4 DISCUSSION

The mechanisms underlying improved allograft function and long-term consequences due to loss of Ccr1 or Ccr5 are not well understood. Therefore, a murine renal transplantation model was utilized, which allows the study of both the acute and the clinically more important phase of chronic allograft rejection without additional immunosuppression [224]. Wildtype BALB/c (H-2^d) mice served as donors of renal allografts, which were transplanted into bilaterally nephrectomized C57BL/6 (H-2^b) mice as recipients resulting in a full MHC-mismatch and a maximal severity of transplant rejection. Besides wildtype, $Ccr1^{-/-}$ and $Ccr5^{-/-}$ recipient mice, mice lacking both chemokine receptors ($Ccr1^{-/-}$/$Ccr5^{-/-}$) were included in this study to analyze potential additive or synergistic effects due to deficiency of both Ccr1 and Ccr5. Recipients of C57BL/6 isografts were used as a control. Effects of chemokine receptor deficiency on allograft function, histopathology, graft infiltration, expression of selected marker genes and humoral rejection were analyzed. $Ccr5^{-/-}$ recipients of renal allografts demonstrated massive alternative activation of intragraft macrophages as compared to wildtype recipients.

To answer the question, whether this phenomenon is specific for renal allograft rejection or if Ccr5 deficiency has a general impact on macrophage polarization the investigation was extended to macrophage populations in the spleen of unchallenged mice and to inflammatory macrophages elicited during experimental peritonitis.

Furthermore, methods to generate bone marrow-derived macrophages (BMDM) were established, which allowed analysis of highly pure macrophage populations.

The first two sections of the discussion (4.1 and 4.2) are intended to provide an overview to the marker genes used in this study and to the current knowledge of how the function of these genes might be related to allograft rejection.

4.1 Chemokines and chemokine receptors in allograft rejection

Chemokines and chemokine receptors play important roles during allograft rejection. In the first hours after transplantation, tissue injury caused by surgical trauma and ischemia-reperfusion triggers a series of events that finally induce the recruitment of leukocytes – mostly T cells and macrophages – from the peripheral blood into the allograft [101, 225]. Stressed allograft cells generate increased amounts of oxygen and nitric oxide radicals [226] which in turn leads to the release of inflammatory mediators such as platelet-activating factor and TNF. Furthermore, adhesion molecules are induced that enhance leukocyte rolling and firm adhesion to the endothelium [101]. In the next step, chemokines secreted by activated endothelial cells or platelets bind to the surface of activated endothelia in the allograft via glucosaminoglycans (GAGs) [227]. Rolling leukocytes that encounter chemokines presented on GAGs are activated resulting in surface expression of integrins and firm adhesion. In addition, chemokines also promote the following events of spreading, diapedesis and migration into the allograft [101, 127]. Interestingly, CCL5 was found to mediate CCR1-dependent firm adhesion, whereas CCR5 seems to be important for the process of leukocyte spreading [228]. During the process of extravasation T cells and monocytes express matrix metalloproteinases (MMPs), which degrade extracellular matrix components and promote leukocyte migration through basement membranes [101]. MMPs are also able to proteolytically cleave chemokines, thereby changing their receptor specificity, affinity or activity. Consequently, MMPs might alter or regulate chemokine signaling and contribute to differential cell recruitment [21, 101, 229].

Additionally, chemokines contribute to allograft rejection by mediating traffic of dendritic cells from the allograft to secondary lymphoid organs (SLOs) [101]. The finding that mice lacking SLOs accept cardiac allografts indefinitely highlights the importance of this mechanism during allograft rejection [230]. Immature dendritic cells express inflammatory chemokine receptors such as CCR1, CCR2, CCR5 and CXCR1 which promote the immigration of dendritic cells into

inflamed tissues [231]. During the maturation process, dendritic cells loose expression of the inflammatory receptors and up-regulate chemokine receptors like CCR4, CCR7 and CXCR4 which guide mature DCs into SLOs where they activate naïve T and B lymphocytes. Interestingly, T cells show a reciprocal chemokine receptor expression pattern. Upon activation by dendritic cells expression of inflammatory chemokine receptors (CCR3, CCR5, CCR8, and CXCR3) increases in naïve T cells, whereas receptors for homing to lymphoid organs (CCR7 and CXCR4) are down-regulated [231]. Thereby, activated T cells acquire the ability to migrate into inflamed tissue and sites of allograft rejection. Besides their role in promoting allograft rejection, dendritic cells have been implicated to play an important role during tolerance induction by several processes including induction of regulatory T cells, polarization of T helper cell populations, depletion of tryptophan needed for T cell activation as well as initiation of T cell anergy and deletion [232, 233]. Furthermore, chemokines are able to selectively control leukocyte influx. While Th1 cells preferentially express the chemokine receptor CCR5 and CXCR3, Th2 polarized cells favorably expresses CCR3 and CCR4 [234]. Additionally, CCR6 expression was recently demonstrated on the newly discovered Th17 cell population and Bromley *et al.* suggested that corresponding to each of these effector Th cell subsets a subset of regulatory T cells exists with a similar pattern of chemokine receptor expression [235].

Besides T cells, chemokines influence migration of circulating monocytes to sites of inflammation. In the mouse, monocytes can be divided into two subsets according to differential expression of chemokine receptors. The first set is characterized by high expression of CX_3CR1 and low expression of CCR1, CCR2, Gr-1 and L-selectin. These are long-lived ‘resident monocytes’ which home to non-inflamed sites and serve as precursors of resident tissue macrophages. A second set of short-lived ‘inflammatory monocytes’ displays low expression of CX_3CR1 and high expression of CCR2 and Gr-1 and is actively recruited to sites of inflammation

[157, 236]. Upon maturation into tissue macrophages expression of CCR1 and CCR5 increases whereas CCR2 expression is lost [45, 157, 237].

Little is known about chemokine receptor expression on activated macrophages. A study by Martinez *et al.* demonstrated that classically (IFN-γ+LPS) activated human macrophages have increased of *CCR7* and diminished *CCR1* mRNA expression, whereas alternative activation by IL-4 led to up-regulation of *CXCR4* [151]. Another study showed that IL-4 inhibits CCR5 expression, while growth factors (M-CSF and GM-CSF) induced CCR5 expression on human macrophages [238]. The immunosuppressive cytokine IL-10 was also shown to induce expression of CCR1, CCR2 and CCR5 in human monocytes [238, 239]. Classically activated macrophages produce chemokines that bind to pro-inflammatory chemokine receptors like CCR5 and CXCR3 expressed on Th1 cells, cytotoxic T cells and NK cells. By contrast, alternatively activated macrophages induce recruitment of basophils, eosinophils, Th2 cells, regulatory T cells and B cells by secretion of chemokines that bind to CCR3, CCR4 and CCR8 [151, 155, 157].

4.2 Role of T helper cells and regulatory T cells in allograft rejection and tolerance induction

Initially, Th1 cells (which produce IL-2, IFN-γ and TNF) – were thought to be the central mediators of rejection by supporting $CD8^+$ cytotoxic T cell development, delayed-type hypersensitivity (DTH) immune responses and macrophage activation as well as promotion of IgG2a isotype switching which facilitates complement activation and antibody-dependent cellular cytotoxicity [240-242]. In turn, activated $CD8^+$ cytotoxic T cells stimulate Th1 differentiation of naïve $CD4^+$ T cells by secretion of IFN-γ and the induction of IL-12 synthesis in alloreactive DCs [243]. On the other hand, Th2-type alloimmune responses have so far been associated with induction of tolerance and prolonged allograft survival due to suppressive effects of Th2 cytokines (IL-4, IL-5 and IL-13) on Th1 polarization and development of $CD8^+$

cytotoxic T cells [240, 242]. Furthermore, maternal tolerance of fetal tissues has been correlated with the presence of Th2 cells and deletion or anergy of $CD8^+$ cytotoxic T cells [242]. However, several lines of evidence suggest that Th2 cells are also able to promote allograft rejection. Neutralization of IFN-γ or IL-12 does not prolong allograft survival and even exacerbates rejection by an increase of eosinophil infiltration which has been associated with Th2-biased immune responses [241]. Additionally, depletion of $CD8^+$ T cells led to increased Th2 cytokine synthesis, eosinophil infiltration and Th2-promoted IgG1 production [244]. Another study showed that Th2 cytokines promote deposition of collagen and fibrous material within the graft thereby contributing to chronic rejection [242]. In contrast, neutralization of IL-4 or depletion of $CCR3^+$ cells (mainly Th2 cells and eosinophils) delays allograft rejection in models of CD4-restricted alloreactivity [241]. Interestingly, IFN-γ might have different functions during allograft rejection, since this cytokine is also required for tolerance induction by regulatory T cells [245-248]. In summary, these findings suggest that Th1 and Th2 cytokines play complex roles during allograft rejection and induction of tolerance [241].

The recent identification of a new pro-inflammatory $CD4^+$ T helper cell population that is distinct from Th1 and Th2 cells might help to explain discrepancies of the Th1/Th2 paradigm in the setting of allograft rejection. This new population is defined by the selective production of IL-17. Th17 cells specifically express the transcription factor RORγτ (gene symbol Rorc) which is induced by STAT3-mediated signaling in response to IL-6, IL-21 or IL-23. Antigen-presenting cells (APCs) produce IL-6 and IL-23 upon stimulation of pattern recognition receptors (*e.g.* TLRs) by "danger" signals. These signals include exogenous ligands (*i.e.* viral or bacterial molecular patterns) as well as endogenous ligands (*e.g.* hyaluronic acid, heparin sulfate, high mobility group box 1 HMGB1, *etc.*) that are released during infections or ischemia/reperfusion injury and transplant handling. Several studies report that TLR activation induces resistance to tolerance induction [241, 249-251]. Moreover, the nature of this signal determines the balance

between IL-12 and IL-23 production by APCs and therefore controls Th1 or Th17 differentiation, respectively [241]. IL-21 is produced by Th17 cells in an autocrine fashion, induces IL-23 receptor expression on Th17 cells and inhibits IFN-γ production by Th1 cells. Additionally, Th1 and Th2 cytokines were shown to suppress Th17 functions or antagonize TGF-β induced Th17 cell differentiation suggesting complex cross-regulation between these T helper cell populations [209]. IL-17 and IL-23 were suggested to induce neutrophil recruitment to sites of inflammation. Interestingly, cardiac allografts of $Ifng^{-/-}$ recipients exhibited increased IL-17 production and neutrophil infiltration indicating that Ifng-deficiency might lift inhibition of Th17 cell differentiation [209, 252]. During allograft rejection, IL-17 antagonism was shown to prolong allograft survival in a number of studies [253-255]. IL-17 blockade seems to prevent acute rejection more efficiently than chronic rejection indicating a role for IL-17 during early rejection [208]. Antonysamy *et al.* suggested that IL-17 promotes the maturation of alloreactive dendritic cells [254].

