Impaired Effector Memory T Cell Regulation Facilitates Graft versus Host Disease in CCR7 Deficient Bone Marrow Transplant Chimeras

Abstract
The development of Graft versus Host Disease (GvHD) is one of the major challenges of bone marrow transplantations. While clinical symptoms of GvHD share many features with autoimmune diseases, the underlying mechanisms remain unclear. Here we examined the effects of hematopoietic CCR7 deficiency on the development of GvHD. Lethally irradiated C57BL/6 mice were transplanted with bone marrow cells derived from wild type or CCR7−/− C57BL/6 donor mice. Unlike littermate controls, CCR7−/− chimeras develop overt symptoms of GvHD within 6 weeks after transplantation. Circulating CD4+ and CD8+ T cell populations of CCR7−/− chimeras were enriched in effector memory T cells. CCR7−/− CD62L− regulatory T cell expansion, which typically occurs after bone marrow transplantation was markedly delayed in CCR7−/− chimeras. Furthermore, GvHD did not occur after co-transplantation of WT and CCR7−/− bone marrow, showing that CCR7 is critically required for tolerance induction and prevention of GvHD. In conclusion, we are the first to demonstrate that lack of CCR7 results in delayed regulatory T cell expansion, subsequent insufficient control of effector memory T cell expansion and eventually in severe tissue damage. Conceivably therapies aimed at boosting CD4+ CD62L+ regulatory T cell expansion after bone marrow transplantation could help to control GvHD.
Chapter 10

Introduction

Graft versus Host Disease (GvHD) occurs when hematopoietic stem cells from a mismatched allogeneic bone marrow transplant cause a pathogenic immune response in recipient organs. The introduction of HLA typing and -matching has resulted in a much better prognosis for recipient patients. Nevertheless, some recipients of HLA matched bone marrow still do develop GvHD, at least partly attributable to mismatches in minor histocompatibility antigens. The latter results in a more chronic GvHD with many features of autoimmune diseases. Chronic GvHD is a late complication of allogeneic stem cell transplantation and typically manifested after more than 100 days following stem cell transplantation in humans and it is characterized by thymic atrophy and lymphocytopenia. Acute GvHD on the other hand is characterized by damage to the skin, liver and the gastrointestinal tract and usually occurs within 100 days post transplantation.

Although GvHD does originate from failing tolerance, the exact mechanisms involved in the initiation and progression of the disorder are still unclear. The retention of autoreactive T cells is currently appreciated as the main cause of GvHD initiation. During the activation phase of GvHD, activated T cells can exit lymphoid tissues and migrate, under influence of for instance chemokines, towards different target organs such as the gastrointestinal tract, liver, lung, and skin. Once migrated into the target organ effector T cells will release inflammatory cytokines and cytotoxic mediators, thereby causing severe tissue damage. As key players in immune cell surveillance and recruitment, chemokines may be relevant in the pathobiology of GvHD. Previously, CC-chemokine receptor 2 (CCR2) expression on donor- non T cell lineages was shown to have protective features in GvHD as CCR2 deficiency resulted in accelerated GvHD due to interferon (IFN) γ-mediated bone marrow aplasia. However the presence of CCR2 in this model normally results in the development of chronic GvHD. The observed switch from chronic GvHD to severe acute GvHD is hypothesized to be a direct result of impaired cross talk between the T cell and non-T cell compartment. On the other hand, CCR2 is also involved in the specific recruitment of CD8+ T cells to host organs, causing GvHD as a result of an intrinsic migratory defect in CCR2- CD8+ T cells.

Another member of the chemokine receptor family, CCR7 is one of the key receptors in T cell biology linked to homing of T cells and central and peripheral tolerance as well as recruitment of T cells into non-lymphoid tissue. CCR7 is required for migration of naive T cells to as well as from the cortex to the medulla of the thymus. Early thymocyte development occurs during the migration to the cortex, whereas positively selected thymocytes migrate from cortex to medulla. A migratory defect will thus lead to accumulation of thymocytes and impaired positive selection of thymocytes. Additionally, a defective T cell receptor (TCR)-signaling response in CCR7 knockout mice resulted in decreased negative selection and attenuated central tolerance. Recent studies suggest a CCR7-dependent immune response in tissue grafting in mice, since CCR7 deficiency of the host resulted in modestly prolonged graft survival of heterotopic heart and skin transplants. This prolonged survival was accompanied by a delay in the cellular infiltration of allografts and T cell accumulation and expansion in the draining lymph nodes in CCR7 recipients was severely impaired. Although these data may suggest the involvement of CCR7 in GvHD its role as well as its mechanisms of action in this disorder is far from clear. In this study we sought to further elucidate the role of CCR7 in GvHD by examining the effect of hematopoietic CCR7-deficiency on the development of this disorder.

