Ccr1$^{-/-}$ and Ccr5$^{-/-}$ recipients compared to wildtype recipients indicating a further attenuation of Th1-type immune responses in acute phase allografts from single-deficient recipients. Surprisingly, mRNA levels of Ifng, Tnf and Cxcl10 were similar in wildtype and Ccr1$^{-/-}$/Ccr5$^{-/-}$ recipients during acute phase. In chronic phase of rejection a significant rise in the amounts of Tnf and Cxl10 mRNA above wildtype level was detected, suggesting ongoing acute phase or delayed Th1-type immune responses in double-deficient recipients. No significant differences were observed for the expression of the Th1-specific transcription factor Tbx21 and Il12a between the individual recipient groups at both time points.

The next panel of markers analyzed consisted of genes with established proinflammatory functions: the chemokines Ccl3 (MIP-1α), Ccl4 (MIP-1β) and Ccl5 (RANTES) [201]. CCL3 and CCL5 are ligands of the chemokine receptors CCR1 and CCR5 while CCL4 only binds to CCR5. Ccr2 and its main ligand Ccl2 (MCP-1) were included in this panel because of their importance for the recruitment of monocytes to sites of inflammation [202]. Furthermore, Il2 mRNA expression was analyzed due to its central role as an immune regulator [203]. IL-2 is an autocrine growth factor for T cells and promotes the proliferation of B cells in the presence of additional factors like IL-4. Furthermore, IL-2 shows Th1-associated functions by stimulating the synthesis of IFNγ and inducing of TNFα secretion. Expression of the IL-2 receptor was also proposed to be diagnostic for acute renal allograft rejection in human patients [204]. In accordance with these data, the expression profile observed for Il2 was similar to those found for Ifng and Tnf (**Figure 20**). Moreover, there was a significant reduction during acute phase in single-deficient recipients whereas mRNA levels at 42 days were considerably lower than at 7 days without any differences between the individual recipient groups.

The mRNA levels for Ccl2 were decreased in renal allografts from Ccr1$^{-/-}$ and Ccr5$^{-/-}$ recipients compared to wildtype recipients at 7 days after transplantation suggesting reduced infiltration by monocytes in allografts of single-deficient recipients during the acute phase of rejection

