by T cells is thought to be mediated by local production of chemokines and the expression of their corresponding receptors on infiltrating T cells. We set out to determine which chemokine/chemokine receptor interaction could be involved in the migration of T cells to the fasciae. Given that chemokine receptors may be rapidly up- or downregulated upon entry of peripheral tissues, we first investigated the chemokine receptor expression by circulating T cells. Flow cytometric analysis of PBMC revealed an increased expression of CCR4 and CCR5 by $CD4^+$ T cells of all 3 patients at the time of fasciitis as compared to 2 control HSCT patients without cGvHD (UPN 589 and UPN 592) and a group of healthy donors (Figure 5). The $CD8^+$ T cells only showed increased expression of CCR5 in one fasciitis patient (Figure 5). All other chemokine receptors studied (CCR1-3, CCR6-10, CXCR1-7) were not differently expressed between patient and control samples (data not shown).

Subsequent immunohistochemical staining of the skin-muscle-fascia biopsies for CCR4 and CCR5 only revealed some *in situ* expression of CCR5 in 1 patient (UPN 612), which is the same patient with the observed high expression of CCR5 on $CD8^+$ T cells. Unexpectedly, this receptor was not expressed by fascia-infiltrating T cells (data not shown). Due to scarcity of biopsied tissue we were not able to investigate additional chemokine receptor expression in the biopsies.

In summary, this study confirms the presence of mainly activated $CD8^+$ T cells in the fasciae of HSCT patients who developed fasciitis as the main symptom of chronic GvHD. We additionally report co-localisation of these activated $CD8^+$ T cells with, presumably host-derived $CD14^+CD163^+$ dermal macrophages and $CD14^+CD163^-$ monocytes or type 1 macrophages. Further studies on the origin of these myeloid cell types and their alloantigen-presenting capacity are needed to further clarify their role in the onset of fasciitis.

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Chapter

7

Summary and General Discussion

Summary

The homing of both immune cells and their malignant counterparts is, amongst others, determined by the interaction between locally produced chemokines and their corresponding receptors expressed by blood-borne cells. The majority of studies described in this thesis have addressed the involvement of distinct chemokine/chemokine receptor combinations in directing this cellular trafficking.
Acute leukaemia in children is often associated with extramedullary infiltration of leukaemic cells leading to relapses at other sites than the bone marrow. In a previous study (Annels *et al.* Blood 2004;103(7):2806-8) we set out to determine whether chemokines and their receptors play a role in determining the site of leukaemia relapse. To this end, chemokine receptor expression by leukaemic blasts in peripheral blood and/or bone marrow aspirates was investigated at the time of diagnosis in 11 T-ALL patients. In one patient, in whom a gut relapse manifested 18 months after diagnosis, malignant cells present in the peripheral blood showed high expression of the gut-homing molecules CCR9 and CD103. The leukaemic cells which entered the gut also expressed CCR9 *in situ*; CCR9 expression was found to co-localise with its specific ligand (CCL25). All other patients showed the same chemokine receptor expression pattern on their leukaemic blasts as compared to normal circulating T cells in age-matched controls. None of these patients presented with a relapse of their leukaemia. These results suggest that screening of leukaemic blasts for chemokine receptor expression at the time of diagnosis may predict the extramedullary leukaemia (EML) risk and location.
To investigate the role of chemokines and their receptors in extramedullary AML, peripheral blood samples, bone marrow aspirates and skin biopsies of 15 paediatric AML patients with proven skin involvement were investigated. As presented in **Chapter 2**, AML blasts detected in the blood of patients who developed EML in the skin, showed a significantly higher expression of CCR2 as compared to control patients. The leukaemic blasts in the skin of these patients were also found to express CCR2. Besides CCR2, these skin-residing blasts also expressed CCR5, CXCR4 and CXCR7 as well as the corresponding ligands CCL3 and CXCL12, respectively. Based on these findings, we hypothesised that circulating blasts are directed to the skin mediated by CCR2, which interacts with an as yet unidentified locally produced chemokine. Subsequent interaction of CCR5/CCL3 and CXCR4/CXCL12 facilitates the retention of the blasts in the skin, whereas, CXCR7/CXCL12 interaction may prolong the extramedullary survival of leukaemic cells.
Omenn syndrome (OS) is an inherited immunodeficiency, characterised by abnormal B and T cell development and a corresponding limited T cell repertoire. These patients display generalised erythrodermia of the skin caused by a massive auto-reactive T cell infiltrate. The main characteristic of OS is the unusual tissue distribution of T cells in skin, gut and liver, which is similar to that of acute Graft-versus-Host Disease (aGvHD) patients (see next paragraph). In **Chapter 3** we investigated the homing of T cells in an OS patient with severe skin involvement. Not only circulating $CD4^+$ but also $CD8^+$ T cells were found to express high levels of the skin-homing molecule CCR10. Additionally, both T cell subsets were clearly present in skin biopsies collected from the patient; these biopsies also displayed abundant expression of

CCL27, one of the two thus far known ligands for CCR10. Topical treatment with Tacrolimus resulted in a significant improvement of the skin problems, followed by a major decline of $CCR10^+$ T cells in the circulation, normalised CCL27 expression and T cell disappearance from the skin. These results suggest an important role for CCR10/CCL27 interactions in the migration of activated $CD4^+$ and $CD8^+$ T cells to the skin in OS patients.

Allogeneic haematopoietic stem cell transplantation (HSCT) is the treatment of choice for the haematological and immunological disorders studied in Chapters 2-3. One of the major drawbacks of this treatment is the occurrence of GvHD. This transplantation-related complication results from homing of activated donor T cells to the skin, liver and gut where these cells induce inflammation and eventually life threatening tissue destruction. In order to investigate whether CCR10/CCL27 interactions also facilitate the homing of donor T cells to GvHD-affected skin, we analysed peripheral blood and skin biopsies of 15 paediatric patients who displayed acute GvHD early after HSCT (**Chapter 4**). Indeed, CCR10 was highly expressed by circulating $CD4^+$ T cells and appeared to correlate with duration of GvHD activity in the skin. $CD4^+CCR10^+$ T cells were clearly present in biopsies of affected sites in the skin, but not in the gut biopsies of the patients that also suffered from intestinal GvHD. The infiltration of $CD4^+CCR10^+$ T cells correlated with an enhanced CCL27 expression in the epidermis of these skin biopsies. These results suggest that, in aGvHD of the skin, particularly $CD4^+$ T cells are recruited through CCR10/CCL27 interactions.