Materials & Methods

Animals

CCR7−/− mice were described previously and were backcrossed at least 8 times to...
C57BL/6. C57BL/6 mice were used as controls and obtained from Taconic (Denmark). All animal work was approved by the regulatory authority of Leiden University and performed in compliance with the Dutch government guidelines and was performed at the Gorlaeus laboratories of the Leiden/Amsterdam Center for Drug Research.

**Bone Marrow Transplantation**

To induce bone marrow aplasia, male recipient C57Bl/6 were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation using an Andrex Smart 225 Röntgen source (XYLON International, Copenhagen, Denmark) with a 6-mm aluminum filter 1 day before the transplantation. Bone marrow was isolated by flushing the femurs and tibias from male CCR7⁻/⁻ or wild-type (WT) littermates. Irradiated recipients received 0.5x10⁷ WT or CCR7⁻/⁻ bone marrow cells by tail vein injection. Mice were maintained on sterilized regular chow (RM3; Special Diet Services). Drinking water was supplied with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymyxin B sulfate) and 6.5 g/L sucrose and provided *ad libitum*.

**Characterization of chronic Graft vs. Host Disease**

Animals were closely monitored for the development of GvHD. Bodyweight was registered weekly. Animals were sacrificed at 6 (early phase) and 10 weeks (late phase) after bone marrow transplantation (BMT). Blood was collected for lymphocyte isolation. Spleen, liver, thymus and lungs were isolated, weighed and fixed for histological analysis. Bone marrow was harvested for analysis of the extent of repopulation and hematopoietic chimerism.

**Assessment of Hematopoietic Chimerism**

The hematopoietic chimerism of transplanted mice was determined by polymerase chain reaction (PCR) on genomic DNA isolated from bone marrow. The forward and reverse primers 5’CGTCTACCTGTGTAAGGGCATC TTTGGCATCTA3’ and 5’GTAGAGCAGCTCCGGGATGGAGAGGAAGAG3’ for murine CCR7 and 5’GGATCTCCTGTATCTCATCCTACCTT3’ and 5’ CAAGCTCTTCAGC AATATCACG 3’ for CCR7 deficiency were used.

**In Vivo Cytotoxicity**

Spleens from WT and CCR7⁻/⁻ animals were harvested and gently squeezed through a 70 μm mesh cell strainer (Beckton Dickinson, San Diego, CA) to obtain single cell suspensions. Subsequently lymphocytes were isolated by density gradient centrifugation with Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) according to manufacturer’s protocol. WT mice were i.v. injected with 1x10⁷ WT or CCR7⁻/⁻ lymphocytes. Three weeks later freshly isolated splenocytes were fluorescently labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes Invitrogen, Breda, the Netherlands) to allow *in vivo* tracing and cytotoxicity assessment. CCR7⁻/⁻ splenocytes were labeled with a high concentration of 20 μM CFSE, while WT splenocytes were labeled with 2 μM CFSE to allow distinction of the two subsets by flow cytometry. After addition of CFSE cells were incubated for 15 minutes at 37°C under constant mild shaking. Dye uptake was quenched by adding 5 volumes of ice cold medium (RPMI, 10% FCS, L-glutamine, pen-strep) and subsequent incubation on ice for 5 minutes. Cells were pelleted and resuspended in PBS. Both cell populations were mixed at an 1:1 ratio and 1x10⁷ labeled cells were injected i.v. into C57BL/6 recipient mice. Twenty four hours and 3 days after injection blood was collected, erythrocytes were ablated and cells were analyzed by flow cytometry.

**Rescue Experiment**

Mice were transplanted with bone marrow from either WT, CCR7⁻/⁻ or a 1:1 mixture of both. To monitor GvHD development mice were weighed three times a week and the GvHD score was determined as described earlier. Four weeks after transplantation animals were sacrificed. Blood was collected for further flow cytometric analysis and
bone marrow was harvested for chimerism assessment.