Besides their interaction with Th1 and Th2 cells, Th17 cells also show complex interactions with regulatory T cells. Treg cells specifically express the transcription factor Foxp3 and suppress Th1 and Th2 alloimmune responses. Foxp3 induces expression of molecules with an immunosuppressive function: CD39 generates adenosine from nucleotides which exerts suppressive effects on activated effector T (Teff) cells. Furthermore, Foxp3 up-regulates the immunosuppressive cytokine IL-35 and CTLA-4 (a negative co-stimulatory molecule) and represses production of cytokines such as IL-2, IL-4, IFN-γ and TNF [241]. Naïve $CD4^+$ T cells develop into Treg cells in the presence of TGF-β. However, in the presence of TGF-β and IL-6 naïve $CD4^+$ T cells develop into Th17 cells. Therefore, IL-6 was suggested to shift Treg differentiation towards Th17 development – a finding important for strategies targeting Treg cells for the induction of transplant tolerance, since IL-6 is induced during acute phase of

inflammation by ischemia/reperfusion injury [256] and has been associated with increased IL-17 levels in lung transplantation [209].

4.3 Effects of Ccr1 and Ccr5 deficiency on tolerance induction by regulatory T cells and inhibition of DTH reactions

Russell *et al.* already described in 1978 that certain donor-recipient combinations of H-2 incompatible mouse strains exhibit extended periods of renal graft survival (>60 days) without any further immunosuppression [224, 233, 257, 258]. Due to the finding, that cardiac allografts in the same donor-recipient strain combination and transplanted in the same location as renal allografts (*i.e.* in the peritoneal cavity) were rejected within 10 days, the authors suggested that renal allografts were accepted spontaneously. However, in this study lesion scores increased from 7 to 42d post transplantation and biochemical parameters (creatinine and BUN) indicate decreasing allograft function over time in all investigated groups compared to isograft recipients. Furthermore, the abundant leukocyte infiltrate, marked glomerulosclerosis and chronic vascular rejection at 42 days after transplantation show that in our experiments renal allografts were not accepted spontaneously in any of the analyzed groups. In summary, these findings indicate ongoing allograft rejection and demonstrate the validity of this model to study renal allograft rejection.

Regulatory T (Treg) cells have been implicated to play an important role during tolerance induction [208]. These cells are able to suppress allograft rejection by activated T cells. Thus, it was tempting to speculate whether Treg cells might be affected by loss of Ccr1 and/or Ccr5. Therefore, intragraft mRNA expression of genes previously associated with Treg cells was determined [235]. Lee *et al.* demonstrated that long-term engraftment of cardiac allografts by costimulatory blockade induces up-regulation of intragraft mRNA for the Treg-specific transcription factor Foxp3 and that the chemokine receptor CCR4 is required for recruitment of

immunosuppressive Treg cells into the graft [214]. Another chemokine receptor required for the *in vivo* function of Treg cells is CCR7. This chemokine receptor has been reported to be required for the homing of Treg cells [259] as well as dendritic cells [213] to SLOs. The immunosuppressive cytokines TGF-β and IL-10 have been shown to play a role in tolerance induction by inhibition of donor-reactive DTH immune responses [258].

Whereas Tgfb1 mRNA remained constantly high throughout the period of observation, intragraft mRNAs for Il10, Foxp3, Ccr4 and Ccr7 decreased over time in all analyzed recipient groups indicating decreased immunosuppression within the allografts itself and supporting the view that allografts were not spontaneously accepted. At 42 days post transplantation Foxp3 and Ccr7 mRNA levels were markedly reduced in all chemokine receptor-deficient compared to wildtype recipients indicating that loss of Ccr1 and/or Ccr5 induces additional reduction of Treg-mediated immunosuppression. Further analyses involving SLOs (*e.g.* spleens) of renal allograft recipients are required to gain a better understanding of the consequences of Ccr1 and Ccr5 deficiency on immune responses that rely on leukocyte traffic between the site of inflammation and SLOs.

DTH reactions contribute to chronic allograft rejection by T cell-dependent activation of macrophages. Although the intragraft mRNA levels of cytokines promoting DTH reactions (Ifng, Tnf and Il2) and Th1-associated chemokines (Ccl2, Ccl3, Ccl4, Ccl5 and Cxcl10) were down-regulated in acute phase $Ccr1^{-/-}$ and $Ccr5^{-/-}$ recipients, recruitment of $F4/80^{+}$ cells (macrophages) and mRNA levels of Nos2 were not differentially regulated in $Ccr1^{-/-}$ and $Ccr5^{-/-}$ recipients compared to wildtype recipients at both time points. Interestingly, decreased numbers of glomerular ($CD4^{+}$ and $CD8^{+}$) and tubulointerstitial ($CD8^{+}$) T cells in chronic phase $Ccr5^{-/-}$ recipients was accompanied by moderately increased expression of Th2 cytokines (Il4 and Il13) and a strong polarization of macrophages towards an alternative activation phenotype (see 4.6). In summary, while ongoing DTH reactions in $Ccr1^{-/-}$ recipients cannot be ruled out

completely on the basis of the underlying results, the findings in $Ccr5^{-/-}$ recipients suggest that the effects of DTH reactions are at least diminished in this recipient group.

4.4 Ccr1 and Ccr5 play different roles during allograft rejection

Histopathologic analysis revealed improvements for vascular and glomerular lesion scores in $Ccr1^{-/-}$ and $Ccr5^{-/-}$ recipients at day 7 after transplantation compared to wildtype recipients. These improvements continued to day 42 and were complemented by reduced tubulointerstitial inflammation and less matrix deposition in both recipient genotypes at the later time point. In spite of these histopathologic similarities found in $Ccr1^{-/-}$ and $Ccr5^{-/-}$ recipients, analysis of leukocyte infiltration and intragraft mRNA expression (see 4.3, 4.5 and 4.6) demonstrated significant differences between $Ccr1^{-/-}$ and $Ccr5^{-/-}$ recipients, suggesting that Ccr5-dependent rejection mechanisms are different from those involving on Ccr1.

Analysis of leukocyte infiltration further supported this view. $Ccr1^{-/-}$ recipients demonstrated reduced acute phase infiltration in glomerular and tubulointerstitial compartments by $CD4^+$, $CD8^+$ and $CD11c^+$ cells, whereas reduced infiltration by these cells in $Ccr5^{-/-}$ recipients was mostly limited to the glomerular compartment in the chronic phase of rejection. Reduced acute tubulointerstitial infiltration by $CD4^+$ and $CD8^+$ T cells observed only in $Ccr1^{-/-}$ recipients might also explain why tubulointerstitial inflammation improved only in $Ccr1^{-/-}$ recipients.

The finding that loss of either Ccr1 or Ccr5 resulted in only moderately reduced numbers of infiltrating $F4/80^+$ cells (monocytes/macrophages) was rather unexpected, since both receptors were reported to be involved in monocyte migration into the graft [101, 108]. However, there was a considerable decrease of acute phase $CD11c^+$ cells in all three analyzed chemokine receptor-deficient recipient groups suggesting that loss of Ccr1 or Ccr5 influences infiltration and/or maturation of dendritic cells ($CD11c^+$) in the graft.

Both single-deficient recipients demonstrated diminished intragraft mRNA expression of pro-inflammatory cytokines (Ifng, Tnf and Il2) and chemokines (Ccl2, Ccl3, Ccl4, Ccl5 and Cxcl10) during acute phase, which in turn might contribute to the observed reductions in leukocyte infiltration in acute phase $Ccr1^{-/-}$ recipients and chronic phase $Ccr5^{-/-}$ recipients. Furthermore, the decreased acute phase production of cytokines and chemokines suggests less activation of infiltrating leukocytes and might therefore also contribute to improvements in acute and chronic lesion scores and matrix deposition observed in mice lacking Ccr1 or Ccr5. Interestingly, certain signature genes for specific T helper cell populations were differentially expressed in $Ccr1^{-/-}$ and $Ccr5^{-/-}$ recipients and loss of Ccr5 induced alternative macrophage activation (detailed discussion in 4.5 and 4.6).

In summary, these findings suggest that Ccr1 and Ccr5 have stage and compartment-specific functions during allograft rejection and that both receptors are involved in different types of alloimmune responses.

4.5 $Ccr1^{-/-}$ recipients show Th17-skewed alloimmune responses and improved allograft outcome

$Ccr1^{-/-}$ recipients displayed gene expression patterns characteristic for Th17-skewed immune responses. Most importantly, $Ccr1^{-/-}$ recipients demonstrated significantly increased mRNA expression of the Th17-specific transcription factor Rorc during acute phase and increased expression of Il17a mRNA during chronic phase as compared to wildtype recipients. Furthermore, decreased acute phase mRNA levels of Th1 cytokines (Ifng, Tnf and Il2) and Th1-associated chemokines (Ccl3, Ccl5 and Cxcl10) might contribute to this shift by reducing the inhibitory signal on Th17 differentiation in $Ccr1^{-/-}$ recipients [260]. In the next step, mRNA expression of two cytokines promoting Th17 development was analyzed: TGF-β and IL-6. TGF-β

promotes differentiation of Treg cells, but in the presence of TGF-β and IL-6 differentiation is shifted towards Th17 development [256, 261, 262].

No genotype-specific differences for the mRNA expression of Tgfb1 were found, but in contrast to Th1 cytokines (Ifng, Tnf and Il2) and Th1-associated chemokines (Ccl3, Ccl5 and Cxcl10) which dropped significantly in all groups between the investigated time points, mRNA levels of Tgfb1 remained consistently high from acute to chronic stage and demonstrated the highest expression levels of all analyzed cytokines during the later phase. Furthermore, Il6 mRNA levels in $Ccr1^{-/-}$ recipients were significantly reduced in acute phase and expression of Ccr6 and Ccl20 – both genes were associated with Th17 cells – showed no differential regulation in the analyzed recipient groups at both time points. However, development of Treg and Th17 cells is a mutually exclusive process. Intriguingly, $Ccr1^{-/-}$ recipients displayed decreased expression of Foxp3 – a transcription factor specific for regulatory T cells – indicating either reduced Treg recruitment or increased differentiation of Treg cells into Th17 cells in allografts of $Ccr1^{-/-}$ recipients. Interestingly, increased Rorc mRNA expression during acute phase was accompanied by significantly reduced Ccr4 mRNA levels, indicating that Treg recruitment might be inhibited in the absence of Ccr1 since Ccr4 was found to be required for Treg recruitment and tolerance induction in cardiac allografts [214].