Depending on the degree of HLA matching between donor and recipient, aGvHD may be caused by activated donor T cells recognising mismatched major (HLA) and/or minor histocompatibility antigens (mHags) expressed by the recipient and not by the donor. The involvement of mHag-specific T cells in the onset of aGvHD after gender mismatched HSCT was studied in **Chapter 5**. To this end, we validated a multivalent staining reagent and visualised, for the first time, the presence of HY-specific T cells, *in situ*, in skin biopsies derived from male recipients of female haematopoietic stem cells. Only limited numbers of these cells could be detected in peripheral blood mononuclear cells (PBMC) of these patients. mRNA analysis of total $CD8^+$ cells in the PBMC revealed expression of the chemokine receptor CX3CR1 (unpublished observations). Unfortunately, we were technically not able to combine HY multimer staining with an antibody specifically binding to this chemokine receptor. The skin-homing mechanism exploited by HY-specific T cells remains, therefore, to be elucidated.

Chemokine receptor expression by T cells in chronic GvHD (cGvHD), another more long-term complication of allogeneic HSCT, was studied in **Chapter 6**. PBMC as well as tissue-infiltrating cells were analysed in 3 patients who presented with fasciitis as the main clinical feature of cGvHD. The fascia infiltrates were characterised by a high percentage of both activated $CD8^+$ T cells and $CD163^+$ cells, most likely dermal macrophages. Although we were not able to identify the precise chemokine/chemokine receptor pair(s) responsible for the migration of the $CD8^+$ T cells to the fasciae, we demonstrated that CCR5 was expressed in these infiltrates albeit this expression did not co-localise with $CD8^+$ T cells. Hence, further studies are required for elucidation of the chemokine(s) and chemokine receptor(s) involved in the attraction of $CD8^+$ T cells to inflamed fasciae.

General Discussion and Future Directions

Extramedullary infiltration of leukaemic cells and local relapses are major complications of acute leukaemia as often observed in affected children. Site directed trafficking of cells is, however, not only relevant in local recurrences of malignancies but also in immunological disorders with characteristic local manifestations such as Omenn syndrome (OS) and transplant-related diseases like Graft-versus-Host Disease (GvHD). Despite advances in the general treatment of haematological and immunological disorders and GvHD, cellular trafficking still causes severe problems. Malignant cells escape treatment by homing to more immune-privileged sites and in immunological reactions and GvHD the overt tissue-specific inflammatory reactions are of great concern. Understanding chemokine receptor/ligand interactions involved in migration of (malignant) immune cells is expected to lead to new approaches for specific interference in these unwanted processes and, hence, improvement of currently existing treatment modalities. The major overall conclusion from the studies described in this thesis, is that tissue-specific cellular trafficking is a complex process which is not directed by a single chemokine receptor/chemokine pair alone.

One of the best studied and described examples of chemokine-mediated metastasis of malignant cells is CXCR4-dependent relapse of breast cancer as described by Müller *et al*. This study clearly showed high expression of active (responsive to its ligand) CXCR4 on human breast cancer cells and distinct expression of the ligand for CXCR4, CXCL12, in the organs affected by metastasis (lymph nodes, lung, liver and bone marrow). Additionally, when mice were injected with a human breast carcinoma cell line and subsequently treated with either anti-human CXCR4 monoclonal antibody or an isotype control antibody, a significant decrease in lung metastasis was seen in the anti-CXCR4-treated mice. These results strongly support the concept that CXCR4/CXCL12 interactions play an important role in directing the location of the metastasis of breast cancer[1]. This study was the starting point for numerous studies investigating the role of chemokines in cancer metastasis[2], including our own.

From the results of our study on the migration patterns of T-ALL cells[3], we indeed concluded that chemokine receptors should be considered as important diagnostic or prognostic markers for predicting extramedullary relapse risk. FACS analysis was used in this study to screen the leukaemic blasts for their chemokine receptor expression profile. Currently, mRNA analysis of leukaemic blasts by PCR or microarray is being used to identify genes that may be involved in relapse of leukaemia, resulting in a better upfront stratification of patients[4]. This elegant approach could also be used to predict the location of relapse[5]. Importantly, such studies should not only focus on chemokine receptors and their ligands, but also on adhesion molecules involved in cell-cell contact and trans-endothelial migration. Collectively, such analyses are expected to provide a more informative and easily applicable method of diagnostic screening in this respect.

The situation might, however, be less clear in the case of extramedullary leukaemia. Chapter 2 describes data obtained from AML patients who presented with extramedullary leukaemia in the skin. Given that CCR10 and CCR4 are well known skin-homing receptors (Chapters 3 and 4), we expected circulating leukaemic blasts to

express the same receptors. Unexpectedly, we rather found that CCR2 is involved in the homing of AML cells to the skin. Once migrated to this site, different chemokine receptors seemed to be involved in retention of the cells (i.e., CCR5 and CXCR4) and their survival (i.e., CXCR4 and CXCR7). These results indicate that chemokines and their receptors play a more complex role in cancer than merely directing the trafficking of cells.

Indeed, evidence exists that, besides involvement of chemokines and their receptors in metastasis of the tumour[6], these interactions are also operational in growth and survival of malignancies. CXCR4 and CXCR7 have been described to be involved in tumour cell survival. Besides CXCR4, CXCR7 is also expressed by a range of primary tumours, many tumour cell lines and activated endothelial cells, but is rarely expressed by non-transformed cells[7]. Unlike other chemokine receptors, CXCR7 lacks the ability to mediate chemotaxis and calcium mobilisation after ligand binding. Yet, CXCR7 is thought to regulate several important biological processes including cell survival, cell clustering and tumour development as shown for prostate, lung and breast cancer cells[8;9]. In gallbladder cancer, cytoplasmic and nuclear expression of CXCR4 was observed, whereas, CXCR7 was only expressed in the cytoplasm of the tumour cells. Nuclear expression of CXCR4 correlated with lymph node metastases, however, cytoplasmic expression of both chemokine receptors was not associated with metastases but separately associated with an advanced tumour stage[10]. Upon binding to its ligand, chemokine receptors are internalised into the cytoplasm and signal transduction pathways are activated leading to cell proliferation and migration. Translocation of the chemokine receptor to the nucleus may serve as a transcriptional regulatory signal, increasing transcription of genes and resulting in increased cell proliferation. In our study described in Chapter 2, we also observed intracellular expression of CXCR4. Further research is needed in order to clarify the roles of extracellular, cytoplasmic and nuclear expression of these different chemokine receptors in survival and migration of tumour cells.