**Flow Cytometry**
Leukocytes from whole blood were isolated by density gradient centrifugation with Lympholyte (Cedarlane Laboratories) according to manufacturer’s protocol. Cell suspensions from blood were incubated with 1% normal mouse serum in PBS and stained for surface markers (0.25 μg Ab/200.000 cells; anti mouse F4/80 antigen, anti mouse CD19, anti mouse CCR7, anti mouse CD62L, anti mouse CD4, (all eBioscience, San Diego, CA), anti mouse CD8 and anti mouse CD45RA, (both BD Pharmingen, San Diego, CA). When appropriate, cells were permeabilized and intracellularly stained for Foxp3, according to the manufacturer’s protocol (mouse regulatory T cell staining kit, eBioscience). Stained cells were subjected to flow cytometric analysis. All data were acquired on a FACSCalibur and were analyzed with CELLQuest software (BD Biosciences).

**Statistical Analysis**
Analysis for statistical significance was performed with a Student’s T-test for comparison of two groups, while ANOVA accompanied by a Bonferroni correction test was used for comparison of three or more groups. A *p* value of less than 0.05 was considered statistical significant. Results are expressed as mean ± SEM.

**Results**

**Generation of CCR7−/− chimeras**
CCR7−/− chimeras were generated by transplanting whole bone marrow from CCR7−/− donor mice (backcrossed > 8 times to C57Bl/6) into WT recipient mice (C57Bl/6). Controls were WT littermate receiving WT bone marrow. Bone marrow was collected from all animals (10 weeks after transplantation). Total bone marrow counts did not differ between groups (41.2 ± 8.6x10⁶ in control vs. 42.6 ± 7.6x10⁶ in CCR7−/−, *p* =0.8, Figure 1A), excluding a suboptimal/incomplete grafting and bone marrow repopulation in the CCR7−/− chimeras. Hematopoietic chimerism as determined by PCR analysis on whole bone marrow was greater than 95% for the CCR7−/− chimeras at 10 weeks after transplantation (Figure 1B).

**Induction of chronic Graft versus Host Disease in CCR7−/− chimeras**
The onset of GvHD was apparent from week 6 after BMT as animals showed clinical symptoms such as weight loss (Figure 1C) and diarrhea. Increased mortality was observed in the CCR7−/− chimeras from day 43 onward, culminating in a striking 73% mortality 10 weeks after transplantation (Figure 1D). During the experiment animals were monitored and GvHD grade was determined based on weight loss, hunching, physical activity, fur condition and the presence of opportunite skin lesions, where grade 1 represents moderate GvHD responses and grade 2 severe GvHD. Animals transplanted with WT bone marrow had an average endpoint GvHD score of 0.13, compared to 0.97 for the CCR7−/− chimeras (*p*<0.001, Figure 1E). The unexpected occurrence of a low GvHD score in WT transplanted animals is likely due to irradiation related symptoms. Animals on a C57BL6 background tend to be more sensitive to irradiation. As a result of the high dose used in our experiment the animals display some minor effects on skin condition, resulting in the minor GvHD score.

**In vivo cytotoxicity**
The CCR7−/− mice were backcrossed to the C57BL/6 background at least eight times in order to minimize GvHD induction due to mismatched strains. To absolutely exclude the possibility that the observed GvHD effects are due to immune responses directed against the host transplant as a result of (minor) mismatches, an *in vivo* cytotoxicity test
was performed. WT animals were primed with 1x10^7 WT or CCR7⁻/⁻ lymphocytes. Three weeks later CFSE labeled mixed lymphocytes were injected intravenously at a ratio of 1:1 (WT-CFSE<sup>low</sup>, CCR7⁻/⁻-CFSE<sup>high</sup>) into both WT and CCR7⁻/⁻ mice and cytotoxicity directed against the injected lymphocytes was assessed by comparing WT and CCR7⁻/⁻ fractions based on fluorescence intensity 1 day and 3 days post infusion. No cytotoxic responses against WT or CCR7⁻/⁻ cells are obvious in WT recipient mice (Figure 1F).