Possibly, the sustained expression of Tgfb1 outweighs the slightly reduced amount of Il6 mRNA observed during acute phase in $Ccr1^{-/-}$ recipients. Therefore, TGF-β/IL-6 promoted Th17 differentiation might dominate in $Ccr1^{-/-}$ recipients due to reduced Th1 polarization and Treg differentiation during acute phase of rejection. Expression analysis of additional Th17-associated genes such as Il21, Il23 and their corresponding receptors, could further corroborate these findings.

Several studies in human allograft recipients pointed to a detrimental effect of Th17-polarized immune responses and the IL-17/IL-23 axis has been associated with increased neutrophil

recruitment into transplants [209, 241]. In contrast to these findings, $Ccr1^{-/-}$ recipients demonstrated improved allograft histopathology and fibrosis. Interestingly, there is striking evidence that neutrophil recruitment into inflamed tissues is dependent – at least in part – on the presence of CCR1 [44, 263-265]. Therefore, loss of Ccr1 might shift Th1 alloimmune responses towards a Th17 response phenotype that is not accompanied by neutrophil-mediated tissue damage. However, additional immunohistologic stainings of neutrophils with appropriate antibodies in sections of renal allografts are required to confirm this idea and to clarify the functional role of CCR1 in Th17 alloimmune responses. These findings also suggest a role for CCR1 in the development of autoimmune diseases, since IL-17 producing T cells have been associated with several pathologic conditions including Crohn's disease, multiple sclerosis, autoimmune diabetes and rheumatoid arthritis [241].

4.6 $Ccr5^{-/-}$ recipients show decreased Th1 responses and increased alternative macrophage activation during chronic phase of rejection

$Ccr5^{-/-}$ recipients demonstrated improved chronic phase histopathology and less graft fibrosis compared to wildtype recipients at this time point. Moreover, Ccr5 deficiency resulted in improved histology as observed by reduced numbers of T cells in the glomerular ($CD4^+$ and $CD8^+$ cells) as well as in the tubulointerstitial ($CD8^+$ cells) compartment. Similar to $Ccr1^{-/-}$ recipients, $allograft^-$ recipients lacking Ccr5 displayed reduced acute phase intragraft mRNA levels of Th1 cytokines (Ifng, Tnf and Il2) and Th1-associated chemokines (Ccl3, Ccl5 and Cxcl10) as compared to wildtype recipients. Additionally, the mRNA levels of these Th1 marker genes and the Th1-specific transcription factor Tbx21 decreased significantly between acute and chronic phases, so that no significant differences remained between wildtype and $Ccr5^{-/-}$ at 42 days post transplantation. Interestingly, the immunosuppressive cytokine Il10 also decreased from acute to chronic phase in Ccr5-deficient recipients. Nevertheless, this finding is in full

agreement with recently published data showing that Th1 cells produce IL-10 under certain conditions to limit immune responses and prevent damage to the host [266].

Taken together, these observations suggest that the beneficial effect of Ccr5 deficiency on renal allograft rejection might be due to decreased Th1 immune responses including acute Th1 cytokine production and chronic infiltration by $CD4^+$ Th1 cells as well as cytotoxic $CD8^+$ T cells. These observations therefore suggest, that Th1-driven DTH reactions and cytotoxic T cell-mediated tissue injury are decreased by loss of Ccr5.

On the one hand, immunosuppression by Treg cells appears to be an unlikely explanation for the observed improvements, since Foxp3 mRNA levels decreased considerable over time in wildtype and $Ccr5^{-/-}$ recipients and were even significantly lower in chronic phase allografts from Ccr5-deficient recipients. Furthermore, Ccr7 mRNA was significantly down-regulated in $Ccr5^{-/-}$ recipients at 42 days post transplantation as compared to wildtype recipients. Schneider *et al.* reported that CCR7 is required for the *in vivo* function of Treg cells [259]. Therefore, this finding further supports the view, that improvements in Ccr5-deficient recipients are not mediated by Treg cells.

Alternatively, a shift in the immune response from a Th1-type to a Th2-type could potentially contribute to the improved allograft outcome observed in $Ccr5^{-/-}$ recipients. Expression of CCR5 on $CD4^+$ T lymphocytes has been associated with a Th1-type immune response [61]. Subsequently it was reported that Ccr5 deficiency results in a shift from a Th1- towards a Th2-type response in mice with dextran sodium sulfate-induced colitis [267]. In a murine islet transplantation model Abdi *et al.* demonstrated, that Ccr5 deficiency skews intragraft alloimmune responses towards the Th2-type [117]. They observed increased Il4 and Il5 as well as decreased Ifng mRNA levels in $Ccr5^{-/-}$ as compared to wildtype recipients. Interestingly, this Th2-shift was not only found within the allograft, but also in the periphery, when the response of splenocytes to donor cells was investigated.

The experiments of this these show that intragraft mRNA levels of Th1-associated marker genes such as Ifng and Tbx21 decreased over time, while expression of Th2-associated cytokines Il4, Il13 and the Th2-specific transcription factor Gata3 increased over time in all analyzed recipient groups. However, mRNA expression of Th2-associated cytokines Il4 and Il13 genes tended to be stronger in allografts from $Ccr5^{-/-}$ recipients. Hence, diminished Th1-driven DTH reactions in parallel with increased Th2 cytokine expression in $Ccr5^{-/-}$ allograft recipients, might generate a microenvironment that affects the polarization of intragraft macrophages and induces the alternative activation pathway in macrophages [160].

Alternatively activated macrophages (AAMs) characteristically show a strong expression of the signature gene Arg1 (arginase 1). Therefore, Arg1 mRNA expression was analyzed in renal allografts. Interestingly, Arg1 was only increased at 42d and only in Ccr5-deficient recipients. The notion that Ccr5 deficiency reprograms macrophages to the AAM phenotype was confirmed by analysis of intragraft expression of additional AAM marker genes including Chi3l3 (chitinase 3-like 3, also called Ym1), Retnla (resistin like alpha, also known as Fizz1 (Found in inflammatory zone 1)) and Mmp12 (matrix metallopeptidase 12) [160, 215, 268]. All three AAM marker genes showed a marked up-regulation in $Ccr5^{-/-}$ recipients at 42d. In addition, determination of the numbers of $Arg1^{+}$, $Mrc1^{+}$ and $Chi313^{+}$ cells within grafts during the chronic phase by immunohistological staining corroborated the findings on mRNA level.

Besides the Th2 cytokines IL-4 and IL-13, the immunosuppressive cytokines IL-10 and TGF-β have been implicated to play a role during alternative macrophage activation [155]. The mRNAs for Il10 and Tgfb1 were highly abundant in chronic allografts of all recipient groups, but AAMs were only observed in $Ccr5^{-/-}$ recipients, suggesting that signaling through CCR5 might inhibit alternative macrophage activation.

The data presented in this thesis provide a possible explanation for the mechanism underlying the beneficial effect of Ccr5 deficiency, which goes beyond a recruitment defect for

inflammatory cells. In the past most investigations have concentrated on quantitative effects that chemokine receptors have on the recruitment of specific leukocyte subpopulations. Qualitative phenotypic changes in leukocytes induced by loss of a specific chemokine receptor have been investigated much less. The number of infiltrating cells can be the same and still a profound effect may be observed when the phenotype of the infiltrating leukocytes has changed. The number of $F4/80^{+}$ cells (mostly macrophages) in the glomerular and the tubulointerstitial compartment did not differ significantly between grafts from wildtype and $Ccr5^{-/-}$ recipients at both time points. Interestingly, Abdi *et al.* made a similar observation in a murine islet transplantation model [117]. While immune responses where shifted towards Th2, numbers of infiltrating mononuclear cells ($CD4^{+}$ and $F4/80^{+}$ cells) remained unchanged between wildtype and $Ccr5^{-/-}$ recipients. Therefore, the authors concluded that immunosuppression due to loss of Ccr5 is not necessarily the result of altered leukocyte infiltration [117]. However, when analyzing numbers of intragraft macrophages one has to take into account that effects due to the presence of donor macrophages and local proliferation might obscure the true number of infiltrating recipient macrophages [269, 270]. In this context it would be interesting to determine intragraft levels of Csf1 (macrophage colony-stimulating factor) mRNA to get a first impression whether macrophage proliferation could be relevant in this model [271, 272].

However, the macrophages found in grafts from $Ccr5^{-/-}$ recipients at 42 days had been reprogrammed to AAMs, which clearly have a different phenotype than the macrophages found in grafts from wildtype recipients. In summary, these data indicate that loss of Ccr5 in the recipient is responsible for the accumulation of AAM and a Th2-type immune response during the chronic phase of transplantation, thereby favoring “repair” rather than progressive inflammation and destruction.

Eosinophil infiltrates have been associated with Th2-polarized alloimmune responses directed against skin grafts and antibody blockade of CCR3 was shown to inhibit graft infiltration by

eosinophils [273-275]. Therefore, intragraft Ccr3 mRNA expression was analyzed, but only very low levels of Ccr3 mRNA near or below detection limit were observed which did not allow accurate analysis (data not shown). Therefore it is conceivable, that eosinophil infiltration does not play a significant role in this experimental model. However, the possibility that small numbers of highly potent eosinophils might affect allograft rejection cannot be ruled out on the basis of these findings. Additional analyses of eosinophil-associated markers (*e.g.* IL-5, major basic protein (MBP) and eosinophil cationic protein (EBP) [276]) and Giemsa-stained sections are required to obtain an answer this question.