Besides using chemokine receptor/ligand interactions for metastasis, retention and survival of tumour cells, the secretion of chemokines by these tumour cells can facilitate the attraction of immune cells, which not necessarily leads to tumour cell damage but, in contrast, may support tumour cell viability. Macrophages, for instance, produce factors that promote angiogenesis and impair immune responses[11]. The recruitment of dendritic cells (DC) into the tumour microenvironment causes immune paralysis[12], whilst attracted regulatory T cells inhibit anti-tumour responses[13;14], correlating with poor prognosis. These observations emphasise the clear and multi-level involvement of chemokine receptor/ligand interactions in tumour pathophysiology.

In the last decade, it has become clear that CXCR4 is the chemokine receptor most abundantly expressed by tumour cells, given that its presence is demonstrated in over 23 different human malignancies[15]. Given that its ligand, CXCL12, is expressed at the most common sites where metastases have thus far been found, CXCR4/CXCL12 interaction is considered one of the most important drivers of tumour cell metastasis[2]. It is, therefore, of great interest to explore the therapeutic potential of CXCR4 inhibiting agents. The CXCR4 antagonist AMD3100 (Plerixafor) has been successfully used in animal models to inhibit growth of breast cancer[16] and of a number of haematological malignancies including non-Hodgkin lymphoma[17] and

AML[18]. Currently, various clinical trials using CXCR4-targetting strategies are being evaluated in ovarian cancer, osteogenic sarcoma, ALL and AML[2;19;20]. Furthermore, AMD3100 is already used as a stem cell mobilisation agent to provoke haematopoietic stem cells to leave the bone marrow and enter the circulation in order to collect them for HSCT procedures via apheresis. This approach to release cells from the bone marrow might also be useful in the treatment of leukaemia. It is known that some leukaemic cells can escape chemotherapy whilst lingering in the bone marrow stromal environment[2;21-23]. CXCR4 antagonists like AMD3100 would drive these cells out of the bone marrow into the circulation, where they might be more susceptible to (chemo) therapy. Although short-term use of AMD3100 may be safe, caution should be taken when applied for prolonged periods. Chemokine receptor antagonists might interfere with essential chemokine receptor/ligand interactions, which are responsible for immune surveillance e.g. B- and T cell development and leukocyte travelling, rendering severe adverse effects. In case of CXCR4 antagonists like AMD3100, the sustained dislocation of the stem cell reserve from the bone marrow may potentially lead to bone marrow aplasia and subsequent haematological and/or immunological complications.

Although CXCR4 antagonists are currently most intensively studied, other chemokine receptors would also provide promising therapeutic targets. Currently, a patent is pending for a CCR10 antagonist, which would be useful in numerous skin diseases including OS and skin GvHD. Furthermore, a CCR5 antagonist (Maraviroc) is now being applied for the treatment of HIV-1 infection, in which CCR5 is used as a co-receptor to enter its target cells. Although its use is safe and well-tolerated, long term risks (>5 years) are yet unknown[24]. Successful pre-clinical tests have been performed with an orally active CCR2 antagonist, preventing glomerulosclerosis and renal failure in type 2 diabetes[25]. These are just a few examples of the current research to develop chemokine receptor antagonists as therapeutic tools.

Another way of interfering with the interaction of chemokines and their receptors for therapeutic purposes could be through the application of signalling pathway inhibitors. In Chapter 3, we observed down regulation of CCL27 and/or CCR10 after topical administration of Tacrolimus in an OS patient suffering from a GvHD-like inflammatory skin reaction with involvement of infiltrating T cells. Tacrolimus belongs to a group of pharmacological agents known as calcineurin inhibitors. These drugs prevent the transcription of several cytokine genes (IL-2, IL-3, IL-4, IL-5, TNF-α and IFN-γ in T cells by inhibiting the translocation of nuclear factor of activated T cells (NFAT)[26]. TNF-α is known to stimulate the production of CCL27 by keratinocytes[27;28]. The TNF-α inhibition by Tacrolimus might decrease CCL27 production and, thereby, reduces the influx of dermal $CCR10^+$ T cells. The reduction of T cells in the skin is not the sole reason for the major decrease in CCR10 expression at this site. It has also been shown that Tacrolimus has a direct effect on chemokine receptor expression by the inhibition of NFAT[29]. The DNA promoter sites of chemokine receptors, however, contain binding sites for multiple transcription factors. The expression of chemokine receptors can, therefore, be regulated by different signalling pathways. Indeed, Tacrolimus has been shown to have an effect on CCR2 and CXCR3, but not on CCR7, expression[30-32]. Besides the hypothesised regulation of CCR10 by Tacrolimus, other ways to intervene with chemokine receptor signalling pathways

