3 RESULTS

As outlined under ”External contributions” (see appendix 7.1), the group of Prof. Gröne at the DKFZ in Heidelberg (Dept. of Cellular and Molecular pathology) contributed to this work by performing orthotopic kidney transplantation (see 2.15), determination of plasma urea and creatinine (3.1.1), histopathology (3.1.2) and immunohistochemistry (3.1.3) of renal allograft sections. The results of these investigations are described to provide comprehensive insight to this complex project. Results supplied by Prof. Gröne’s group are indicated in section headlines by DKFZ in brackets to unambiguously indicate the origin of contribution.

3.1 Analysis of renal allograft rejection in chemokine receptor-deficient mice

3.1.1 Functional analysis of renal allografts (DKFZ)

In order to assess renal allograft function creatinine and urea levels were determined in plasma samples collected at days 7 (acute rejection phase) and 42 (chronic rejection phase) after transplantation of a single kidney into bilaterally nephrectomized wildtype, $Ccr1^{-/-}$, $Ccr5^{-/-}$ and $Ccr1^{-/-}/Ccr5^{-/-}$ recipients.

While the organism catabolizes carbohydrates and lipids almost entirely to CO_2 and H_2O, nitrogen from amino acid, protein and nucleotide catabolism is excreted in the form of urea and to a minor extent as creatinine and other nitrogen compounds. During the urea cycle amino groups from ammonia and L-aspartate are converted to urea, using L-ornithine, citrulline, L-argininosuccinate and L-arginine as intermediates. Although the production of urea is an energy dependent anabolic process it is necessary to detoxify ammonia which would raise intracellular pH to toxic levels. Creatinine is a metabolic waste product of muscular tissues and its plasma concentrations are relatively constant in healthy individuals. Creatinine as well as urea is excreted by the kidney as components of the urine. Increased concentrations of creatinine and urea in the blood indicate defective glomerular filtration and renal dysfunction

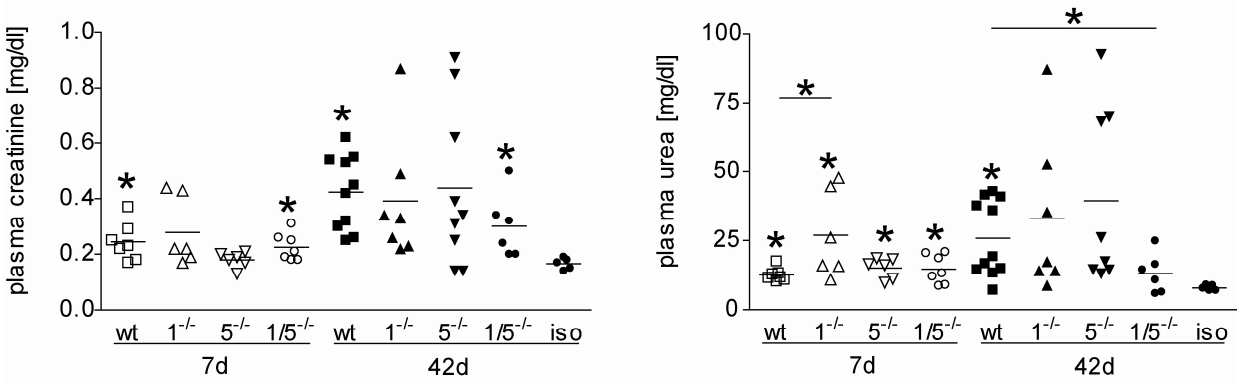

Figure 14. Effect of chemokine receptor deficiency on renal allograft function. Plasma from wildtype, $Ccr1^{-/-}$, $Ccr5^{-/-}$ and $Ccr1^{-/-}/Ccr5^{-/-}$ renal allograft recipients was collected 7 and 42 days after transplantation and used to assess allograft function by determining creatinine and urea levels. Each symbol represents the corresponding value from an individual mouse. Mean values for each group are indicated by a horizontal line. The levels in isografts at 42d are also shown. Asterisks above individual columns indicate significance against isograft whereas asterisks on horizontal lines indicate significance between groups of recipients. * $p<0.05$.

[200]. Creatinine and urea levels increased moderately between 7 and 42 days in all recipient groups (**Figure 14** and **Table 4**) demonstrating deterioration of renal allograft function over time. At 42 days after transplantation recipients of fully MHC-mismatched allografts showed higher creatinine and urea levels as recipients in the isograft group indicating ongoing allograft rejection. No differences were found for creatinine levels between the individual recipient groups at both investigated time points. However, plasma urea levels were significantly increased in $Ccr1^{-/-}$ recipients at 7 days post transplantation, but this increase disappeared at 42 days. Remarkably, $Ccr1^{-/-}/Ccr5^{-/-}$ recipients showed significantly decreased plasma urea concentrations at this time point whereas single deficient recipients had moderately increased mean plasma urea levels.