In 2004, our group demonstrated that Ccr5 has a role in carotid artery allograft rejection [277]. Ccr5-deficient recipients showed a significantly reduced infiltration with $CD3^+$ T cells and a marked reduction in neointima formation. Although transplant-associated arteriosclerosis represents a special case of vasculopathy caused by an activation of the immune system, these findings are compatible with the hypothesis that Ccr5 might also be involved in the pathogenesis of “classical” atherosclerosis. This disease is nowadays considered to represent a chronic inflammatory Th1-cell driven disease with macrophages playing a key role in the disease process [278]. In cooperation with the groups of François Mach from Geneva and Christian Weber from Aachen our group studied the role of Ccr5 in a high-fat diet induced atherosclerosis model. It was demonstrated, that deletion of Ccr5 in Apoe-deficient mice protected the animals from diet-induced atherosclerosis [279]. This phenotype was associated with decreased atherosclerotic lesion extent, reduced mononuclear cell infiltration and attenuated Th1 immune responses. Thus, the beneficial effect of Ccr5 deficiency on atherosclerosis might be caused by the presence of alternatively activated macrophages in the atherosclerotic lesions.

4.7 $Ccr5^{-/-}$ recipients do not show increased humoral rejection

Several transplantation studies in $Ccr5^{-/-}$ recipient mice including heart [111, 112], islet [116, 117] and carotid artery [112] allografts, demonstrated a beneficial effect of Ccr5 deficiency on allograft rejection leading to prolonged survival times. In contrast, two recent studies by Fairchild and colleagues using $Ccr5^{-/-}$ mice as recipients of cardiac [113] or renal [114] allografts showed, that loss of Ccr5 does not prolong cardiac allograft survival and results in accelerated rejection of renal allografts. In both studies, the authors explained these results by increased acute humoral rejection occurring in $Ccr5^{-/-}$ mice. This explanation was supported by increased deposition of the complement split product C3d and markedly elevated alloreactive antibody serum titers in $Ccr5^{-/-}$ recipients [113, 114]. In the renal transplantation experiments described in this thesis a comparable model of murine renal allograft rejection was performed, which resulted in long-term graft survival, though with evidence of chronic graft nephropathy. This was demonstrated by moderately increased serum creatinine and BUN levels as well as histopathologic changes consistent with mild to moderate chronic rejection. Furthermore, antibody levels did not increase in our fully backcrossed $Ccr5^{-/-}$ recipient mice and histopathologic analysis revealed significant improvements compared to wildtype recipients. A possible explanation for the differences obtained between our renal allograft model and the transplantation studies by Fairchild *et al.* could be the use of different donor-recipient strain combinations. In this thesis BALB/c ($H\text{-}2^{d}$) mice were utilized as donors of renal allografts, while Fairchild *et al.* used A/J mice ($H\text{-}2^{a}$) as donors of cardiac and renal allografts. Furthermore, different Ccr5-deficient recipient mouse lines were used ($Ccr5^{tm1Blck}$ (our group) *vs.* $Ccr5^{tm1Kuz}$ (Fairchild)) and our mice had acquired a true congenic status. For both studies, Fairchild *et al.* purchased Ccr5-deficient mice from The Jackson Laboratory. Fully backcrossed (N10) Ccr5-deficient mice in the C57BL/6 background are available from The Jackson Laboratory only since November 2005 (personal communication, Technical Service, The Jackson Laboratory). Thus, at

least the mice used for the cardiac transplantation study (received for publication in March 2004 [113]) were not appropriately backcrossed and therefore on a mixed genetic background at the time of the experiment. The backcrossing status of the mice used in the subsequent renal allograft study is not mentioned in their publication [114].

Another explanation for the differences between our study and the results of Fairchild *et al.* may be, that donor-reactive antibody levels are highly variable over time showing significant fluctuations above and below detection limits [280]. Cornell *et al.* suggested that this phenomenon is the result of a cycle of complement- and antibody-mediated injury, endothelial activation and repair [280].

4.8 $Ccr1^{-/-}/Ccr5^{-/-}$ recipients resemble wildtype recipients in certain aspects

Targeted deletion of a single inflammatory chemokine or chemokine receptor gene in mice often results in a mildly developed or no apparent phenotype. This phenomenon has been attributed to redundancy and promiscuity of the chemokine network [101, 281]. A single chemokine may bind to different receptors and a single chemokine receptor can bind different chemokines (see **Figure 1**). For instance, CCL3 and CCL5 bind to CCR1 and CCR5. As a result, it is thought that loss of one chemokine receptor can be compensated for by another receptor [282, 283], explaining the mild phenotypes often found in knockout mice. On the other hand, an agonist for one receptor might exhibit an antagonistic behavior on another receptor [19, 20]. These complex interactions confer a high degree of flexibility to the chemokine network and allow the fine-tuning of immune responses.

Previous studies showed that deficiency of the chemokine receptor Ccr1 or Ccr5 has beneficial effects on survival of cardiac, carotid, corneal and islet allograft in mice. Furthermore, it is known that both chemokine receptors are co-regulated, share ligands and are frequently expressed by the same cell types. These findings indicate potential redundant functions of CCR1

and CCR5 and suggest that loss of both receptors might be accompanied by additional or synergistic improvements in allograft recipients. Moreover, Ccr1/Ccr5 double-deficient mice serve as an important tool to answer the question whether redundant ligand-binding specificities observed *in vitro* are also present *in vivo*. Finally, small molecule antagonists used to block chemokine receptor function have come into the focus of the pharmaceutical industry to treat diseases that are currently beyond remedy. However, the potential redundancy of the chemokine system challenges the validity of chemokine receptors as therapeutic targets [284]. These important questions prompted us to generate Ccr1/Ccr5 double-deficient mice. To our best knowledge, these mice are only available in our laboratory to date. $Ccr1^{-/-}/Ccr5^{-/-}$ recipients were included in this study to answer the question whether redundancy exists between these receptors which might result in additive or synergistic effects in double-deficient recipients.

$Ccr1^{-/-}$ and $Ccr5^{-/-}$ single-deficient transplant recipients demonstrated significantly decreased allograft rejection compared to wildtype recipients during acute and chronic phase of rejection. Although $Ccr1^{-/-}/Ccr5^{-/-}$ double-deficient recipients showed improved graft histology and less leukocyte infiltration at both time points compared to wildtype recipients, additional improvements due to Ccr1/Ccr5 double deficiency were surprisingly limited to certain parameters during the chronic phase of allograft rejection. These effects were striking and nearly reached isograft levels, but were only observed in vascular (chronic vascular damage) and glomerular (transplant glomerulopathy) compartments. Double deficiency resulted in significantly reduced plasma urea levels and less collagen deposition – effects that were less pronounced in single-deficient recipients. Other improvements in double-deficient recipients were substantial, but did not excel the degree observed in single-deficient hosts. These improvements included acute vascular rejection and glomerular damage, chronic tubulointerstitial inflammation and actin (α-SMA) deposition.

Furthermore, numbers of infiltrating $CD8^+$, $CD11c^+$ and $F4/80^+$ cells were considerably diminished by loss of both chemokine receptors, but additional effects remained limited to chronic $CD11c^+$ cells in the tubulointerstitium and were only moderately pronounced. F4/80 and CD11c are markers typically expressed by cells of monocytic origin. Monocytes were shown to require the chemokine receptor CCR2 for emigration from the bone marrow [202]. Remarkably, intragraft Ccr2 mRNA levels were significantly reduced in acute phase double-deficient recipients, whereas levels of Ccl2 mRNA (encoding an important ligand of CCR2) were unchanged between wildtype and $Ccr1^{-/-}/Ccr5^{-/-}$ recipients. Thus, reduced infiltration by monocytic cells in allografts from double-deficient recipients might result in the decreased numbers of macrophages/dendritic cells observed in this study. CCR1 as well as CCR5 are known to play important roles during monocyte diapedesis. Weber and colleagues demonstrated that CCR1 is predominantly required for firm adhesion of monocytes and Th1-like T cells, while CCR5 mainly contributes to spreading in shear flow. Additionally, the authors showed that both receptors mediate transendothelial chemotaxis towards CCL5 [228]. However, reduced Ccr2 expression in monocytic bone marrow precursor cells from $Ccr1^{-/-}/Ccr5^{-/-}$ mice might also contribute to the observed reductions of graft infiltrating $F4/80^+$ and $CD11c^+$ cells – a possibility that requires further examination. Nevertheless, additional effects on chronic infiltration by $CD11^+$ cells were only marginal developed in double-deficient recipients and no additional effect on acute phase infiltration by $F4/80^+$ cells was observed. In summary, these data indicate that CCR1 and CCR5 may not share redundant functions during renal allograft rejection.

Interestingly, numbers of $CD4^+$ T cells were not affected by Ccr1/Ccr5 double deficiency in both renal compartments during acute and chronic phase, contrasting the findings from single-deficient recipients where loss of Ccr1 reduced the numbers of $CD4^+$ T cells during acute phase, while Ccr5 deficiency diminished chronic phase numbers of $CD4^+$ T cells. However, these results

correlate well with the observation that acute phase mRNA levels of Th1 cytokines (Ifng, Tnf and Il2) remained unchanged in double-deficient recipients compared to wildtype recipients. Thus, Th1 cytokine production by graft infiltrating $CD4^+$ T cells might not be affected in double-deficient recipients, while single deficiency had a marked impact on these parameters.

Acute phase Ccr1 and Ccr5 single-deficient recipients demonstrated reduced Th1 cytokine (Ifng, Tnf and Il2) mRNA expression that was paralleled by diminished mRNA levels of pro-inflammatory chemokines Ccl3, Ccl4 and Ccl5 compared to wildtype recipients. Remarkably, mRNA expression of these genes was restored to wildtype levels in acute phase $Ccr1^{-/-}/Ccr5^{-/-}$ recipients. Therefore, loss of both receptors might be compensated for by increased cytokine and chemokine production, in turn leading to increased leukocyte activation and/or infiltration compared to single-deficient recipients. On the other hand, double-deficient recipients showed ambiguous gene expression patterns in chronic phase of rejection: Th1-associated genes Tnf, Ccl3 and Cxcl10 showed significant up-regulation and the Th2-specific transcription factor Gata3 was also increased considerably. Therefore, it appears as if different immune response types reached a stalemate at this time point in double-deficient recipients. These findings could explain why additional effects due to double deficiency remained limited to certain parameters in chronic phase of rejection. Furthermore, a chemokine might function as an agonist for one receptor, while exhibiting antagonistic behavior on another receptor [19, 20]. Thus, it is conceivable that the observed restoration of wildtype chemokine levels (Ccl3-5) in double-deficient recipients induces contrary effects on immune responses mediated by chemokine receptors which share ligand binding specificity with CCR1 and CCR5 [9].