need to be investigated further.
Therapeutic measures that interfere with the chemokine (receptor) system in one way or the other, remain difficult to apply in a safe way. To circumvent the adverse effects of chemokine receptor antagonists, the chemokine system could also be used to promote anti-tumour immune responses. Local injection of chemokine(s) at the tumour site can facilitate recruitment of CTLs, NK cells or immature DC, initiating a tumour-specific immune response without interfering with normal homeostatic interactions[33]. In short, chemokine receptor/ligand interactions offer a attractive therapeutic option for numerous diseases including cancer, but should be investigated more extensively to prevent adverse effects of therapy.
Chemokines and their receptors are not only interesting for their therapeutic implications, but can also help us to understand the pathophysiology of diseases. Especially the migration of lymphocytes in immunological disorders such as OS, will help us to discover underlying mechanisms. A hallmark of OS is the peculiar tissue distribution of T cells; the T cells typically accumulate in skin and gut as seen in aGvHD[34]. One of the underlying genetic defects in OS is a hypomorphic mutation in one of the RAG genes, which severely impairs the function of the gene products in DNA recombination processes, resulting in maturation and expansion of only a restricted number of T cell clones[35]. A near absence of T cells in the thymus of OS patients affects maturation of thymic epithelial cells and dendritic cells. Consequently, auto-reactive T cells are not eliminated and generation of central tolerance is impaired. Together with increased antigen exposure and a defect in antigen clearance, as described for immunodeficient patients, this may result in the proliferation of auto-reactive T cells. Skin and gut belong to the first line of defence against pathogens and immune surveillance in these organs is, therefore, crucial. Auto-antigens derived from these organs are normally highly represented in the thymus to induce T cell-tolerance towards these organs. Consequently, it is not surprising that these organs are affected by auto-reactive T cells in OS. The same holds true for GvHD patients given that T cell-tolerance in the donor is essentially different from central tolerance in the recipient, even in the case of HLA identical sibling donors. This will result in the recognition of recipient-specific antigens by donor T cells. Again, skin and gut are mainly involved due to the abovementioned reason. Skin-specific homing of T cells is known to be facilitated by, amongst others, CCR10/CCL27 interaction. Indeed, we observed a correlation between CCR10 expression on T cells (in peripheral blood and skin) and disease activity in both acute GvHD and OS.
In contrast to the expression of CCR10 on both $CD4^+$ and $CD8^+$ T cells in OS, only $CD4^+$ T cells express CCR10 in aGvHD of the skin, as described in Chapter 4. Peripheral $CD8^+$ T cells also did not express other known skin-homing molecules such as Cutaneous Lymphocyte Antigen (CLA) and CCR4. As only a few of the $CD8^+$ T cells detected in the affected skin co-express CCR10, the remaining $CD8^+$ T cells apparently use a skin homing mechanism different from $CD4^+$ T cells in this setting. Increasing evidence emerges that CXCR3 expression plays an important role in aGvHD. Murine models have shown that CXCR3 expressing $CD8^+$ T cells are responsible for tissue damage in aGvHD[36]. In the human setting, Piper *et al*. have observed CXCR3 expressing T cells in the affected skin of GvHD patients, although expression of this chemokine receptor was absent on peripheral blood T cells[37]. In

our experimental approach, the chemokine receptor expression pattern on PBMC isolated from the blood of GvHD patients was investigated, followed by staining of the skin biopsies for the thus defined chemokine receptors. With this approach, we might have missed some of the chemokine receptors that were present in the skin, but to a lesser extent in the peripheral blood of aGvHD patients. Confirmatively, we have observed that the majority of T cells infiltrating GvHD (both $CD4^+$ and $CD8^+$) also expressed CXCR3 (unpublished data).

The fact that the cellular composition in peripheral blood and tissue can be different is further supported by results described in Chapter 5; focussing on $CD8^+$ T cells in the skin of male aGvHD patients transplanted with a female graft, which are specific for Y chromosome-encoded minor histocompatibility antigens (HY antigens). The peripheral blood of these patients hardly contained HY-specific T cells, whereas they were present in the skin of these patients. PCR analysis of mRNA isolated from the $CD8^+$ T cells sorted from PBMC, showed an increased expression of CCR1, CCR2, CCR3 and CX3CR1 mRNA (data not shown). However, this expression was not observed on the HY-specific $CD8^+$ T cells. CX3CR1 seems to play a role in inflammatory skin disorders like AD and psoriasis. The percentage of CX3CR1 expressing $CD8^+$ T cells was decreased in the peripheral blood of AD and psoriasis patients, but was increased in the skin of these patients[38], suggesting that CX3CR1 is responsible for the homing of $CD8^+$ T cells to psoriatic skin. As samples sizes and precursor frequencies might be too small to allow for substantial analysis, combined staining of tetramers and chemokine receptors might provide us with a better understanding of the migration of specific subsets of $CD8^+$ T cells to the skin.

Due to limited patient material it was not possible to investigate the expression of all thus far known chemokine receptors in the skin of these aGvHD patients. This would have shed more light on the molecules involved in $CD8^+$ T cell migration to the skin. *Ex vivo* analysis on fresh biopsies would be ideal as these biopsies could be used for enrichment of the cell population of interest by FACS sorting followed by mRNA array analysis of the whole spectrum of chemokines and their ligands. Candidate receptors could be subsequently stained on a formalin-fixed paraffin-embedded biopsy collected in parallel for histopathological evaluation. Immunofluorescent staining of aGvHD skin biopsies of the patients in this particular study, did reveal CX3CR1 expression, but no evidence was obtained for co-localisation of CX3CR1 and CD8 staining (data not shown).

CX3CR1 is known to be expressed by $CD16^+$ NK cells and CD45RA expressing effector memory $CD8^+$ T cells (T_{EMRA})[39]. Some evidence exists that $CD14^+$ monocytes use CX3CR1 for migration to the skin in cGvHD patients[40]. Additionally, tissue DC may originate from a distinct monocyte subset, expressing CD14, CD16 and CX3CR1[41;42]. Consequently, the CX3CR1 expressing cells observed in the skin of aGvHD patients might in fact represent monocytes attracted from the peripheral blood into the tissues. Here, these precursor cells will replace resident tissue DC-like dermal macrophages. Investigation of these $CX3CR1^+$ monocytes with regard to recipient or donor origin and their interaction with T cells is needed to further clarify their role in GvHD.

Another disease in which $CD8^+$ T cells play an important role is chronic GvHD, which still displays a poorly understood pathophysiology as compared to aGvHD. One of

the clinical features of cGvHD can be fasciitis of the extremities. As discussed in Chapter 6, the lymphocytic infiltrates in the skin of these fasciitis patients mainly contained $CD8^+$ T cells. Flow cytometric analysis of chemokine receptor expression of $CD8^+$ T cells in the peripheral blood of these patients did not give an obvious clue for the mechanism of migration of these cells to the fasciae. Multicolour staining of the biopsies could have indicated the chemokine receptors involved in $CD8^+$ T cell-specific homing, however, this was hampered by limited patient material. Again, a technical approach as described in the previous section might be helpful in overcoming this problem.

