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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 disorders¹. 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 liver². 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 complication³.

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 proteins⁴. 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 receptors⁵, 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 GvHD⁶⁻⁸, 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\textsuperscript{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\textsuperscript{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\textsuperscript{11,12}. Although CLA mediates specific tethering of the T cells, the activation and subsequent diapedesis is due to specific chemokines\textsuperscript{13}. Several chemokines and their receptors are associated with skin-homing CD4\textsuperscript{+} 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\textsuperscript{14}, while CCL27/CTACK is produced by keratinocytes\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{+}CCR10\textsuperscript{+} 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/m\textsuperscript{2}, 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\textsuperscript{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, just before treatment was started. The biopsies were then frozen in TissueTek and stored at -80°C or paraffin embedded.

For chimaerasism analyses, mononuclear cells and granulocytes were isolated from the bone marrow and peripheral blood at regular intervals post-transplant. Percentages of donor-host chimaerasism 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 chimaerasism 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;
<table>
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<th>Conditioning</th>
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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% CO₂ in the presence of 5 lµ/ml Brefeldin A (Sigma-Aldrich). Cells were then aliquoted and stained with anti-CCR10 (DNAX), followed by mlgG-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; 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.
Chapter 4

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 ≤ 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.

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.
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\textsuperscript{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\textsuperscript{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\textsuperscript{+}CCR10\textsuperscript{+} T cells in GvHD patients*

To try and elucidate the possible function of the CD4\textsuperscript{+}CCR10\textsuperscript{+} 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\textsuperscript{+}CCR10\textsuperscript{+} 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\textsuperscript{+}CCR10\textsuperscript{+} 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\textsuperscript{+}CCR10\textsuperscript{-} T cells, as shown in the FACS plots of a representative patient in Figure 6C. The overall production of cytokines by CD4\textsuperscript{+} T cells (both CCR10\textsuperscript{+} and CCR10\textsuperscript{-}) 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\textsuperscript{+}CCR10\textsuperscript{-} nor CD4\textsuperscript{+}CCR10\textsuperscript{-} 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. 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. 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., 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. The expression of CCL27/CTACK has previously been reported to be increased in inflammatory conditions, such as atopic dermatitis, psoriasis and contact dermatitis. 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, 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. 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. 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. 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.

In GvHD, the presentation of alloantigens of host origin induces the activation of donor T cells and the subsequent production of cytokines. The TH1 cytokines are preferentially produced and have been implicated in the pathophysiology of acute GvHD. 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 TH1-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. 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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References