The observed phenomenon of restored cytokine and chemokine production might also be relevant for pharmaceutical approaches targeting chemokine receptors with small molecule antagonists directed against more than one receptor [53]. Use of such antagonists might affect

opposing immune responses. In this thesis, loss of Ccr1 induced a Th17-polarized immune response, while Ccr5 deficiency led to Th2-shifted immune responses and accumulation of AAM in the allograft. Therefore, a combined blockade of both receptors by antagonistic drugs might induce a phenotype similar to the observed phenotype of Ccr1/Ccr5 double-deficient recipients resembling wildtype recipients in many aspects.

In conclusion, no indication was found for redundant functions of CCR1 and CCR5 during renal allograft rejection. Moreover, effects of the different immune responses, which induced improved allograft outcome in single-deficient recipients, appear to neutralize each other in double-deficient recipients and might hinder the development of additive or synergistic effects in Ccr1/Ccr5 double-deficient recipients.

Although loss of Ccr1 or Ccr5 had beneficial effects on allograft rejection, different studies showed that loss of Ccr1 or Ccr5 is not necessarily accompanied by beneficial effects on disease outcome. Zernecke *et al.* showed that Ccr5 but not Ccr1 deficiency reduced neointima formation upon wire injury in atherosclerosis-prone mice [58]. In cooperation with the groups of François Mach from Geneva and Christian Weber from Aachen our group analyzed the effect of Ccr1 and Ccr5 deficiency in a high-fat diet induced atherosclerosis model [279]. Ccr5 deficiency was demonstrated to reduce diet-induced atherosclerosis, while loss of Ccr1 induced an opposing phenotype with increased plaque size and T cell infiltration. Moreover, our own unpublished results show that loss of Ccr1 aggravates lupus nephritis in MRL/lpr mice, while Ccr5 deficiency had a beneficial effect on the disease course compared to wildtype mice. On the other hand, Ccr1-deficient mice exhibited reduced numbers of infiltrating leukocytes and less fibrosis in a unilateral ureter obstruction model, while loss of Ccr5 had no effect on these parameters compared to wildtype mice [263]. Therefore, it appears that the effects due to the loss of one of these receptors are strongly dependent on the disease model analyzed. Frequently, loss of Ccr1 or Ccr5 induced contrary effects on disease outcome. Such contrary

effects might also explain why additional effects due to double deficiency remained limited and why Ccr1/Ccr5 double-deficient recipients resembled wildtype recipients in several aspects in the model of renal allograft rejection analyzed in this thesis.

4.9 Effects of Ccr5 deficiency on macrophage polarization are not limited to renal allograft rejection

The massive accumulation of AAM in grafts from $Ccr5^{-/-}$ recipients during the chronic phase of rejection raised the question, whether this change in macrophage phenotype was restricted to this particular disease model or whether Ccr5 deficiency has a general effect on macrophage polarization. In a first attempt to obtain an answer to this important question, macrophage polarization was analyzed in spleens and cultivated splenocytes from unchallenged wildtype and $Ccr5^{-/-}$ mice.

The increased expression of the AAM signature genes observed in spleens (Arg1, Mmp12, Mrc1 and Mrc2) and cultivated splenocytes (Arg1, Chi3l3, Msr1 and Retnla) from unchallenged $Ccr5^{-/-}$ mice suggested that Ccr5 deficiency indeed reprograms macrophages to an alternatively activated, anti-inflammatory phenotype already under basal conditions. These results are corroborated on protein level by increased frequencies of $CD206^{+}$ cells in $Ccr5^{-/-}$ splenocytes. However, additional experiments are required to identify the $CD206^{+}$ splenic macrophage subpopulation, since $CD206^{+}$ cells did not show surface expression of the common monocyte/macrophage marker CD11b. Interestingly, expression of CD11b (gene symbol Itgam) was up-regulated on mRNA level and frequencies of $CD11b^{+}$ cells were increased in cultivated $Ccr5^{-/-}$ splenocytes compared to wildtype splenocytes. Lloyd *et al.* suggested that $CD11b^{+}$ cells in spleens of unchallenged mice represent either neutrophils, monocytes or cells of monocytic origin [285]. Furthermore, it is conceivable that expression of the phagocytic mannose receptor C type 1 (CD206) occurs upon maturation of monocytes to tissue macrophages, explaining why

$CD206^+$ cells did not co-express CD11b. To date, at least four populations of splenic macrophages have been described based on different localization, function and expression of surface markers: white pulp macrophages (MOMA-2^+), red pulp macrophages ($F4/80^+$), marginal zone macrophages (SIGN-$R1^+$) and marginal metallophilic macrophages (MOMA-1^+) [286]. These markers could be used in additional experiments to identify the $CD206^+$ splenic macrophage population. Such an analysis might also provide new insights into the functional role of CCR5 in splenic macrophages.

Several subgroups have been defined for alternatively activated macrophages depending on the nature of the activating stimuli. While IL-4-activated AAM show a wound-healing phenotype, stimulation with various stimuli including IL-10, glucocorticoids or apoptotic cells, induces AAM with a regulatory phenotype. These so-called regulatory macrophages produce high levels of IL-10 and TGF-β and suppress immune functions [2, 155, 287]. Since IL-10 and TGF-β were shown to induce expression of CCR5 on human monocytes [67, 239] and human blood-derived DCs [68], CCR5 might as well have a function in regulatory macrophages.

Indeed, increased AAM marker expression (Arg1, Mmp12, Mrc1 and Mrc2) in spleens of $Ccr5^{-/-}$ mice was accompanied by augmented mRNA levels of Il10 and Tgfb1 supporting the view that macrophages in the spleen of $Ccr5^{-/-}$ mice might be shifted towards a regulatory phenotype compared to wildtype macrophages.

In addition to these findings in unchallenged mice, the effect of Ccr5 deficiency on cultivated macrophages obtained by thioglycollate-induced peritonitis was also analyzed. In this model, dramatically increased mRNA expression of Arg1 (~65x) was observed in untreated peritoneal macrophages from $Ccr5^{-/-}$ mice compared to wildtype mice. Two other AAM signature genes Chi3l3 and Retnla were not differentially regulated in untreated and IL-4-stimulated peritoneal macrophages while differences for Arg1 mRNA expression remained upon IL-4 stimulation. These results also contribute to the hypothesis that loss of Ccr5 induces an alternative

activation phenotype in macrophages. Several reasons might explain why the effect of Ccr5 deficiency was limited to Arg1 in this model. First, alternatively activated macrophages represent a highly heterogeneous cell population with different marker expression characteristics [163]. While IL-4-induced AAM express high amounts of Chi3l3 and Retnla mRNAs, AAMs activated by IL-10 did not express Chi3l3 or Retnla mRNA [288, 289]. Since the peritoneal macrophages used in the experiment shown in this thesis were harvested 4 days after thioglycollate-injection these cells might possibly be derived from a resolution phase micromillieu resembling more closely the phenotype of regulatory macrophages than the typical IL-4 induced alternatively activated macrophages.

Second, experiments performed in this study (see **Figure 29** and 4.10) showed that the time course of mRNA expression varies between different AAM marker genes - a result which is confirmed by the findings of Loke *et al.* [290]. Furthermore, it is unclear whether loss of Ccr5 delays or accelerates induction of these markers. Third, complex cytokine production by elicited peritoneal macrophages might also contribute to the modulation of AAM marker expression and result in altered macrophage activation compared to the situation found in renal allografts or cultivated splenocytes. Additional analyses on protein and functional level are required to confirm the findings from peritoneal macrophages and spleen cells. The established methods to determine arginase enzyme activity and measure NO production will be very useful to investigate differences on a functional level. Determining AAM marker protein expression by ELISA (cytokine expression in supernatants), Western blot and flow cytometric approaches offer additional methods to study the effect of Ccr5 deficiency on macrophage polarization.

In summary, the observations made in renal allografts, in spleens of unchallenged mice, in cultivated splenocytes and peritoneal macrophages reveal a novel phenotype in $Ccr5^{-/-}$ mice favoring alternative activation of macrophages. This finding may account for the improved transplant survival described in Ccr5-deficient mice [111, 112, 116, 117], in primates treated

with CCR5 antagonists [291], and in humans homozygous for the *CCR5Δ32* allele [8]. Furthermore, this observation may prove to be of considerable significance for the progression of chronic inflammatory and fibrosing disease processes.

4.10 Analysis of macrophage polarization in BMDM generated in Petri dishes

Due to the limitations and difficulties experienced with macrophages obtained by experimental peritonitis a method to generate pure macrophage populations was established. In a first approach, macrophages were generated by cultivation of bone marrow cells in Petri dishes using medium containing murine recombinant M-CSF. This method yielded 10-15x10^6 bone marrow-derived macrophages (BMDM) from a single mouse and ≥90% of these cells stained positive for the macrophage marker F4/80. Under basal conditions BMDM from C57BL/6 mice expressed high amounts of mRNAs typically expressed by macrophages (Csf1r and Mmp12) but did not display any genotype-specific differences for macrophage activation markers. Additionally, Nos2 and Ifng mRNA levels were below the detection limit (data not shown) in wildtype and $Ccr5^{-/-}$ BMDM. These results and the dramatic increase of AAM marker gene expression upon IL-4 stimulation suggest that wildtype and $Ccr5^{-/-}$ BMDM were unbiased regarding their phenotypic polarization under basal conditions. However, it is conceivable that $Ccr5^{-/-}$ BMDM show an altered time course of AAM marker expression compared to wildtype BMDM – a possibility which has not been analyzed in this study. The slightly decreased mRNA expression levels of Chi3l3 and Retnla in $Ccr5^{-/-}$ BMDM (C57BL/6) at 48h after IL-4 stimulation do therefore not exclude the possibility that $Ccr5^{-/-}$ BMDM show increased AAM marker expression at an earlier time point but might indicate an accelerated expression of Chi3l3 and Retnla in $Ccr5^{-/-}$ compared to wildtype BMDM. On a functional level, $Ccr5^{-/-}$ BMDM displayed significantly increased arginase enzyme activity compared to wildtype BMDM under unstimulated conditions. However, the increase under basal conditions was only moderate

(~1.27x) and disappeared upon IL-4 stimulation. On the other hand, the amount of NO produced under unstimulated or IL-4-stimulated conditions remained unchanged in wildtype and $Ccr5^{-/-}$ BMDM.

BMDM from wildtype and $Ccr5^{-/-}$ mice were pre-stimulated to analyze whether an activation/priming step might be necessary to obtain differential macrophage polarization. Interestingly, only pre-stimulation with LPS followed by IL-4 stimulation induced a significant difference in Arg1 mRNA expression while expression of other AAM marker genes (Chi3l3 and Retnla) remained unchanged.