The question which remains is why the same type of immune cells, i.e. $CD8^+$ T cells, uses different ways to reach the same location in the body in different clinical situations. One possible reason is that up regulation of chemokine receptors is influenced by different locally produced triggers. The microenvironment, including the cellular constituents (such as the various types of antigen presenting cells) and the cytokine milieus, in which T cells are activated is, at least partly, responsible for the chemokine receptor expression pattern of cells upon activation. For instance, the activation requirements differ between the $CD4^+$ and $CD8^+$ T cell subsets. Additionally, constitutive chemokine receptor expression differs between the different types of T cells, suggesting that different chemokine/chemokine receptor interactions can be involved in homing to the same location. It is important to note that most studies, including our own, focus on expression of separate receptors and their ligands. This is an oversimplification of the in vivo situation in which site-directed migration most probably is orchestrated by simultaneous and sequential involvement of various chemokine receptors and adhesion molecules. This is exemplified in the next section.

Besides the expression of CCR10 by skin-infiltrating T cells in aGvHD and OS, these cells also expressed CLA. This is a skin-specific adhesion molecule that together with E-selectin, causes tethering of the cells along the endothelial wall. Although we focussed on skin GvHD, the clinical manifestation of acute GvHD of the gut can be more severe, and is more often refractory to steroid treatment. The homing mechanism of T cells to the gut is less clear than that to the skin. Unpublished data from our group shows that, compared to HSCT recipients without GvHD, the peripheral blood of patients displaying gut GvHD contains higher percentages CD103 expressing T cells; this integrin is specific for T cell homing to the gut[43]. These $CD103^+$ T cells (both $CD4^+$ and $CD8^+$) also expressed high levels of the chemokine receptor CCR3 and multicolour staining of gut biopsies showed that CCR3 was also expressed by T cells in the gut of these GvHD patients. Together with the high expression of CCL28, the ligand for CCR3, in the biopsies, these data suggest that CD103 and CCR3/CCL28 interaction are involved in the homing of T cells to the gut. However, these results were only found in a few patients and should be confirmed in an extended patient cohort.

In retrospect, our observation regarding the correlation between the presence of $CCR10^+$ T cells in the peripheral blood of patients with acute skin GvHD at the same time as their appearance in the skin should be considered as a unique set of data. Most likely, we have been extremely lucky that the patients in question were sampled at the right time points, as skin-homing T cells may only be present in the circulation for a very limited amount of time. Overall, our results indicate that the expression of

chemokine receptors in the peripheral blood does not necessarily represent their expression pattern on cells present in inflamed tissue. Acute GvHD mainly occurs within 100 days post transplant, when immune reconstitution is generally far from complete. In this lymphopenic setting, it is much easier to find deviations than when reconstitution is (nearly) complete, as is the case in most patients who manifest with cGvHD. In OS, the highly restricted T cell receptor repertoire mimics a lymphopenic setting, which may explain our finding of skin-homing $CCR10^+$ T cells amongst the T cells present in PBMC collected from this patient. As mentioned before, a promising approach to obtain a better understanding of the homing processes in GvHD would be by multicolour staining or mRNA analysis of tissue infiltrating T cells instead of looking at surface expression levels in peripheral blood cells of these patients.

In order to fully unravel the pathophysiology of GvHD, not only the effector cells should be investigated but also the antigen presenting cells (APC) involved in activation of these cells. Some types of APC such as DC are actively on the move which allows them to patrol the body, whereas other APC such as macrophages are sessile in tissues. The kinetics of APC turnover from recipient to donor origin after HSCT probably plays a decisive role in the pathophysiology and tissue specificity of GvHD. In Chapter 6 we describe the “per exclusionem” finding that host-derived dermal macrophages are major constituents of the fasciae infiltrates. As described before[44], recipient dermal macrophages can persist for a long time post transplant. These cells are probably not involved in the initiation of GvHD but could sustain the response of previously activated allo-reactive T cells. In the epidermis, Langerhans cells (LCs) self-renew and are only replaced by bone marrow-derived precursors upon inflammation[45], which might also hold true for dermal macrophages. APC in other organs than the ones affected in GvHD, might have faster turnover rates and, therefore, these organs are not affected by donor T cells.

In contrast to Haniffa *et al*., we attempted to investigate the kinetics of LCs in the skin *in situ*, using XY-FISH in combination with fluorescent labelling of CD3 and CD1a. However, we found contradictory results comparing frozen and paraffin embedded biopsies (unpublished data). A larger study, looking at different organs, applying better sampling schemes, using fresh material and another way to combine LC markers with techniques to discriminate between donor and recipient, could provide a better understanding of LC turnover kinetics and the impact on the initiation and persistence of GvHD.

The role of chemokines and their receptors in the pathophysiology of GvHD remains to be elucidated. Recently, CCR6 has been described as an important chemokine receptor in cGvHD. Disparities in SNP’s in the CCR6 gene between donor and recipient may result in a cGvHD protective genotype[46]. It is hypothesised that the protective genotype is a result of low CCR6 expression levels by donor cells. The explanation for this is two-fold. First, immature DC express, amongst others, CCR6 to enable recruitment of DC to inflammatory sites[47]. Interestingly, the sole ligand for CCR6, CCL20, is constitutively expressed in skin, gut, liver, colon and lung[48], corresponding with the GvHD target organs. Impaired homing of immature DC may thus result in reduced allo-reactive T cell responses. Second, T_H17 cells are characterised by expression of $CD4^+CD161^+CCR4^+CCR6^+$[49]. As T_H17 cells are involved in the occurrence of GvHD[49], low levels of CCR6 on these cells would impair

their homing to GvHD target tissues.
Recently, a distinct $CD8^+$ T cell subset has been described, expressing CD161. These cells also express CCR6 and can produce IFN-γ and IL-17, which are GvHD-associated cytokines. Reconstitution of these cells after HSCT is rapid, probably due to their unresponsiveness to CsA, which is used as GvHD prophylaxis. Decreased levels of this subset in the PBMC of HSCT patients correlated with the occurrence of GvHD. These results suggest that $CD8^+CD161^+CCR6^+$ T cells specifically migrate to the CCL20 expressing GvHD target organs (Figure 1) and indicate a role in GvHD pathophysiology (A.G.S. van Halteren, personal communication). It would be worthwhile to investigate the expression of CD161 and CCR6 on the $CD8^+$ T cells observed in fasciitis and by the HY-specific $CD8^+$ T cells described in Chapters 5 and 6 of this thesis, to see whether they belong to this specific $CD8^+$ T cell subset. If so, the expression of CCL20 would also be of interested in the affected lesions .