3.1.2 Histopathologic analysis of renal allografts (DKFZ)

To assess allograft damage periodic acid-Schiff (PAS)-stained sections from renal allografts of all recipient genotypes (wildtype, $Ccr1^{-/-}$, $Ccr5^{-/-}$ and $Ccr1^{-/-}/Ccr5^{-/-}$) and both time points (7d, 42d) were evaluated by light microscopy using lesion scores as defined in Materials and Methods.

Representative micrographs are shown in **Figure 15**. In general, all acute damage scores as well as the score for tubulointerstitial inflammation increased between 7 and 42 days after transplantation (**Table 4**) indicating ongoing rejection.

The lesion score for acute vascular rejection was significantly reduced in allografts from $Ccr1^{-/-}$ and $Ccr5^{-/-}$ recipients at day 7. Although, double-deficient recipients showed significantly reduced acute vascular rejection compared to wildtype recipients there was no additional decrease compared to single-deficient recipients at this time point. During the chronic rejection phase (42d postTX) a significant reduction of acute vascular rejection was observed only in $Ccr5^{-/-}$ recipients and remained above isograft level in all recipient groups. A chronic vascular rejection score was calculated for allografts at day 42 and significant reductions were observed in $Ccr1^{-/-}$, $Ccr5^{-/-}$ and $Ccr1^{-/-}/Ccr5^{-/-}$ recipients. Notably, the chronic vascular rejection score was reduced to the level of isograft recipients in double-deficient recipients.

Acute and chronic tubulointerstitial damage scores showed reductions in all analyzed chemokine receptor-deficient recipients but these differences did not reach statistical significance at both time points. At 7 days post transplantation tubulointerstitial inflammation was significantly reduced only in $Ccr1^{-/-}$ recipients whereas all recipient groups showed reduced tubulointerstitial inflammation compared to wildtype recipients at 42 days post transplantation. No additional reduction was found for double-deficient recipients and all recipient groups displayed tubulointerstitial inflammation above isograft level at 42 days.

Similar to acute vascular rejection at 7 days the lesion score for acute glomerular damage was significantly reduced for allografts from $Ccr1^{-/-}$, $Ccr5^{-/-}$ and $Ccr1^{-/-}/Ccr5^{-/-}$ recipients during acute rejection. At 42 days after transplantation the score for acute glomerular damage was significantly reduced in $Ccr1^{-/-}$ and $Ccr5^{-/-}$ recipients. Again, no additional reduction was found for double-deficient recipients and all recipient groups displayed acute glomerular damage scores above isograft level at 42 days. The lesion score for transplant glomerulopathy was

