The handle http://hdl.handle.net/1887/19745 holds various files of this Leiden University dissertation.

Author: Faaij, Claudia Margaretha Johanna Maria  
Title: Cellular trafficking in haematological and immunological disorders  
Issue Date: 2012-09-05
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.
Chapter 7

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. This study was the starting point for numerous studies investigating the role of chemokines in cancer metastasis, including our own.

From the results of our study on the migration patterns of T-ALL cells, 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. This elegant approach could also be used to predict the location of relapse. 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, 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. 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. 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.

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. The recruitment of dendritic cells (DC) into the tumour microenvironment causes immune paralysis, whilst attracted regulatory T cells inhibit anti-tumour responses, 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. 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. 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 and of a number of haematological malignancies including non-Hodgkin lymphoma.
AML<sup>18</sup>. Currently, various clinical trials using CXCR4-targetting strategies are being evaluated in ovarian cancer, osteogenic sarcoma, ALL and AML<sup>2,19,20</sup>. 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<sup>2,21-23</sup>. 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<sup>24</sup>. Successful pre-clinical tests have been performed with an orally active CCR2 antagonist, preventing glomerulosclerosis and renal failure in type 2 diabetes<sup>25</sup>. 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)<sup>26</sup>. TNF-α is known to stimulate the production of CCL27 by keratinocytes<sup>27,28</sup>. 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<sup>29</sup>. 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<sup>30-32</sup>. 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 patients38, 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 (TEMRA)38. Some evidence exists that CD14+ monocytes use CX3CR1 for migration to the skin in cGvHD patients40. Additionally, tissue DC may originate from a distinct monocyte subset, expressing CD14, CD16 and CX3CR141,42. 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
Chapter 7

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 milieu, 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 gut43. 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, 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, 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. 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. Interestingly, the sole ligand for CCR6, CCL20, is constitutively expressed in skin, gut, liver, colon and lung, corresponding with the GvHD target organs. Impaired homing of immature DC may thus result in reduced allo-reactive T cell responses. Second, T<sub>h</sub>17 cells are characterised by expression of CD4<sup>+</sup>CD161<sup>+</sup>CCR4<sup>+</sup>CCR6<sup>+</sup>. As T<sub>h</sub>17 cells are involved in the occurrence of GvHD, low levels of CCR6 on these cells would impair
Chapter 7

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) and gut (epithelial cells). This increased expression of CCL20 induces the recruitment of immature DC (iDC), Th17 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), TH17 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
References

Chapter 7