Figure 1: Induction of chronic Graft versus Host Disease in CCR7⁻/⁻ chimeras. A) Total number of bone marrow cells from WT controls (black) and CCR7⁻/⁻ chimeras (white). B) Chimerism assessment 10 weeks after bone marrow transplantation. C) Weight loss in CCR7⁻/⁻ chimeras (white dots) and WT littermates (black dots). D) Survival curve of CCR7⁻/⁻ chimeras (black line) and wt controls (dashed line). E) Average Graft versus Host Disease grade at endpoint in WT littermates (black) and CCR7⁻/⁻ chimeras (white). F) In Vivo cytotoxicity assay; WT animals were primed with WT or CCR7⁻/⁻ cells. Three weeks later animals were injected with CFSE labeled lymphocytes to assess cytotoxicity responses. Depicted is the ratio of WT and CCR7⁻/⁻ cells (1 and 3 days after a 1:1 ratio infusion of WT and CCR7⁻/⁻ splenocytes) for WT (black) and CCR7⁻/⁻ recipients (white), with representative dot plots for WT primed (left panel) and CCR7⁻/⁻ primed mice (right panel) 3 days after transplantation. **p<0.01.

Histological Changes
Interestingly, hematopoietic CCR7 deficiency caused a dramatic decrease in thymus size 6 weeks after transplantation (-69%, p<10⁻⁴) even when corrected for changes in body weight (-54%, p<10⁻⁴, Figure 2A,B,C). Microscopic analysis of the thymi reveals an
overall atrophy of both medulla and cortex (Figure 2D) and a general hypo cellularity (Figure 2D). Interestingly, patchy leukocytes infiltrates were present in lungs from CCR7-/- chimeras. (Figure 3A). Although the spleen size was unchanged 6 weeks after transplantation, germinal centers were almost completely absent in CCR7-/- chimeras (Figure 3B), suggestive of impaired expansion of activated B/T-cells. Moreover, liver specimens showed clear signs of injury represented by influx of inflammatory cells, massive necrosis of hepatocytes and overall “structural disintegration of the hepatic sinusoids” (Figure 3C,D).

Figure 2: Severely decreased thymus size in CCR7-/- chimeras. Thymus weight (A) as a percentage of total body weight (B) in control and CCR7-/- chimeras, 6 weeks after transplantation. Values are expressed as mean ± SEM. C) Representative pictures of the thymus. Image acquisition was performed on a Olympus S2XG microscope connected to a standard digital camera (both Olympus Nederland B.V., Zoeterwoude, the Netherlands). D) Cross sections of the thymus reveal a normal distribution of cortex and medulla (25x magnification). Image acquisition was performed on a Leica DMRE microscope equipped with a Leica DC500 digital camera (Leica Microsystems, Rijswijk, the Netherlands); *** p<10^-4.

Assessment of total blood lymphocytes:
In the early phase (6 weeks) of GvHD the number of circulating B cells was dramatically reduced (reduced by 82%, p=0.008) in CCR7-/- chimeras compared to WT controls, while the number of circulating macrophages and CD4 and CD8 T cells were unaffected (Table 1). During the late phase (10 weeks), CD8 T cells were slightly, although not significantly, induced (2.3 fold, p=0.065). No effects were observed on B cells, macrophages and CD4 T cells (Table 1).

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<td>B cells</td>
<td>7.4 ± 1.8</td>
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Table 1: Percentages of circulating Peripheral Blood Mononuclear Cells 6 and 10 weeks after transplantation. Values are expressed as mean ± SEM. **p=0.008 compared to WT.
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Figure 3: Massive tissue damage in CCR7-/- chimeras. A) Cellular influxes in lung tissue of littermate and CCR7-/- chimeras (100x magnification). B) Loss of germinal centers in spleens from CCR7-/- chimeras (25x magnification) Liver damage is represented by cellular influxes (C) and loss of hepatocytes and sinusoid deformation (D; both 200x magnification). Image acquisition was performed on a Leica DMRE microscope equipped with a Leica DC500 digital camera.