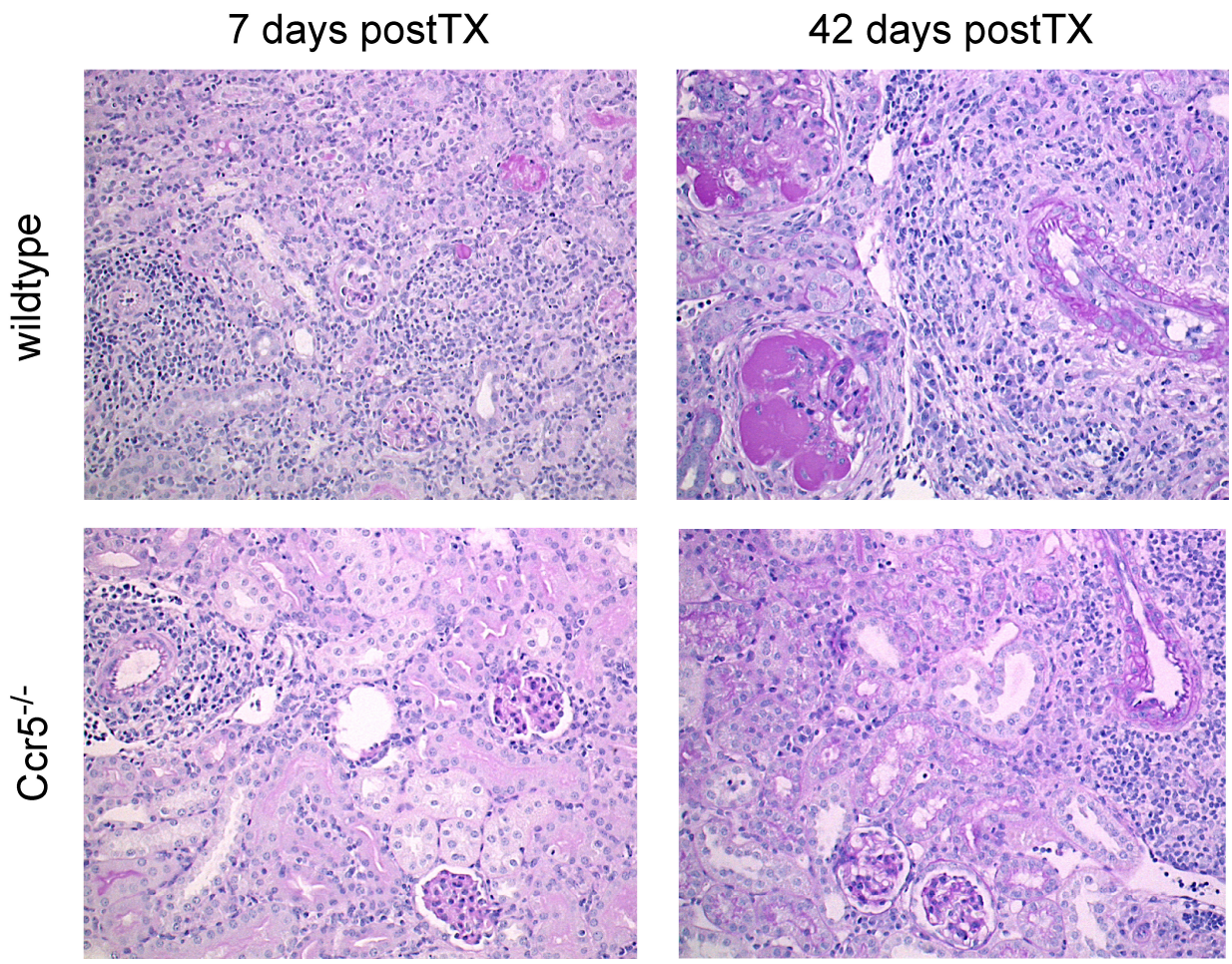

Figure 15. Effects of chemokine receptor deficiency on the histology of renal allografts. Representative micrographs of PAS-stained sections of renal allografts from wildtype and $Ccr5^{-/-}$ recipients from 7d and 42d post transplantation are shown (200x). Due to the exemplary character of these illustrations only wildtype and $Ccr5^{-/-}$ micrographs are shown here.

reduced in all analyzed chemokine receptor-deficient recipients at 42 days after transplantation but reached statistical significance only in double-deficient recipients.

Graft fibrosis was analyzed to assess transplant damage. Fibrosis is the formation of excess fibrous connective tissue. This process is either due to a healing process during the restoration of injured tissue by scar tissue or an abnormal condition where normal tissue is replaced by fibrous tissue. During allograft rejection fibrosis is induced by acute immune responses directed against the transplant that lead to cellular necrosis. Infiltrating lymphocytes within the vessel wall of the allograft are activated by antigen and stimulate macrophages to produce smooth muscle growth factors. Eventually, proliferation of smooth muscle cells results in thickening,

Table 4. Compilation of functional and histopathologic data from renal allograft recipients. Pairwise comparisons between wt and $Ccr1^{-/-}$, $Ccr5^{-/-}$ or $Ccr1^{-/-}/Ccr5^{-/-}$ at either 7d or 42d using Student's T-test or Mann-Whitney-U test were appropriate. Significant improvements are highlighted in light grey. Fields in dark grey highlight additional improvements in $Ccr1^{-/-}/Ccr5^{-/-}$ recipients (mean±SD; *: p<0.05; **: p<0.01; ***: p<0.001).