It is known for years, that different mouse strains exhibit different or even opposite immunological responses to the same pathogen. For example upon infection with the intracellular pathogen *Leishmania major* C57BL/6 mice show Th1 responses whereas BALB/c mice show Th2 reactions. In case of *Leishmania* infection the Th1 response results in a resistant phenotype whereas BALB/c mice are rendered susceptible to this intracellular pathogen by induction of a Th2 response [292]. Interestingly, these strain differences occur already without priming through infection. Mills *et al.* found that macrophages from C57BL/6 and BALB/c strains differ in their capability to produce nitrite and ornithine in response to IFN-γ, LPS or both. While macrophages from Th1 responders (C57BL/6) predominantly produced nitrite and citrulline (products of classically activated macrophages [7]), Th2 macrophages (BALB/c) preferentially generated ornithine – an amino acid precursor that is predominantly produced by alternatively activated macrophages [7, 293]. In this thesis, a shift in macrophage polarization towards AAM was observed in renal BALB/c allografts transplanted in $Ccr5^{-/-}$ C57BL/6 recipients. Wyburn *et al.* showed that macrophages in the renal allograft are a mixed population consisting of infiltrating recipient macrophages and donor tissue macrophages which proliferate in response to inflammatory stimuli induced by ischemia/reperfusion injury of the transplanted kidney [270]. Therefore it is conceivable, that BALB/c donor macrophages are present at least during the

acute phase of rejection and might influence allograft rejection profoundly. Accordingly, the impact of Ccr5 deficiency on macrophage polarization in BMDM from BALB/c mice was analyzed. Upon stimulation with increasing amounts of IL-4 the dose-dependent increase of AAM marker gene expression (Arg1 and Chi3l3) was more pronounced in $Ccr5^{-/-}$ than in wildtype BMDM. In parallel, increased arginase enzyme activity and reduced NO production were observed in $Ccr5^{-/-}$ BMDM. In summary, while C57BL/6 $Ccr5^{-/-}$ BMDM did not show relevant differences under unstimulated and IL-4-stimulated conditions, BALB/c $Ccr5^{-/-}$ BMDM displayed increased alternative activation upon IL-4 stimulation compared to BALB/c wildtype BMDM. However, pre-stimulation by LPS induced a slight increase of AAM marker expression in $Ccr5^{-/-}$ compared to wildtype BMDM from C57BL/6 mice.

In conclusion, the Th2-polarized micromillieu of BALB/c allografts potentially supports alternative activation of C57BL/6 $Ccr5^{-/-}$ recipient macrophages and suggests complex strain dependent influences on macrophage polarization. Additionally, classical activation stimuli might induce AAMs in the absence of Ccr5 in C57BL/6 mice.

4.11 Analysis of macrophage polarization in BMDM generated in Teflon bags

BMDM generated in Teflon bags provide several advantages compared to conventional BMDM generated in Petri dishes. While the use of Petri dishes allowed the generation of $10\text{-}15x10^6$ BMDM/mouse, the Teflon bag method increased the amount of BMDM obtainable from a single mouse to $80\text{-}100x10^6$. Furthermore, use of proteases or scraping is not required to detach BMDM from the hydrophobic Teflon surface thereby reducing stress and damage to cells during harvesting. Additionally, generation of BMDM in Teflon bags is less time consuming and laborious, since it is not necessary to change medium during the differentiation period.

Morphologic and flow cytometric analysis revealed that the cell population generated by the Teflon bag culture method consists of highly pure macrophages. Over 98% of these cells

expressed the macrophage marker proteins CD11b and F4/80, while expression of granulocyte (Gr-1), T cell (CD3ε) and NK cell (NK1.1) markers was not detectable. BMDM from Teflon bags also showed weak expression of DC (CD11c) and B cell (CD19) marker proteins. Interestingly, CD11c as well as CD19 are subunits of the complement receptor complexes CR4 and CR2, respectively, which recognize cleavage products of C3b [1]. The presence of CD11c and CD19 on BMDM is in accordance with recent findings showing that both markers are also expressed by macrophage subsets. On the one hand, evidence is accumulating that CD11c – previously considered to be a specific marker of dendritic cells – is also expressed on macrophage subsets [285, 294] and the concept of dendritic cells and macrophages representing different cell types is being challenged [144]. On the other hand, a particular subset of $CD19^{+}$ myeloid progenitor cells ($CD45R^{-}CD19^{+}$) was demonstrated to generate either B cells or macrophages in mice [295]. The interesting observation that CD11c and CD19 were differentially expressed in BMDM from wildtype and $Ccr5^{-/-}$ mice requires additional experiments due to the low number of animals analyzed.

Next, the impact of Ccr5 deficiency on macrophage polarization was analyzed by stimulation of BMDM from Teflon bags with IFN-γ+LPS or IL-4+IL-13, thus inducing classical or alternative activation, respectively. While alternative activation drastically induced expression of AAM marker genes Arg1 (~138x), Chi3l3 (~1200x) and Retnla (~8300x) differential expression of these genes was not observed between wildtype and $Ccr5^{-/-}$ BMDM. However, IFN-γ+LPS-stimulated $Ccr5^{-/-}$ BMDM showed significantly increased expression of Arg1 and Mmp12 as well as a 5x increase of Retnla mRNA compared to wildtype BMDM. The increase of Arg1 mRNA upon IFN-γ+LPS stimulation compared to unstimulated BMDM is in accordance with earlier observations by Munder *et al.* showing that LPS induces Arginase 1 protein expression and enzyme activity in BMDM [191].

Together, these findings suggest that classical activation might induce a phenotype resembling alternative macrophage activation in $Ccr5^{-/-}$ BMDM from C57BL/6 mice. Further investigations including time course and dose/response analyses using classical activation stimuli are required to confirm these findings.

An interesting interpretation of the findings from BMDM generated in Petri dishes and Teflon bags is that Ccr5 deficiency reduces the capacity of macrophages to appropriately respond to Th1-associated stimuli. Instead, impaired signaling in $Ccr5^{-/-}$ macrophages may induce an alternative activation-like program under circumstances normally inducing classical macrophage activation. This notion is supported by the finding that CCR5 is generally expressed by Th1 lymphocytes [61] and might therefore be associated with classical rather than alternative macrophage activation.

The experiments provided in this thesis showed that renal allografts from $Cc5^{-/-}$ recipients, spleens and peritoneal lavage cells of $Ccr5^{-/-}$ mice exhibit a marked increase of AAM marker expression compared to their wildtype counterparts. However, pure macrophage populations derived from bone marrow cells of $Ccr5^{-/-}$ C57BL/6 mice showed only slight increases of AMM signature gene levels compared to wildtype BMDM. These results suggest that reproducing the effect of Ccr5 on macrophage polarization *in vitro* might be more complex than previously anticipated. Interestingly, Tiemessen *et al.* reported that Tregs induce alternative activation of co-cultivated human macrophages [296]. Thus, co-cultivation of Tregs and BMDM might be a promising approach to induce differential macrophage polarization in wildtype and $Ccr5^{-/-}$ BMDM.

5 OUTLOOK

The novel observation, that loss of Ccr5 induces alternative macrophage activation during renal allograft rejection in mice provides an explanation, why human transplant patients homozygous for a *CCR5Δ32* null allele show significantly prolonged survival of renal transplants. This hypothesis has not yet been tested. However, once optimal conditions have been found to induce differential macrophage polarization in BMDM from wildtype and $Ccr5^{-/-}$ mice these settings might be applied to macrophages derived from peripheral blood of human wildtype and $CCR5^{\Delta 32/\Delta 32}$ donors. This study established basic methods (generation of BMDM, functional assays for macrophage polarization and determination of AAM marker expression by flow cytometry) that could improve our understanding of the role of CCR5 during macrophage polarization. Nevertheless, species-specific differences might pose a serious hurdle when it comes to transfer the settings from the murine to the human system. For instance, while iNOS and ARG1 are readily inducible in murine macrophages upon stimulation with classical or alternative stimuli, respectively, detection of Nos2 expression in human macrophages is difficult *in vitro* [297] and ARG1 is expressed only in human granulocytes [298]. Moreover, the murine AAM signature genes Chi3l3 and Retnla lack human homologs, whereas Mrc1 expression indicates alternative activation in both species [160]. To date, most of our knowledge about gene expression in alternatively activated macrophages is derived from murine models, whereas studies using human macrophages remain scarce [160]. Although differences exist for macrophage polarization in mice and men, sufficient data should be available to distinguish between classical and alternative activation phenotypes on the basis of marker expression in human macrophages [160].

The role of Th17 immune responses in allograft rejection is not well understood, but it was shown that Il17 mRNA expression is increased in rejecting human transplants [299, 300] and

that IL-17 antagonism prolongs allograft survival in rats [208, 241, 253]. The experiments of this thesis show that loss of Ccr1 induces a shift away from Treg- and towards Th17-mediated immune responses. Paradoxically, $Ccr1^{-/-}$ recipients showed improved chronic phase lesion scores and less fibrosis as compared to wildtype recipients – a finding which is in accordance with previously reported results from other groups [102, 103]. The IL23/IL17 axis is known to induce neutrophil recruitment into the allograft [301] – a process that probably requires the presence of CCR1 [44, 263-265]. Hence, improvements in Ccr1-deficient recipients might – at least in part – be due to decreased neutrophil recruitment in a Th17-shifted microenvironment. To further corroborate these findings, expression of Th17 marker genes should be determined on the protein level which might be done by Western blot or flow cytometric analysis of allograft cell suspensions. Detection of IL-17 levels in urine might also be of interest. Additionally, investigation of allograft infiltration by neutrophils could be of importance.

Th17 immune responses have been implicated to play a role in several autoimmune pathologies. Thus, investigation of the impact of Ccr1 deficiency on autoimmune disease progression might shed new light into the function of CCR1 in this context. A first step into this direction might be the analysis of MRL/lpr $Ccr1^{-/-}$ mice which have already been generated in our laboratory.

6 ZUSAMMENFASSUNG

HINTERGRUND

Die Nierentransplantation ist die weltweit am häufigsten durchgeführte Art der Organtransplantation am Menschen [167]. Doch trotz der Fortschritte zur Behandlung der akuten Abstoßungsreaktion, werden immer noch mehr als 40% der transplantierten Organe durch chronische Abstoßungsreaktionen so stark geschädigt, dass sie schließlich durch ein neues Transplantat ersetzt werden müssen [168].