Altered percentages of circulating Memory T cell subsets in CCR7-/- chimeras
As specific memory T cell subsets, most notably effector memory T cells, have been implicated in the pathobiology of GvHD26,27, blood lymphocytes were analyzed for the presence of different memory T cell subsets. Kinetics of these memory T cell subsets displayed diverse patterns in WT control and CCR7-/- chimeras. CD8+ effector memory RA T cells (CCR7-/CD45RA+) were increased at 6 weeks, but returned to baseline levels 10 weeks after WT bone marrow transplantation when compared with non-irradiated mice (Figure 4A, black bars). CD4+ effector memory T cells (CCR7- CD62L-) showed a somewhat different pattern in the WT chimeras as levels were similar to baseline at 6 weeks, but down regulated 10 weeks after transplantation (Figure 4B, black bars). In the CCR7-/- chimeras however, CD8+ effector memory RA T cells (CCR7- /CD45RA+) were significantly reduced compared to WT chimeras but similar to non-irradiated mice during the early phase of GvHD (1.2 ± 0.17 % in WT vs. 0.48 ± 0.09 % in CCR7-/- chimeras; p=0.003, Figure 4A, white bars).This subset was significantly upregulated in CCR7-/- chimeras 10 weeks after transplantation (0.82 ± 0.22 % in WT vs. 4.2 ± 1.1 % in CCR7-/- chimeras, p=0.01 Figure 3A). Within the CD4+ memory T cell populations quite a different pattern is evident. In the early phase there is a strong induction of CD4+ effector memory T cells (CCR7+ /CD62L+) in the CCR7-/- chimeras (1.90 ± 0.24 % in WT vs. 6.76 ± 0.96 % in CCR7-/- chimeras), which is down to non-irradiated baseline levels at 10 weeks (Figure 4B, white bars). Central memory T cells are characterized by (among other markers) the expression of CCR7 and thus could not be fairly compared between WT and CCR7-/- chimeras. Although it is possible that central memory T cells lacking CCR7 are attenuated with regard to number and/or function, these cells normally only represent a small proportion (approximately 5%) of the memory T cells and are therefore not likely to affect the induction or progression of GvHD.

Accumulation of Regulatory T Cells in the circulation of CCR7-/- chimeras
The presence of regulatory T cells was initially analyzed by expression of the markers CD4, CD25 and Foxp3. Based on these markers, regulatory T cell numbers were unchanged in the CCR7-/- chimeras compared to WT controls 6 and 10 weeks after transplantation, but lower than non-irradiated mice 6 weeks after transplantation. (3.2
± 0.4 % in non irradiated mice compared to 1.9 ± 0.2 % in WT (p<0.05) and 2.1 ± 0.2 % in CCR7-/- chimeras (n.s., data not shown). However when CD62L was induced as a marker for a specific subset of regulatory T cells with specific migratory responses towards chemokines and prolonged suppression properties, significant differences were observed. At 6 weeks after transplantation the percentages of CD4+ CCR7- CD62L+ Foxp3+ cells in both WT and CCR7-/- chimeras are slightly declined compared to non-irradiated mice (0.79 ± 0.08 in non-irradiated mice compared to 0.46 ± 0.05 % in WT and to 0.26 ± 0.06 in CCR7-/-; p=0.005 and p=0.0005 respectively, Figure 3C). Interestingly, CD4+ CCR7- CD62L+ Foxp3+ percentages are further enhanced (0.31 ± 0.13 % in WT compared to 1.26 ± 0.4% in CCR7-/- chimeras; p=0.028, Figure 4C) during the late phase (10 weeks) of GvHD in the CCR7-/- chimeras. This pattern was further supported when performing a more extensive analysis of the total CD4+ T cell population, which revealed a disturbed regulatory T cell (Treg) homing or expansion in the CCR7-/- chimeras. Regulatory T cells were relatively enriched within the CD4 population in WT transplanted animals, when compared to non-irradiated WT mice as well as CCR7-/- chimeras (Figure 4D). This enrichment significantly declines over time in WT chimeras (62.8 ± 2.1% at 6 weeks compared to 23.6 ± 4.3% at 10 weeks; p=0.00001, Figure 3D), whereas the CCR7-/- chimeras display an opposite pattern (28.1 ± 2.5% at 6 weeks vs. 49.1 ± 8.1% at 10 weeks; p=0.03, Figure 4D). Taken together, these data indicate a disturbed Treg response in the CCR7-/- chimeras during disease progression. Thus suggesting a delayed Treg expansion or defective Treg homing to secondary lymphoid organs in the absence of CCR7.