	acute rejection (7d postTX)				chronic rejection (42d postTX)				
	wildtype	**$Ccr1^{-/-}$**	**$Ccr5^{-/-}$**	**$Ccr1/5^{-/-}$**	**wildtype**	**$Ccr1^{-/-}$**	**$Ccr5^{-/-}$**	**$Ccr1/5^{-/-}$**	**isograft**
functional data									
plasma creatinine [mg/dl]	0.24 ± 0.07	0.28 ± 0.12	0.18 ± 0.03	0.23 ± 0.05	0.4 ± 0.16	0.39 ± 0.23	0.44 ± 0.29	0.3 ± 0.11	0.17 ± 0.01
plasma urea [mg/dl]	12.6 ± 2.4	26.9 ± 15.8	15.1 ± 3.7	14.7 ± 5.2	25.8 ± 13.7	32.8 ± 28.5	39.5 ± 32	*** 13.2 ± 7.1**	7.92 ± 0.43
lesion scores									
acute vascular rejection	29.8 ± 9.2	**** 11 ± 6.9**	*** 16 ± 3**	**** 12.6 ± 6**	42 ± 25	30 ± 29	**** 16 ± 9**	25 ± 7	1.40 ± 0.91
chronic vascular rejection	-	-	-	-	18.8 ± 12.9	**** 3.4 ± 6.4**	**** 1.7 ± 3.4**	**0 ± 0**	0 ± 0
acute tubulointerstitial damage	3.2 ± 2.4	2.5 ± 4.2	3.8 ± 3.8	1.7 ± 1.3	12 ± 8	6.8 ± 4.9	6.4 ± 5	7.5 ± 6.7	-
chronic tubulointerstitial damage	-	-	-	-	5.3 ± 2.3	3.4 ± 3.3	4 ± 3.4	2.9 ± 2.7	-
tubulointerstitial inflammation	187 ± 47	*** 109 ± 40**	129 ± 33	126 ± 50	241 ± 43	*** 166 ± 58**	*** 166 ± 84**	*** 173 ± 56**	6.00 ± 2.92
acute glomerular damage	19.5 ± 4.1	**** 7.3 ± 3.4**	**** 8.1 ± 3.5**	***** 7.3 ± 2.9**	86 ± 66	*** 26 ± 11**	*** 28 ± 26**	27 ± 15	3.58 ± 0.74
transplant glomerulopathy	-	-	-	-	51 ± 37	37 ± 22	20 ± 11	**** 8 ± 8**	0 ± 0
matrix deposition									
α-SMA	-	-	-	-	34 ± 16.6	**** 12.5 ± 5**	**** 11.6 ± 2.4**	*** 17.3 ± 7.3**	-
Coll I/III	-	-	-	-	125 ± 19	*** 103 ± 19**	107 ± 15	**** 85 ± 22**	-

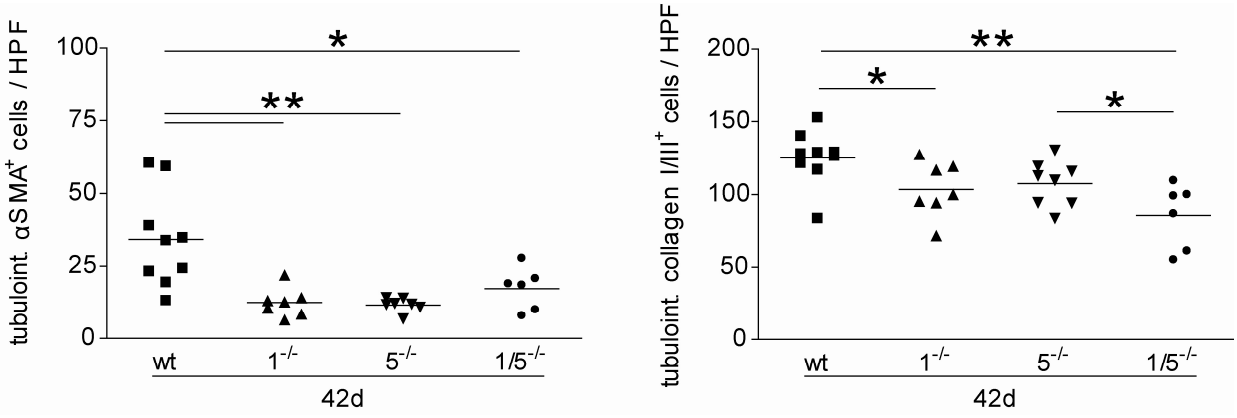

Figure 16. Effects of chemokine receptor deficiency on matrix deposition. αSMA$^+$ and collagen I/III$^+$ cells were counted in sections of renal allograft recipients at day 42 after transplantation. Each symbol represents the corresponding value from an individual mouse. Mean values for each group are indicated by a horizontal line. Asterisks on horizontal lines indicate significance between groups of recipients (*: $p<0.05$; **: $p<0.01$).

hardening and loss of elasticity of graft vessels – a process also seen in arteriosclerosis.

Graft fibrosis was determined at day 42 after transplantation in the tubulointerstitial compartment by immunohistochemical staining for alpha-smooth muscle actin (α-SMA) and collagen I/III (**Figure 16** and **Table 4**). The number of α-SMA$^+$ cells was significantly reduced in allografts from Ccr1$^{-/-}$, Ccr5$^{-/-}$ and Ccr1$^{-/-}$/Ccr5$^{-/-}$ recipients compared to wildtype recipients but no additional reduction was found in double-deficient recipient mice. Ccr1$^{-/-}$ and Ccr1$^{-/-}$/Ccr5$^{-/-}$ recipients showed a significant decrease of collagen I/III deposition in comparison to wildtype recipients. The staining for collagen I/III in Ccr5$^{-/-}$ recipients tended to decrease in comparison to wildtype recipients but did not reach statistical significance.

In summary, pathological analysis revealed significantly reduced lesion scores in Ccr1$^{-/-}$ and Ccr5$^{-/-}$ recipients (glomerular damage, vascular rejection and tubulointerstitial inflammation) at the investigated time points but additional improvements due to double deficiency were limited to certain parameters during the chronic phase of rejection (transplant glomerulopathy and chronic vascular damage).