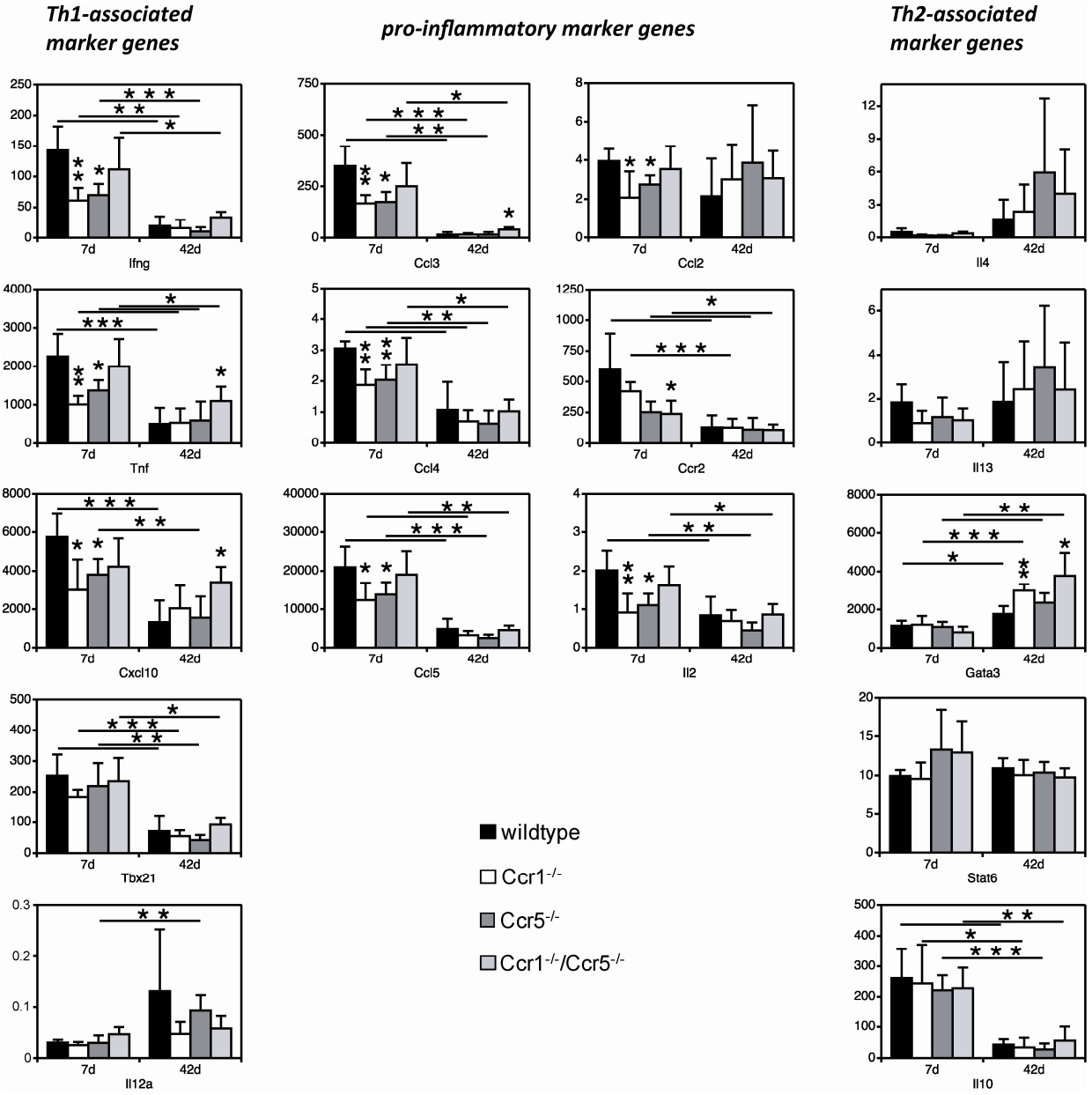

Figure 20. Effect of chemokine receptor deficiency on mRNA levels of selected Th1 (Ifng, Tnf, Cxcl10, Tbx21 and Il12a), pro-inflammatory (Il2, Ccl3, Ccl4, Ccl5, Ccl2 and Ccr2) and Th2 (IL-4, Il10, Il13, Gata3, Stat6) marker gene sets in renal allografts. Real-time RT-PCR was used to quantify intragraft mRNA levels for marker genes. Bar diagrams show mean values ± SD of 18S rRNA normalized expression for allografts from wildtype, $Ccr1^{-/-}$, $Ccr5^{-/-}$ and $Ccr1^{-/-}/Ccr5^{-/-}$ recipients at 7 and 42 days after transplantation. Asterisks on top of a horizontal line indicate statistically significant differences between the acute and the chronic phase for animals with the same genotype, whereas asterisks on top of an error bar indicate statistically significant differences between animals with different genotypes at the same time point (either 7d or 42d). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

(**Figure 20**). Additionally, a trend towards decreased Ccr2 mRNA was observed at this early time point in $Ccr1^{-/-}$ and $Ccr5^{-/-}$ recipients, supporting the idea of reduced monocyte infiltration.

While Ccl2 levels in allografts of $Ccr1^{-/-}/Ccr5^{-/-}$ recipients were comparable to wildtype levels at 7 days, expression of Ccr2 mRNA was significantly reduced in double-deficient recipient allografts at this time point. At 42 days post transplantation the levels of Ccr2 were noticeably lower in all recipient groups than at 7 days and differences in the mRNA expression of Ccr2 and Ccl2 disappeared between the recipient groups.