Vorangehende Studien legen eine Beteiligung des Chemokinrezeptors CCR5 bei der Abstoßung humaner Transplantate nahe. So ist bekannt, dass Nierentransplantate, die abgestoßen werden, eine erhöhte Expression des Chemokins CCL5/RANTES und des zugehörigen Rezeptors CCR5 aufweisen [125, 130]. In der kaukasischen Bevölkerung findet man relativ häufig ein Mutationsallel für *CCR5*, das *CCR5Δ32* genannt wird. Diese Mutation führt zur Expression eines verkürzten, nicht-funktionellen Rezeptorproteins, das im endoplasmatischen Retikulum verbleibt und nicht an die Zelloberfläche transportiert wird. Homozygote Träger dieses Allels stellen deshalb einen humanen „knockout“ für *CCR5* dar [4]. Unser Labor bestimmte im Jahr 2001 im Rahmen einer retrospektiven Studie den CCR5 Genotyp von Patienten mit Nierentransplantation und korrelierte den Genotyp mit dem Transplantatüberleben. Von 1227 untersuchten Patienten waren 21 homozygot für *CCR5Δ32* und diese Patientengruppe zeigte ein signifikant verlängertes Transplantatüberleben [8]. In einer Reihe von Tierversuchen mit gentechnisch veränderten Mäusen konnte außerdem gezeigt werden, dass der Verlust von Ccr5 oder des nahe verwandten Chemokinrezeptors Ccr1 in einer verminderten Abstoßungsreaktion von Herz-, Karotiden-, Kornea- und Inselzelltransplantaten resultiert [102, 103, 111, 112, 116, 117]. Die Chemokinrezeptoren CCR1 und CCR5 zeigen außerdem deutliche Überschneidungen in ihrer Ligandenspezifität, werden häufig gemeinsam reguliert und von den gleichen Zelltypen

exprimiert, was auf eine gewisse Redundanz in der Funktion dieser Rezeptoren hinzudeuten scheint [6, 45, 302].

ZIELE

Zum einen sollte geklärt werden, ob der Verlust von Ccr5 auch im Falle der Nierentransplantation in der Maus Verbesserungen mit sich bringt und welche Mechanismen für die beobachtete Verminderung der chronischen Transplantatabstossung in Patienten mit einem *CCR5*$^{\Delta 32/\Delta 32}$ Genotyp in Betracht gezogen werden können. Auf Grund der positiven Befunde aus Transplantationsversuchen mit CCR1-defizienten Mäusen, sollte zum anderen geklärt werden, ob sich der Verlust dieses nahe verwandten Rezeptors ebenso in einer Verminderung der Abstoßungsreaktion von transplantierten Nieren auswirkt und ob dabei ähnliche Mechanismen wie bei einem Verlust von Ccr5 beteiligt sind. In vorangegangenen Studien wurde beobachtet, dass der Verlust oder eine Blockade von CCR1 oder CCR5 im Empfängertier zu einer Verminderung der Abstoßungsreaktion führt [102, 103, 111, 112, 116, 117]. Außerdem sind beide Rezeptoren häufig koreguliert, besitzen ein überlappendes Ligandenspektrum und werden von den gleichen Zelltypen exprimiert. Diese Befunde veranlassten uns, Ccr1$^{-/-}$/Ccr5$^{-/-}$ doppelt-defiziente Mäuse zu generieren, um zu klären, ob CCR1 und CCR5 redundante Funktionen während der Transplantatabstoßung ausüben. Der Verlust beider Rezeptoren würde in diesem Fall additive oder synergistische Auswirkungen auf die Abstoßungsreaktion haben und könnte so das Transplantatüberleben zusätzlich begünstigen.

METHODEN

Um die Ursachen der verringerten Transplantatabstossung besser zu verstehen, wurde ein allogenes Nierentransplantationsmodell in der Maus genutzt, das sowohl die Analyse der akuten als auch der klinisch relevanteren chronischen Abstoßungsreaktion erlaubt, ohne dabei

immunsuppressive Medikamente einsetzen zu müssen, deren toxische Nebenwirkungen die zu untersuchenden Prozesse beeinflussen könnten.

Neben Wildtyp, Ccr1$^{-/-}$ und Ccr5$^{-/-}$ Mäusen dienten „doppelt-defiziente“ (Ccr1$^{-/-}$/Ccr5$^{-/-}$) C57BL/6 Mäuse als Empfänger von allogenen Wildtyp BALB/c Nierentransplantaten. Den Empfängertieren wurden zuvor beide Nieren mikrochirurgisch entfernt, so dass der eingesetzten Spenderniere eine lebenserhaltende Funktion zukam. Durch die Kombination von BALB/c (H-2^{d}) Spender- und C57BL/6 (H-2^{b}) Empfängertieren ergibt sich eine maximale MHC-Inkompatibilität und damit eine starke Abstoßungsintensität. Als Kontrollen dienten Wildtyp C57BL/6 Nieren, die in C57BL/6 Wildtypempfänger eingepflanzt wurden („Isograft“-Gruppe). Um mögliche Unterschiede in der akuten und chronischen Phase der Transplantatabstoßung analysieren zu können, wurden die Spendernieren 7 bzw. 42 Tage nach der Transplantation entnommen und mit verschiedenen Methoden untersucht. Außerdem wurden zu beiden Zeitpunkten Harnstoff- und Kreatininspiegel im Plasma bestimmt, um die Transplantatfunktion beurteilen zu können. An PAS-gefärbten Schnitten wurde die Transplantatschädigung mittels verschiedener Schädigungsindizes bewertet. Die Infiltration tubulointerstitieller und glomerulärer Kompartimente der Nierentransplantate durch verschiedene Leukozyten-Subpopulationen wurde mit Hilfe immunhistochemischer Färbungen (CD4^{+}, CD8^{+}, CD11c^{+} und F4/80^{+} Zellen) analysiert. Zur Bestimmung fibrotischer Prozesse wurden „smooth muscle actin (α-SMA)“ und Collagen I/III immunhistochemisch nachgewiesen. Die Expression ausgewählter Markergene in den Transplantaten wurde mittels quantitativer real-time RT-PCR bestimmt. Vorangehende Untersuchungen einer anderen Arbeitsgruppe deuteten darauf hin, dass sich der Verlust von Ccr5 auf die humorale Abstoßungsreaktion auswirken könnte [113, 114]. Daher wurden die Plasmaspiegel alloreaktiver Antikörper in Wildtyp und Ccr5-defizienten Mäusen bestimmt, die zuvor entweder mit BALB/c Milzzellen immunisiert wurden oder ein BALB/c Transplantat erhalten hatten.

Ccr5-defiziente Empfängertiere entwickelten im Verlauf der Abstoßungsreaktion erhebliche Unterschiede hinsichtlich des Aktivierungsphänotyps von Makrophagen im Transplantat. Um zu untersuchen, ob der Verlust von Ccr5 die Makrophagenpolarisierung allgemein beeinflusst, oder ob die beobachteten Effekte auf die Abstoßungsreaktion beschränkt sind, wurde der Aktivierungsphänotyp von Makrophagen aus Wildtyp und $Ccr5^{-/-}$ Mäusen bestimmt. Eine erste Versuchsreihe mit relativ heterogenen Zellpopulationen, die zum einen aus der Milz und zum anderen durch Thioglycolat-induzierte Peritonitis gewonnen wurden, wurde durch Versuche mit Knochenmark-abgeleiteten Makrophagen (BMDM) ergänzt. Neben der Expressionsanalyse von Signaturgenen mittels quantitativer real-time RT-PCR und Durchflusszytometrie, kamen dabei auch Methoden zur Bestimmung des Makrophagenphänotyps auf funktioneller Ebene (Bestimmung von Nitrat-Produktion und Arginaseenzymaktivität) zum Einsatz.

ERGEBNISSE

Die Funktion des Transplantats verschlechterte sich in allen Empfängergruppen, außer bei Ccr1/Ccr5 doppelt-defizienten Mäusen, deren Konzentrationen an Plasmaharnstoff zwischen 7 und 42 Tagen nach Transplantation konstant blieben. Die histopathologische Untersuchungen der Nierentransplantate ergaben bei $Ccr1^{-/-}$ und $Ccr5^{-/-}$ Empfängertieren signifikant verringerte Schädigungsindizes schon während der akuten Phase der Abstoßung und verringerte Fibrose während der chronischen Phase. Zusätzliche Effekte in doppelt-defizienten Empfängern waren auf verbesserte Schädigungsindizes (Transplantatglomerulpathie und chronisch-vaskulären Schäden) in der chronischen Phase beschränkt. $Ccr1^{-/-}$ Empfänger zeigten signifikant verringerte Infiltration durch $CD4^{+}$, $CD8^{+}$ und $CD11c^{+}$ Zellen nur während der akuten Abstoßungsphase, während die Infiltration des Transplantats mit diesen Zelltypen in $Ccr5^{-/-}$ Empfängertieren erst in der chronischen Phase und hauptsächlich im glomerulären Kompartiment der Niere signifikant reduziert war. Zusätzliche Effekte durch das Fehlen beider Rezeptoren waren

erstaunlicherweise auf die Infiltration mit F4/80^{+} Zellen bei 7 Tagen und auf CD11c^{+} Zellen bei 42 Tagen nach Transplantation beschränkt. Die Anzahl CD4^{+} T-Zellen in Transplantaten von doppelt-defizienten Empfängern wies im Gegensatz zu einfach-defizienten Empfängern keine Verringerung im Vergleich zu Wildtyp Empfängern auf. In vorangehenden Studien konnte gezeigt werden, dass CCR1 und CCR5 von Monozyten und Makrophagen exprimiert werden [45] und eine Rolle während der Diapedese von Monozyten spielen [101, 228]. Jedoch war die Anzahl F4/80^{+} Zellen in Transplantaten von Ccr1$^{-/-}$ und Ccr5$^{-/-}$ Empfängern gegenüber Wildtyp Empfängern erstaunlicherweise nicht verändert. Keine Unterschiede wurden für die Plasmaspiegel alloreaktiver Antikörper von Wildtyp und Ccr5$^{-/-}$ Mäusen gefunden, die mit BALB/c Milzzellen immunisiert wurden oder eine BALB/c Spenderniere erhalten hatten.