3.1.3 Analysis of leukocyte infiltration in renal allografts (DKFZ)

Immunohistochemical staining for CD4, CD8, CD11c and F4/80 was performed on renal allograft sections to determine the number of infiltrating leukocytes (T cells, dendritic cells and macrophages) in the glomerular and tubulointerstitial compartment. Statistical evaluation of immunohistologic results is shown in **Figures 17 and 18.** An overview of these results is shown in **Table 5**. Representative micrographs are shown in **Figure 34-37** (see appendix 7.2).

In all recipient groups the number of tubulointerstitial $CD4^+$ and $CD8^+$ cells declined between 7 and 42 days after transplantation and glomerular $CD8^+$ cells showed a similar trend. Interestingly, $CD4^+$ cells in the glomerular compartment showed a different behavior (**Figure 17**). Wildtype, $Ccr1^{-/-}$ and $Ccr1^{-/-}/Ccr5^{-/-}$ recipients displayed higher numbers of glomerular $CD4^+$ cells at day 42 compared to day 7 after transplantation, whereas the number of glomerular $CD4^+$ cells in $Ccr5^{-/-}$ recipients remained constant over time. The number of glomerular and tubulointerstitial $CD4^+$ cells was significantly reduced in the $Ccr1^{-/-}$ group at day 7 after transplantation and $Ccr5^{-/-}$ recipients showed reduced numbers of glomerular $CD4^+$ cells at day 42. $CD8^+$ cells in both compartments were reduced during acute phase in $Ccr1^{-/-}$ recipients while $Ccr5^{-/-}$ recipients again showed a significant reduction during chronic phase of rejection. Double-deficient recipients had reduced numbers of glomerular $CD8^+$ at days 7 and 42 as well as a reduction of tubulointerstitial $CD8^+$ cells at day 42. However, the observed reductions showed no additional effect in double-deficient recipients when compared to single-deficient recipients. There was no clear trend for a reduction of $CD11c^+$ infiltrating leukocytes between 7 and 42 days after transplantation. While tubulointerstitial $CD11c^+$ cells from wildtype recipients decreased from 7 to 42 days, the number of glomerular $CD11c^+$ cells was constant over time in recipients with wildtype genotype (**Figure 17**).

Numbers of glomerular $CD11c^+$ cells increased over time in $Ccr1^{-/-}$ recipients while tubulointerstitial $CD11c^+$ cells in $Ccr1^{-/-}$ recipients and $CD11c^+$ cells of $Ccr5^{-/-}$ and $Ccr1^{-/-}/Ccr5^{-/-}$