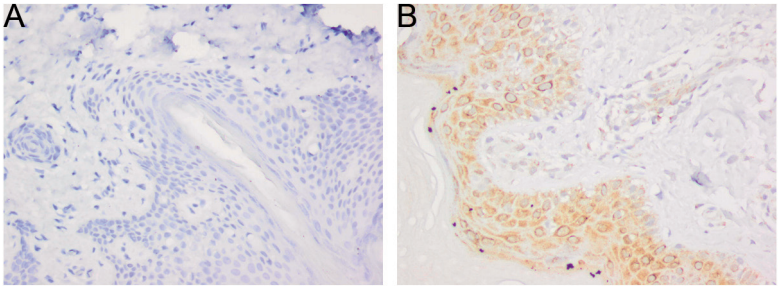

Figure 1. CCL20 expression in the skin of an acute GvHD patient.
Single enzymatic staining for CCL20 (detected by the red/brown colour, B) showed clear expression of this chemokine in a representative GvHD skin biopsy. Omission of the primary antibody was used to show the specificity of the staining in a foreskin biopsy (A).

Based on these data, the model for the pathophysiology of GvHD as depicted in Figure 7, Chapter 1, can be extended (Figure 2). TBI and chemotherapy induce tissue damage, resulting in the secretion of IL-1 and TNF-α. Besides their effect on HLA expression and the expression of adhesion molecules, they also induce the up-regulation of CCL20 by host tissues as skin (keratinocytes)[51;52] and gut (epithelial cells)[52;53]. This increased expression of CCL20 induces the recruitment of immature DC (iDC), T_H17 cells and $CD8^+CD161^+CCR6^+$ T cells. Other donor T cell subsets become activated and their influx is facilitated by tissue-specific chemokine/chemokine receptor interactions e.g. CCR10/CCL27.
Altogether, the major drawback that the investigations described this thesis have in common, is the limited availability of fresh patient material. This could be overcome by better sampling schemes and compliance to these schemes. As described in this thesis, cellular trafficking in haematological and immunological disorders is complicated and, apart from involvement of additional interaction pathways, the chemokine/chemokine receptor system in itself is too heterogeneous to apply

as a single diagnostic or therapeutic tool. However, in diseases like leukaemia, it is still worthwhile to screen for chemokine receptors at diagnosis, and further investigation of migration mechanisms will certainly be instrumental in unravelling the pathophysiology of haematological and immunological disorders.

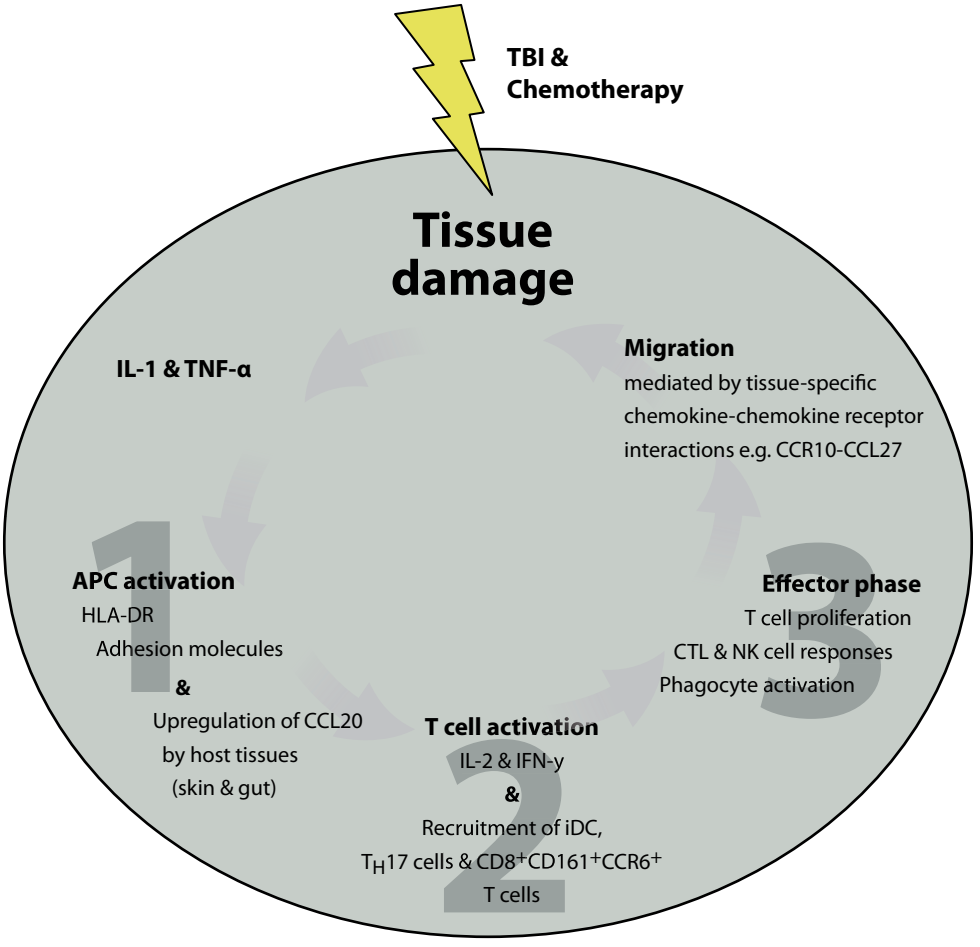

Figure 2. Extended model for the pathophysiology of acute GvHD.
TBI and chemotherapy induce tissue damage, resulting in the secretion of IL-1 and TNF-α. Besides their effect on HLA expression and the expression of adhesion molecules, they also induce the up-regulation of CCL20 by host tissues as skin (keratinocytes) and gut (epithelial cells). This increased expression of CCL20 induces the recruitment of immature DC (iDC), T_H17 cells and $CD8^+CD161^+CCR6^+$ T cells. Other donor T cell subsets become activated and their influx is facilitated by tissue-specific chemokine/chemokine receptor interactions e.g. CCR10/CCL27