Die Expressionsanalyse ausgewählter Markergene zeigte, dass sowohl Transplantate von Ccr1$^{-/-}$ als auch Ccr5$^{-/-}$ Empfängertieren signifikant verringerte mRNA-Spiegel für Th1-Zytokine (Ifng und Tnf) sowie für Th1-assoziierte Chemokine (Ccl2-5 und Cxcl10) in der akuten Phase der Abstoßungsreaktion aufweisen. Überraschenderweise zeigten doppelt-defiziente Empfänger in der akuten Phase keine Veränderung der mRNA-Spiegel dieser Gene im Vergleich zu Wildtypempfängern. Allerdings war die mRNA Expression von Ccr2 nur in doppelt-defizienten Empfängern am Tag 7 nach Transplantation signifikant verringert. Die mRNA-Spiegel von Ccr7 waren am Tag 42 in allen knock-out Empfängergruppen signifikant reduziert.

Ccr1-defiziente Empfänger zeigten erhöhte Expression der Th17-assoziierten Gene Rorc und Il17a, während Gene, die mit der Funktion regulatorischer T-Zellen (Treg) in Verbindung gebracht werden (Foxp3 und Ccr4), eine signifikant verringerte Expression aufwiesen. Im Vergleich zu diesen Ergebnissen, zeigten Ccr5-defiziente Empfänger deutlich unterschiedliche Expressionsmuster. Der Verlust von Ccr5 führte zu einem Anstieg der mRNA Expression von Th2 Zytokinen (Il4 und Il13) in der chronischen Phase der Abstoßungsreaktion, der von einer dramatischen Erhöhung der mRNA-Spiegel von Signaturgenen (Arg1, Chi3l3 und Retnla) für

alternative aktivierte Makrophagen (AAM) begleitet war. Die Anreicherung von AAM in Transplantaten von $Ccr5^{-/-}$ Empfängern wurde auf Proteinebene durch immunhistologische Färbung von $Arg1^{+}$, $Chi3l3^{+}$ und $Mrc1^{+}$ Zellen bestätigt. Zusätzliche Experimente mit C57BL/6 Mäusen zeigten, dass der Verlust von Ccr5 auch in Milzen sowie *in vitro* kultivierten Milzzellen und Makrophagen, die durch Thioglycolat-induzierte Peritonitis gewonnen wurden, eine erhöhte Expression von AAM Signaturgenen bewirkt. Ein ähnlicher, wenn auch weniger ausgeprägter Effekt auf die Makrophagenpolarisierung durch den Verlust von Ccr5 konnte dagegen in einer einheitlicheren Makrophagen-Population, die aus Knochenmarkszellen von C57BL/6 Tieren hergestellt wurde, bisher nur nach Stimulation mit IFN-γ+LPS bzw. Vorstimulation mit LPS und nachfolgender Stimulation mit IL-4, nachgewiesen werden. Allerdings zeigten $Ccr5^{-/-}$ BALB/c BMDM verstärkte, dosisabhängige Expression von AAM Signaturgenen, erhöhte Arginaseenzymaktivität und verringerte NO-Produktion nach Stimulation mit IL-4 im Vergleich zu Wildtyp BALB/c BMDM.

SCHLUSSFOLGERUNGEN

Im Vergleich zu Wildtyp Empfängern zeigten Ccr1- und Ccr5-defiziente Empfängertiere Verbesserungen bei akuten sowie chronischen Schädigungsindizes, verringerte Fibrose und geringere mRNA Expression von Th1-assoziierten Zytokinen und Chemokinen in der akuten Phase.

Die beobachtete Verminderung der Transplantatabstoßung lässt sich nicht auf eine spontane Akzeptanz der Transplantate zurückführen, da sich zum einen die Transplantatfunktion (Plasmakreatinin- und Harnstoffkonzentration) und die meisten Schädigungsindizes in allen Empfängergruppen zwischen 7 und 42 Tagen nach der Transplantation verschlechterten. Zum anderen bestätigt die Anwesenheit eines ausgeprägten leukozytären Infiltrates während der chronischen Phase in allen Empfängergruppen das Andauern von Abstoßungsreaktionen und

unterstreicht somit die Gültigkeit dieses Modells für die Untersuchung von Abstoßungsreaktionen in der transplantierten Niere. Außerdem konnten die verminderten Abstoßungsreaktionen in keiner der Chemokinrezeptor-defizienten Gruppen mit einer verstärkten Immunsuppression durch regulatorische T Zellen (Treg) in Verbindung gebracht werden.

Obwohl die Abstoßungsreaktion in Ccr1- als auch in Ccr5-defizienten Empfängern vermindert war (reduzierte Fibrose, verringerte Leukozyteninfiltration und Verbesserung der Transplantathistopathologie), scheinen diese Effekte durch unterschiedliche Mechanismen vermittelt zu werden. So resultierte der Verlust von Ccr1 in einer verringerten glomerulären und tubulointerstiellen Einwanderung von $CD4^+$, $CD8^+$ und $CD11c^+$ Zellen in der akuten Phase, während Verringerungen in der Anzahl dieser infiltrierenden Leukozyten nach Verlust von CCR5 eher auf das glomeruläre Kompartiment und die chronische Phase beschränkt sind. Dieser Befund könnte auch Einfluss auf den zeitlichen Einsatz von Antagonisten am Menschen haben.

Desweiteren zeigten Ccr1- und Ccr5-defiziente Empfänger wesentliche Unterschiede bei der Art der ausgeprägten Immunantwort. Erstaunlicherweise zeigten Ccr1-defiziente Empfängertiere trotz einer Th17-polarisierten Immunantwort, die mit erniedrigter mRNA Expression Treg-assoziierter Gene einherging, verbesserte Transplantathistopathologie und weniger Fibrose. Dieser widersprüchliche Befund ist möglicherweise auf verringerte Infiltration durch neutrophile Granulozyten zurückzuführen, die eine Rolle bei Th17-vermittelten Pathologien spielen [209, 241] und deren Einwanderung an Entzündungsherde in vielen Fällen von CCR1 abhängt [44, 263-265]. Deshalb könnte der Verlust von Ccr1 die Abstoßungsreaktion in Richtung Th17 polarisieren, ohne dabei Schädigungen durch einwandernde Neutrophile auszulösen. Weitere Analysen sind notwendig, um diese Vermutung zu untermauern.

Die Ergebnisse der vorliegenden Arbeit deuten darauf hin, dass der Verlust von Ccr5 die alternative Aktivierung von Makrophagen (AAM) begünstigt. Dies konnte in

Nierentransplantaten Ccr5-defizienter Empfängertiere, in $Ccr5^{-/-}$ Milzen und kultivierten Milzzellen sowie in kultivierten Peritoneallavage-Makrophagen aus $Ccr5^{-/-}$ Mäusen gezeigt werden. Daher besteht die Möglichkeit, dass dieser Effekt nicht auf Prozesse während der Transplantatabstoßung begrenzt ist, sondern auch bei chronischen und entzündlichen Erkrankungen eine wichtige Rolle spielt. Im Gegensatz zu klassisch aktivierten Makrophagen (sog. „killer macrophages“) besitzen alternativ aktivierte Makrophagen einen „wound-healing“ Phänotyp, der sich positiv auf das Transplantatüberleben auswirken könnte. Interessanterweise unterschied sich die Anzahl infiltrierender $F4/80^{+}$ Zellen (hauptsächlich Makrophagen) nicht signifikant zwischen Wildtyp und $Ccr5^{-/-}$ Empfängern, aber der Verlust von Ccr5 führte zu einer dramatischen Veränderung des Aktivierungsphänotyps, der für die beobachteten Verbesserungen verantwortlich sein könnte und auch eine mögliche Erklärung für die beobachtete Verlängerung des Transplantatüberlebens in Patienten mit $CCR5^{\Delta 32/\Delta 32}$ Genotyp darstellt. Allerdings müssen weitere Analysen am Menschen zeigen, ob sich die in der Maus gewonnen Erkenntnisse auf den Menschen übertragen lassen. Interessanterweise, zeigten Experimente mit BMDM aus verschiedenen Mausstämmen, dass die Polarisierbarkeit von Makrophagen stark vom eingesetzten Mausstamm abhängig ist, und legen den Schluss nahe, dass das BALB/c Spenderorgan möglicherweise die alternative Aktivierung von Makrophagen begünstigt.

Obwohl der Verlust von CCR1 oder CCR5 in Transplantatempfängern zu einer Abschwächung der Abstoßungsreaktion führte, waren zusätzliche Effekte in Ccr1/Ccr5 doppelt-defizienten Empfängern auf einige wenige Parameter in der chronischen Phase beschränkt. Außerdem war die mRNA Expression Th1-assoziierter Zytokine und Chemokine in der akuten Phase mit Wildtyp Empfängern vergleichbar. Im Gegensatz zu Ccr1- und Ccr5-defizienten Empfängern blieb auch die Anzahl $CD4^{+}$ T-Zellen, in doppelt-defizienten Empfängern zu beiden Zeitpunkten im Vergleich zu Wildtyp Empfängern konstant, wodurch möglicherweise die unveränderte

Expression Th1-assoziierter Gene in der akuten Phase erklärt werden kann. Jedoch zeigten Transplantate doppelt-defizienter Empfänger verringerte Infiltration durch F4/80^{+} Zellen (hauptsächlich Makrophagen) in der akuten und durch CD11c^{+} Zellen (Dendritische Zellen) in der chronischen Phase. Ein Befund der sich auf mRNA-Ebene in einer verringerten Expression von Ccr2 (notwendig für die Auswanderung von Monozyten-Vorläuferzellen aus dem Knochenmark) und Ccr7 (notwendig für die Einwanderung Antigen-präsentierender Dendritischen Zellen in sekundäre lymphatische Organe) wiederspiegelt. Allerdings war das Ausmaß zusätzlicher Verbesserungen in Transplantaten doppelt-defizienter Empfänger relativ gering und auf wenige Parameter beschränkt. Daher legen diese Befunde die Vermutung nahe, dass CCR1 und CCR5 keine redundanten Funktionen während der Transplantatabstoßung ausüben. Die in den einfach-defizienten Empfängertieren beobachteten unterschiedlich polarisierten Immunantworten (Th17 bei Ccr1$^{-/-}$ *vs.* Th2/AAM bei Ccr5$^{-/-}$ Empfängern) heben sich in doppelt-defizienten Empfängern möglicherweise gegenseitig auf und führen so zu einem Phänotyp, der in vielen Aspekten dem der Wildtyp Empfänger entspricht.