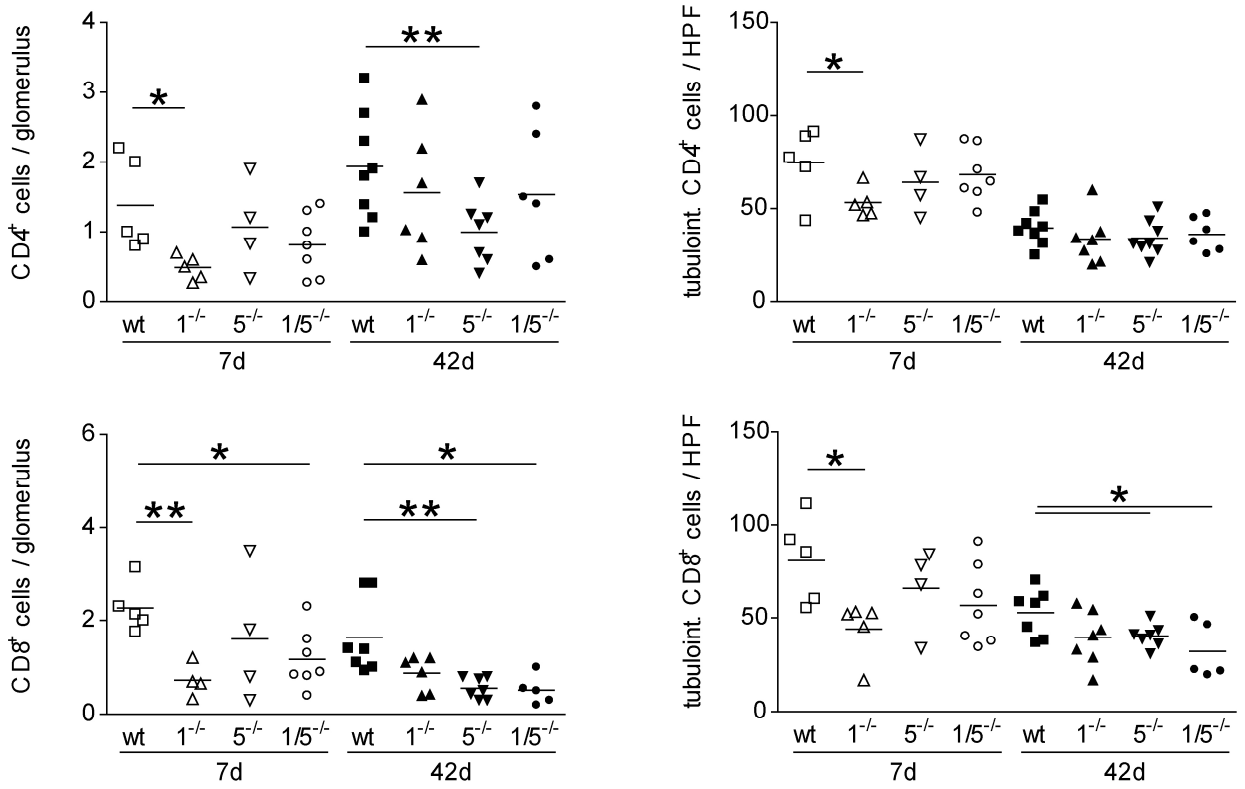

Figure 17. Quantitative evaluation of allograft infiltration by CD4$^+$ and CD8$^+$ cells. Cells stained for either CD4 or CD8 were counted in sections of renal allografts at day 42 after transplantation. Glomerular and tubulointerstitial (tubint.) infiltration are depicted in spate diagrams. Each symbol represents the corresponding value from an individual mouse. Mean values for each group are indicated by a horizontal line. Asterisks on horizontal lines indicate significance between groups of recipients (*: p<0.05; **: p<0.01). HPF: high power field.

recipients showed only moderate reductions in both compartments (**Figure 18**). Compared to wildtype recipients CD11c$^+$ cells were decreased in Ccr1$^{-/-}$ recipients in both compartments and in Ccr5$^{-/-}$ recipients in the tubulointerstitium at 7 days. At the later time point this pattern was inverted and significant reductions were only observed for glomerular CD11c$^+$ cells in Ccr5$^{-/-}$ recipients. Although Ccr1$^{-/-}$/Ccr5$^{-/-}$ recipients had reduced glomerular CD11c$^+$ cells in chronic phase and a reduction in tubulointerstitial CD11c$^+$ cells in acute and chronic phase, only the reduction of tubulointerstitial CD11c$^+$ cells at 42 days was attributable to an additional effect of double deficiency. However, this additional effect was only moderately pronounced. While infiltration by F4/80$^+$ cells in the glomerular compartment increased from 7 to 42 days in all