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Nederlandse Samenvatting

De migratie van cellen van het afweersysteem en hun kwaadaardige tegenhangers, leukemische cellen, van en naar de verschillende weefsels en organen wordt o.a. bepaald door de interactie van locaal geproduceerde chemokinen (kleine eiwitten) met receptoren die op deze cellen tot expressie komen en de chemokinen specifiek herkennen. Het merendeel van het in dit proefschrift beschreven onderzoek heeft zich gericht op de betrokkenheid van specifieke chemokine/chemokine receptor combinaties in het sturen van dit cellulaire verkeer.
Acute leukemie bij kinderen gaat vaak gepaard met infiltratie van leukemische cellen, die ontstaan in het beenmerg, in weefsels buiten het beenmerg (extramedullair). Na behandeling kan dit leiden tot het locaal terugkomen van de leukemie (locaal recidief). In een eerdere studie (Annels *et al.* Blood 2004; 103(7):2806-8) hebben we onderzocht of chemokinen en hun receptoren een rol spelen bij het bepalen van de uiteindelijke locatie van het recidief. Hiervoor werd de chemokine receptor expressie op leukemische cellen in het perifere bloed en/of beenmerg van 11 patiënten met een acute T-cel leukemie (T-ALL) onderzocht ten tijde van diagnose. In één patiënt, die 18 maanden na diagnose een recidief in de darm kreeg, vertoonden de leukemische cellen in het bloed bij diagnose al een hoge expressie van de darm-specifieke chemokine receptor CCR9 en adhesiemolecuul CD103. De leukemische cellen bij het latere recidief in de darm brachten inderdaad CCR9 tot expressie, samen met CCL25, het chemokine dat door CCR9 wordt herkend. De andere 10 patiënten brachten dezelfde chemokine receptoren tot expressie als de normale T cellen van gezonde kinderen van dezelfde leeftijd. Geen van deze patiënten presenteerde zich later met een (locaal) recidief van de leukemie. Deze resultaten suggereren dat stelselmatig onderzoek van leukemische cellen bij diagnose van de leukemie voor afwijkende expressie van chemokine receptoren de mogelijke relaps locatie kan voorspellen.
Om de rol van chemokinen en hun receptoren in extramedullaire acute myeloide leukemie (AML) te onderzoeken werden perifeer bloed, beenmerg en huid biopten van 15 kinderen met AML cellen in het beenmerg en de huid onderzocht. Dit is beschreven in **Hoofdstuk 2**. AML cellen uit het bloed van de patiënten met extramedullaire leukemie (EML) in de huid vertoonden, vergeleken met AML cellen uit het bloed van patiënten zonder leukemische cellen in de huid, een hogere expressie van de chemokine receptor CCR2. De leukemische cellen in de huid van deze patiënten brachten ook CCR2 tot expressie. Behalve CCR2, hadden deze cellen niet alleen de chemokine receptoren CCR5, CXCR4 en CXCR7 op hun celmembraan, maar expresseerden deze AML cellen ook de chemokinen CCL3 en CXCL12 die specifiek door deze receptoren worden herkend. Gebaseerd op deze bevindingen denken we dat AML cellen in de circulatie door CCR2 en een nog niet geïdentificeerd chemokine gedirigeerd worden naar de huid. Interacties tussen CCR5/CCL3 en CXCR4/CXCL12 zorgen hierna voor het vasthouden van de leukemische cellen in de huid, waarna CXCR7/CXCL12 interacties zorgen voor de verlenging van de overlevingskansen van de leukemische cellen.
Omenn syndroom (OS) is een erfelijke stoornis van de immunologische afweer (immuundeficiëntie) die wordt gekarakteriseerd door abnormale ontwikkeling van

B en T cellen. De T cellen hebben bovendien een beperkt repertoire van antigeen-specifieke receptoren. Deze patiënten vertonen een rode verkleuring van de huid (erytrodermie) veroorzaakt door een enorm infiltraat van auto-reactieve T cellen. Het voornaamste symptoom van OS is de uitzonderlijke aanwezigheid van T cellen in de huid, darmen en lever, zoals ook gezien wordt bij patiënten met acute Graft-versus-Host ziekte (aGvHD, zie volgende paragraaf). In **Hoofdstuk 3** hebben we de migratie van T cellen onderzocht in een OS patiënt met ernstige huidproblemen. De huid-specifieke chemokine receptor CCR10 werd niet alleen in hoge mate tot expressie gebracht op $CD4^+$ en $CD8^+$ T cellen in de circulatie, maar beide T cel populaties waren ook duidelijk aantoonbaar in huidbiopten, kleine stukjes weefsel van de aangedane huid, van de patiënt. In deze biopten was bovendien erg veel CCL27 aanwezig, een van de twee chemokinen die door CCR10 herkend worden. Locale behandeling van de huid met het middel Tacrolimus resulteerde in een significante verbetering van de huidproblemen, gevolgd door een enorme afname van $CCR10^+$ T cellen in de circulatie en een vermindering van CCL27 expressie en T cel infiltratie in de huid. Deze resultaten suggereren dat CCR10/CCL27 interactie een belangrijke rol speelt in de migratie van geactiveerde $CD4^+$ en $CD8^+$ T cellen naar de huid van OS patiënten.

Hematopoëtische stamcel transplantatie (HSCT) is een vaak gebruikte behandeling voor hematologische en immunologische aandoeningen zoals besproken in de hoofdstukken 2-3. Een van de grootste nadelen van deze behandeling is het optreden van “Graft-versus Host Disease” (GvHD). Hierbij reageren T cellen afkomstig uit het stamcel transplantaat van de donor tegen de huid, darmen en lever van de ontvanger van het transplantaat, met als gevolg beschadiging van deze weefsels. Om te onderzoeken of CCR10/CCL27 interactie, zoals in hoofdstuk 3 gevonden is bij een patiënt met Omenn syndroom en huidproblemen, ook van belang is voor de migratie van donor T cellen naar de huid bij patiënten met GvHD, hebben we het perifere bloed en huidbiopten van 15 kinderen met acute GvHD vroeg na HSCT onderzocht (**Hoofdstuk 4**). CCR10 werd inderdaad hoog tot expressie gebracht op $CD4^+$ T cellen in het bloed en dit leek te correleren met de duur van de huidproblemen. $CD4^+CCR10^+$ T cellen waren duidelijk aanwezig in de huidbiopten maar niet in de darmbiopten van de patiënten die ook last hadden van GvHD in de darm. De aanwezigheid van $CD4^+CCR10^+$ T cellen in de huid correleerde met een verhoogde expressie van het chemokine CCL27 in de epidermis van deze huidbiopten. Deze resultaten suggereren dat in GvHD voornamelijk de $CD4^+$ T cellen naar de huid migreren door het optreden van CCR10/CCL27 interacties.