**Figure 4:** Altered percentages of circulating Memory T cell subsets and accumulating Regulatory T cells in CCR7-/- chimeras. A) Distribution pattern of CD8 T effector memory RA cells in WT (black) and CCR7-/- chimeras (white) 6 and 10 weeks after transplantation. B) Percentages of CD4+ T effector memory cells in WT (black) and CCR7-/- chimeras (white) 6 and 10 weeks after transplantation. C) Partition of CD4+ CCR7- CD62L+ Foxp3+ regulatory T cells in WT (black) and CCR7-/- chimeras (white) 6 and 10 weeks after transplantation. D) Relative distribution of CCR7- CD62L+ Foxp3+ regulatory T cells within the CD4+ T cell population in WT (black) and CCR7-/- (white) 6 and 10 weeks after transplantation. The dotted grey line represents baseline levels in non-irradiated control mice. *p<0.05, **p<0.01, ***p<0.001.

**CCR7 deficiency elicited GvHD is rescued by co-transplantation of WT bone marrow**

We argued that if GvHD in the CCR7 chimeras are a direct result of CCR7 deficiency,
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Co-transfer of CCR7+ progenitor cells should be able to rescue the GvHD phenotype. Irradiated recipients were injected with a 1:1 mixture of WT and CCR7-/- bone marrow. To establish if the 1:1 ratio of WT and CCR7-/- bone marrow persisted after engraftment, bone marrow was assessed for chimerism 4 weeks after transplantation. Co-transfer of CCR7+ bone marrow cells resulted in 44.3 ± 0.10 % CCR7-/- cells and 54.0 ± 0.10 % of WT cells 4 weeks after transplantation, clearly establishing the mixed chimerism (Figure 5A/B).

![Figure 5: Transplanting WT+CCR7-/- mixed bone marrow rescues from GvHD effects seen in CCR7-/- chimeras. Rescue experiment by infusion of mixed bone marrow. A) Chimerism assessment in bone marrow 4 weeks after transplantation. Image is compiled from different parts of the same gel, including a standard curve from WT and CCR7-/- DNA dilutions and representative CCR7-/-, mixed and WT chimeras. B) Ratio of CCR7-/- bone marrow in WT (black), CCR7-/- (white) and mixed chimeras (grey). C) Bodyweight curves for WT (black dots), CCR7-/- (white dots) and 1:1 mixed infusion chimeras (grey dots). D) Average Graft versus Host Disease Grade at endpoint for WT (black), CCR7-/- chimeras (white) and mixed chimeras (grey). E) Thymus weight as a percentage of total body weight for WT (black), CCR7-/- chimeras (white) and mixed chimeras (grey), at 4 weeks after transplantation. F) Relative distribution of CCR7-/-CD62L+FoxP3+ regulatory T cells within the CD4+ T cell population in WT (black), CCR7-/- (white) and mixed chimeras (grey) 4 weeks after transplantation. The dotted grey line represents baseline levels in non-irradiated control mice. *p<0.05, **p<0.01.

Mixed transfer clearly protected animals from GvHD development (Figure 5C). Illustratively, the weight loss, seen in full CCR7-/- chimeras, was much less pronounced in
combined chimeras (Figure 5C). Furthermore the GvHD score of these mice was much lower that that of CCR7-/- transplanted chimeras (0.15 in mixed chimeras compared to 0.96 in CCR7-/- chimeras; \(p<0.001\), Figure 5D) and their thymus weight was similar to that of WT transplanted animals (Figure 5E). In agreement with the earlier observed data, the proportion of CCR7 CD62L+ Foxp3+ cells within the total CD4+ T cell population was significantly lower in CCR7-/- chimeras (63.9 ± 9.8% in WT chimeras compared to 35.5 ± 4.5% in CCR7-/- chimeras; \(p=0.01\), Figure 5F). Co-transplantation of WT bone marrow tended to partly normalize CCR7-/- CD62L+ Foxp3+ cell numbers (35.5 ± 4.5% in CCR7-/- chimeras vs. 48.6 ± 1.9% in mixed chimeras, \(p=0.08\) compared to CCR7-/- chimeras, \(p=0.16\) compared to WT, Figure 5F).