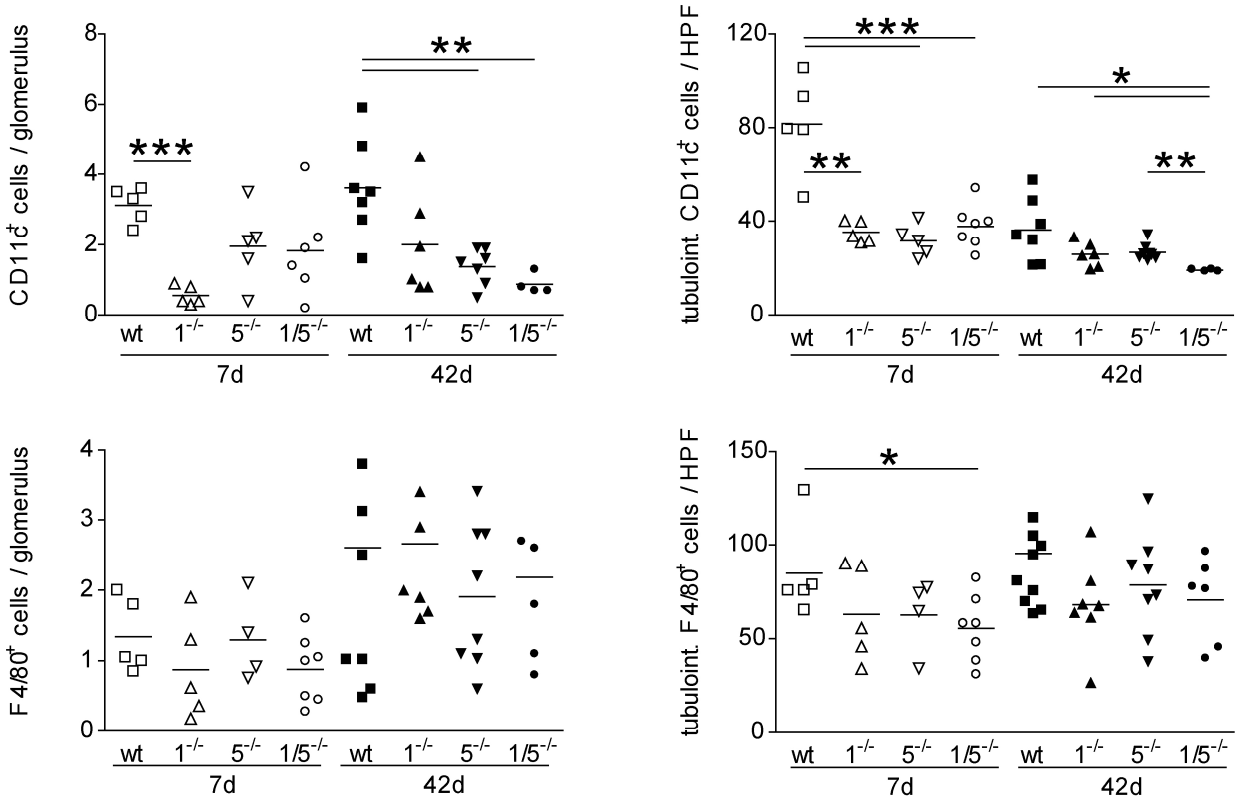

Figure 18. Quantitative evaluation of allograft infiltration by CD11c$^+$ and F4/80$^+$ cells. Cells stained for either CD11c or F4/80 were counted in sections of renal allografts at day 42 after transplantation. Glomerular and tubulointerstitial (tubint.) infiltration are depicted in spate diagrams. Each symbol represents the corresponding value from an individual mouse. Mean values for each group are indicated by a horizontal line. Asterisks on horizontal lines indicate significance between groups of recipients (*: p<0.05; **: p<0.01; ***: p<0.001). HPF: high power field.

recipient groups, numbers of tubulointerstitial F4/80$^+$ cells were more or less unchanged within this period. Remarkably, the only significant reduction found for infiltration by F4/80$^+$ cells was observed in Ccr1$^{-/-}$/Ccr5$^{-/-}$ recipients in the tubulointerstitium during the acute phase of rejection (**Figure 18**).

In summary, deficiency in Ccr1 caused reduced leukocyte infiltration during acute phase (less CD4$^+$, CD8$^+$ and CD11c$^+$ cells in both compartments), while Ccr5 deficiency resulted in reduced numbers of infiltrating leukocytes in the chronic phase of rejection (less glomerular CD4$^+$, CD8$^+$ and CD11c$^+$ cells as well as decreased tubulointerstitial CD8$^+$ cells) (**Table 5**). Surprisingly, infiltration by F4/80$^+$ cells in Ccr1$^{-/-}$ and Ccr5$^{-/-}$ recipient allografts (both compartments and time

Table 5. Summary of immunohistologic results from renal allograft recipients. Pairwise comparisons between wildtype and Ccr1$^{-/-}$, Ccr5$^{-/-}$ or Ccr1$^{-/-}$/Ccr5$^{-/-}$ at either 7d or 42d post transplantation using Student's T-test. Significant improvements against wildtype recipients are highlighted in light grey. Fields in dark grey highlight additional improvements in Ccr1$^{-/-}$/Ccr5$^{-/-}$ recipients (mean ± SD; *: p<0.05; **: p<0.01; ***: p<0.001).

	acute rejection (7d postTX)				chronic rejection (42d postTX)			
	wildtype	Ccr1$^{-/-}$	Ccr5$^{-/-}$	Ccr1/5$^{-/-}$	wildtype	Ccr1$^{-/-}$	Ccr5$^{-/-}$	Ccr1/5$^{-/-}$
glomerular								
CD4+	1.4 ± 0.7	*** 0.5 ± 0.2**	1.1 ± 0.7	0.8 ± 0.5	1.9 ± 0.8	1.6 ± 0.9	*** 1.0 ± 0.4**	1.5 ± 0.9
CD8+	2.3 ± 0.5	**** 0.7 ± 0.4**	1.6 ± 1.4	**** 1.2 ± 0.6**	1.6 ± 0.8	0.9 ± 0.4	*** 0.6 ± 0.2**	*** 0.5 ± 0.3**
CD11c+	3.1 ± 0.5	***** 0.6 ± 0.3**	2 ± 1.1	1.8 ± 1.4	3.6 ± 1.4	2 ± 1.5	**** 1.4 ± 0.5**	**** 0.9 ± 0.3**
F4/80+	1.3 ± 0.5	0.9 ± 0.7	1.3 ± 0.6	0.9 ± 0.5	2.6 ± 2	2.7 ± 1.3	1.9 ± 1	2.2 ± 1.2
tubulointerstitial								
CD4+	75 ± 19	*** 53 ± 8**	64 ± 18	68 ± 15	39 ± 9	34 ± 13	34 ± 10	36 ± 9
CD8+	81 ± 23	*** 44 ± 16**	66 ± 23	57 ± 22	53 ± 13	39 ± 14	*** 40 ± 6**	*** 32 ± 15**
CD11c+	82 ± 21	**** 35 ± 4**	**** 32 ± 7**	**** 38 ± 9**	36 ± 13	26 ± 5	27 ± 3	*** 19 ± 0.4**
F4/80+	85 ± 25	63 ± 25	63 ± 20	*** 55 ± 18**	95 ± 37	68 ± 24	78 ± 27	71 ± 23