GvHD wordt veroorzaakt door de activatie van donor T cellen die de verschillende humane leukocyten antigenen (HLA) en/of minor HLA (mHags) moleculen herkennen die wel door de ontvanger maar niet door de donor tot expressie gebracht worden. HLA en mHAgs moleculen worden door (een gedeelte van) de kernhoudende cellen tot expressie gebracht. In **Hoofdstuk 5** werd de betrokkenheid van T cellen, die specifiek een bepaald mHAg molecuul (namelijk HY dat door mannelijk cellen tot expressie kan worden gebracht) herkennen, bij het ontstaan van aGvHD na HSCT tussen twee mensen van een verschillend geslacht onderzocht. Hiervoor hebben we een speciaal reagens op zijn bruikbaarheid getest. Door gebruik van dit reagens hebben we, voor de eerste keer, in door GvHD aangedane huidbiopten van manneli-

jke ontvangers van vrouwelijke hematopoëtische stamcellen de aanwezigheid van HY-specifieke T cellen zichtbaar kunnen maken. Hoewel maar een beperkt aantal van deze cellen gedetecteerd kon worden in de perifere bloed mononucleaire cellen (PBMC) van deze patiënten, bracht analyse van deze cellen de expressie van de chemokine receptor CX3CR1 aan het licht. Helaas was het technisch niet mogelijk om de HY kleuring van cellen in de huid the combineren met een aankleuring voor chemokine receptoren. Hierdoor blijft het mechanisme waarmee HY-specifieke T cellen naar de huid migreren nog onduidelijk.

In **Hoofdstuk 6** werd de rol van T cellen in chronische GvHD (cGvHD) onderzocht; chronische GvHD is een lange-termijn complicatie van HSCT en een bekende oorzaak van morbiditeit en mortaliteit na transplantatie. Bij 3 patiënten met een ontsteking van het bindweefsel (fasciitis) als voornaamste symptoom van cGvHD werden PBMC en weefsels geanalyseerd. De cellen die te vinden zijn in de fascia (het bindweefsel) bevatten relatief veel geactiveerde $CD8^+$ T cellen en $CD163^+$ cellen, mogelijk dermale (huid) macrofagen. Hoewel we de chemokine/chemokine receptor paren verantwoordelijk voor de migratie van $CD8^+$ T cellen niet konden identificeren, konden we wel aantonen dat de chemokine receptor CCR5 aanwezig was op de plaatsen waar cellen de fascia waren binnengedrongen, maar dat deze expressie niet samenviel met de exacte locatie van de $CD8^+$ T cellen.

Curriculum Vitae

Claudia Margaretha Johanna Maria Faaij was born on the 26th April 1979 in The hague, The Netherlands. She attended secondary school (VWO) at the Ring van Putten in Spijkenisse. After her graduation in 1998, she started Biomedical Sciences at the University of Leiden. During this study she did her practical training at the Biomedical Primate Research Centre in Rijswijk at the Virology department. Under supervision of Peter ten Haaft PhD and Prof. Jonathan Heeney she analysed the possible recombination between HIV-1 and SIV_{CPZ} in chimpanzees. This research was followed by a more clinical internship investigating the long-term effects of the treatment for *M. Cushing*. This was done at the Endocrinology department of the Leiden University Medical Centre (LUMC) under supervision of Alberto Pereira PhD and Prof. Romijn. At her final research internship, she investigated the homing capabilities of paediatric leukaemias at the Immunology laboratory of the Paediatrics Department of the LUMC under supervision of Nicola Annels PhD and Maarten van Tol PhD. In August 2002 she received her doctoral degree in Biomedical Sciences.
After a short detour at the Immunology Laboratory of the Erasmus Medical Centre she came back to the Paediatrics Department in 2003, first as a research technician and later on as PhD student. Under the supervision of Nicola Annels PhD, Astrid van Halteren PhD and Maarten van Tol PhD, she carried out the research presented in this thesis.
From June 2009 until September 2010 she worked as an Application Manager for Astellas Pharma via CLS Services, for which she now acts as a Recruitment Consultant. She is married to Guido Erwich and proud mother of Bente (2010).

Publications

1. Kim YH, Faaij CM, van Halteren AG, Schrama E, de Jong TA, Schøller J, Egeler RM, Pavel S, Vyth-Dreese FA, van Tol MJ, Goulmy E, Spierings E. In Situ Detection of HY-Specific T Cells in Acute Graft-versus-Host Disease-Affected Male Skin after Sex-Mismatched Stem Cell Transplantation. Biol Blood Marrow Transplant. 2011 Nov 4. [Epub ahead of print]

2. Claudia M.J.M. Faaij, Annemieke J. Willemze, Tom Révész, Melania Balzarolo, Cornelis P. Tensen, Manja Hoogeboom, Maarten H. Vermeer, Elisabeth van Wering, Christian M. Zwaan, Gertjan J.L. Kaspers, Colin Story, Astrid G.S. van Halteren, Jaak M. Vossen, R. Maarten Egeler, Maarten J.D. van Tol and Nicola E. Annels. The role of CCR2, CCR5, CXCR4 and CXCR7 in extramedullary leukaemia of the skin and on leukaemic blast survival in childhood AML. Pediatr Blood Cancer. 2010 Aug;55(2):344-8.

3. Faaij CM, Annels NE, Ruigrok G, van der Burg M, Ball LM, Bredius RG, van Tol MJ, Lankester AC. Decrease of skin infiltrating and circulating CCR10+ T cells coincides with clinical improvement after topical tacrolimus in Omenn syndrome.J Invest Dermatol. 2010 Jan;130(1):308-11. Epub

4. Claudia M.J.M. Faaij, Arjan C. Lankester, Eric Spierings, Manja Hoogeboom, Edward P. Bowman, Marc Bierings, Tom Révész, R. Maarten Egeler, Maarten J.D. van Tol, Nicola E. Annels. CCL27/CTACK-CCR10 interaction plays an important role in recruiting CD4+ T cells to skin in human Graft-versus-Host Disease. Br J Haematol. 2006 Jun;133(5):538-49.

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