**Discussion**

In the current study we describe the role of hematopoietic CCR7 deficiency in allogeneic BMT in mice. We show that CCR7 deficiency of donor bone marrow results in the development of GvHD in the WT host as judged by the high incidence of diarrhea, thymus atrophy, loss of bodyweight, organ damage and high mortality rates in CCR7-/- chimeras. This phenotype could be largely rescued by co-transplantation with CCR7+ bone marrow. GvHD in CCR7-/- chimeras was accompanied by a delayed expansion of a particular subset of CD4+ Foxp3+ CD62L+ CCR7+ Treg cells and a concomitant early expansion of CD4+ effector memory and CD8+ central memory cells, supportive of a perturbed Treg/T effector cell (Teff) balance and failing tolerance induction in CCR7-/- chimeras during BMT.

The aforementioned phenomena in CCR7-/- chimeras, were accompanied by thymus atrophy, affecting both cortical and medullar areas, as has previously also been reported for CCR7 knock-out mice. Conceivably this is a result of defective T cell selection and/or defective migration of T cell precursors from the bone marrow to the thymus. Lymphocyte depletion (observed in spleen) and liver dysfunction, two further symptoms frequently encountered in GvHD, were also observed in the CCR7-/- chimeras. Liver histology revealed distinct signs of progressive liver disease typified by massive cellular influx, hepatocyte necrosis, and disintegration of sinusoid architecture. Taken together these data confirm that the observed pathology resembles that of GvHD.

Several subsets of memory T cells have been implicated in transplant rejection or GvHD. Yamashita and colleagues described an enrichment of donor alloreactive effector memory CD4+ T cells in transplanted patients. Moreover the observed overrepresentation of this subset appears to be a unique feature of chronic GvHD. This notion is strengthened by previous findings of D’Asaro and colleagues showing selective increase in CD8 effector memory RA+ cells in patients with chronic GvHD, while central memory T cells were reduced. In agreement with this, we observed a similar (early) induction of CD4 effector memory T cells and a (late) induction of CD8+ effector memory T cell function in CCR7 deficient chimeras.

Further analysis of the CD4+ T cell subsets revealed the involvement of CD4+ regulatory T cells in the pathogenesis of GvHD. Although the total number of CD4+CD25+FoxP3+ regulatory T cells in circulation did not differ between the groups, one specific subset of regulatory T cells, which was reported to be more responsive to chemokine driven migration and to have more persistent suppressive function, namely CD62L+ Tregs, were sharply reduced in the circulation of CCR7-/- chimeras in the early phase (6 weeks) of disease. During disease progression however, this subpopulation robustly expands (by 10 weeks) in the CCR7-/- chimeras. This delayed regulatory T cell expansion in CCR7-/- chimeras is consistent with the fact that CCR7 is required for proper migration and function of regulatory T cells. On the other hand the observed delay of this Treg subset can also be a direct result of impaired Dendritic Cell migration in CCR7-/- chimeras, resulting in inhibition of DC-T cell interaction and subsequent T cell
We therefore conclude that lack of CCR7 leads to a delayed expansion and impairment of regulatory T cells, subsequently resulting in loss of tolerance and GvHD induction.

Seemingly in contrast to our data, Sasaki and colleagues showed that blocking one of the CCR7 ligands CCL21 by treatment with CCL21-IgG fusion protein protected animals from experimental GvHD. Moreover their model of experimental GvHD differed from ours, as it was based on infusion of mismatched cells to fully competent recipients rather than to recipients with ablated hematopoiesis. Still, CCL21-IgG treatment was seen to negatively affect B cell numbers and antibody production only during very early initiation of GvHD which concurs with our findings. CCL21 antibody therapy impaired but did not prevent GvHD related mortality, compared to untreated controls. However, it should be noted that CCL21 is not the only CCR7 ligand and in fact the other known ligand, CCL19, binds with higher affinity to the receptor. Plausibly the modest effects of CCL21 inhibition on GvHD related mortality might be directly related to fully functional CCL19 mediated T cell responses. Clearly CCR7 exerts a complex role in GvHD as it may have inducing or inhibitory effects in GvHD depending on hematopoietic context, degree of mismatching and stage of disease progression.

Summarizing, the development of GvHD critically depends on a delicate spatial and temporal balance between regulation and expansion of memory T cell subsets. Although rare, genetic variants of CCR7 have been identified, and it might be interesting to investigate whether a direct correlation exists between transplant rejection and CCR7 haplotype. It is conceivable that therapeutic strategies aimed at improving CCR7 function in donor cells, or at augmenting CD4+ CD62L+ regulatory T cell function immediately after bone marrow transplantation may have potential in preventing GvHD in bone marrow recipients.
References