points) did not show genotype specific differences and a further reduction of allograft infiltration due to double deficiency was restricted to CD11c^{+} cells in the tubulointerstitium at 42d post transplantation. Representative micrographs are shown in **Figures 34-37** (see appendix 7.2).

3.1.4 Analysis of alloreactive antibody titers in renal allograft recipients

Two recent publications suggested that Ccr5 deficiency in allograft recipients is leading to increased humoral rejection [113, 114]. Therefore, the levels of alloreactive IgG and IgM antibodies were measured in plasma samples from C57BL/6 wildtype and Ccr5$^{-/-}$ mice, which had either been immunized by intraperitoneal injection with BALB/c splenocytes or which had received a BALB/c renal allograft 42 days ago. Plasma from naive mice was used to determine baseline levels. Immunization with splenocytes as well as allografting induced a significant increase in alloreactive antibody levels, which was unaffected by the presence or absence of Ccr5 (**Figure 19**). At least for renal allografting experiments with the strain combination BALB/c

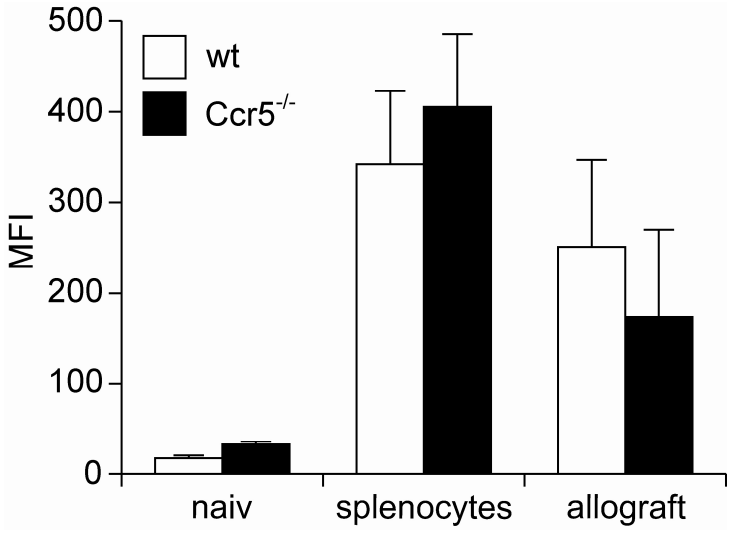

Figure 19. Effect of Ccr5 deficiency on alloreactive antibody titers. Plasma from C57BL/6 wt (n=3) and $Ccr5^{-/-}$ (n=3) mice immunized with BALB/c splenocytes or plasma from C57BL/6 wt (n=5) and $Ccr5^{-/-}$ (n=3) renal allograft recipients 42 days after transplantation was used to determine alloreactive antibody levels by flow cytometry as described in Materials and Methods. Plasma from naive mice was used as control. MFI, mean fluorescence intensity.

donors and C57BL/6 recipients no evidence was found for increased humoral rejection in Ccr5-deficient recipients at 42 days after transplantation.

3.1.5 Gene expression analysis in renal allografts

To further investigate the role of Ccr1 and Ccr5 in renal allograft rejection, mRNA levels of selected marker genes were determined by real-time RT-PCR in grafts from wildtype, $Ccr1^{-/-}$, $Ccr5^{-/-}$ and $Ccr1^{-/-}$/$Ccr5^{-/-}$ recipients at 7d and 42d after transplantation (**Figures 20-23**). A first set of markers analyzed included the cytokines Ifng and Tnf, the chemokine Cxcl10 (interferon-inducible protein 10, IP-10), the Th1-specific transcription factor Tbx21 (T-bet) and the interleukin Il12a (encoding the p35 subunit of IL-12). These genes have been implicated in proinflammatory and Th1-driven immune responses (**Figure 20**). The observed expression profiles – except for Il12a – reflected this common feature: a high expression during the acute phase was followed by a considerable lower level of expression during the chronic phase of rejection. In contrast, Il12a showed relatively low expression levels and increased moderately between 7 and 42 days. These changes demonstrate an overall reduction of the proinflammatory/Th1-type milieu within the allografts from acute to chronic phase of rejection. In addition, acute phase mRNA levels of Ifng, Tnf and Cxcl10 were significantly decreased in