Ccl3 and Ccl5 are ligands of both CCR1 and CCR5 and have been implicated in Th1-type immune responses [205]. Expression profiles for these two genes resembled those found for the Th1-type immune response associated genes Ifng and Tnf: high expression during acute phase with decreased levels in $Ccr1^{-/-}$ and $Ccr5^{-/-}$ recipients, comparable levels between wildtype and double-deficient recipients and drastically reduced expression in chronic phase without differences amongst the individual recipient groups except for case of Ccl3 where double-deficient recipients expressed significantly higher levels than wildtype recipients. Interestingly, Ccl4, a ligand of CCR5, displayed the same expression profile as Ccl3 and Ccl5 suggesting Ccl4 to be a Th1-type immune response gene – at least in the setting of renal allograft rejection (**Figure 20**).

The marker panel for Th2-type immune response-associated genes included the cytokines IL-4, Il10 and Il13 which are primarily produced by Th2 cells, the Th2 cell-specific transcription factor Gata3 and also Stat6, a transcription factor mediating of IL-4 and IL13 signaling [206, 207]. IL-4 and Il13 mRNAs had comparable expression profiles. While both genes showed only low mRNA expression during acute phase without any differences between individual recipient groups, expression increased during chronic phase with the highest levels in the group of $Ccr5^{-/-}$ recipients. However, none of these differences reached statistical significance due to large intragroup variations. Gata3 mRNA showed a significant increase in all recipient groups from 7 to 42 days and was significantly increased in $Ccr1^{-/-}$ and $Ccr1^{-/-}/Ccr5^{-/-}$ recipients at 42 days post transplantation. In contrast to the aforementioned Th2-type markers mRNA expression of the

transcription factor Stat6 remained constant over time and differences between the recipient groups were not observed. Interestingly, the mRNA expression profile of Il10 resembled those observed for Th1-type marker genes and did not show differences among individual recipient groups (**Figure 20**).

In summary, the acute phase of rejection is characterized by an increased Th1-type gene expression pattern, whereas Th2-type marker genes are increased during the chronic phase of rejection but did not reach the levels of Th1-type genes during acute rejection. While single-deficient recipients showed decreased Th1-type marker expression during acute phase (Ifng, Tnf, Cxcl10, Il2, Ccl3 and Ccl5), significant differences in double-deficient recipients were limited to decreased expression of Ccr2 in acute phase and increased expression of Tnf, Cxcl10 and Ccl3 during chronic phase. In contrast to single-deficient recipients, double-deficient recipients often showed expression levels for Th1-marker genes (Ifng, Tnf, Cxcl10, Il2, Ccl3 and Ccl5) that were comparable to wildtype recipients during acute phase. During chronic phase a trend towards Th2-type immune responses was observed for $Ccr5^{-/-}$ recipients by increased expression of IL-4 and Il13 (**Figure 20**).

Skewing of immune responses to Th17-type has been implicated in the development of acute transplant rejection in humans [208, 209]. Therefore, the effect of Ccr1-, Ccr5- and double deficiency on Th17-type immune response-associated marker gene expression was determined in our murine renal allograft model (**Figure 21**). The marker gene set for this purpose consisted of the cytokine Il17a (produced by Th17 cells), the Th17-specific transcription factor Rorc, the cytokines Tgfb1, Il6 and Il23 which induce Th17 cell proliferation and the chemokine Ccl20 along with its corresponding receptor Ccr6 that is expressed by the majority of IL17 producing cells [210, 211]. The expression of Il17a mRNA was remarkably reduced in $Ccr1^{-/-}$, $Ccr5^{-/-}$ and $Ccr1^{-/-}$/$Ccr5^{-/-}$ recipients at 7 days after transplantation but did not reach statistical significance (**Figure 21**). Furthermore, at 42 days Il17a expression levels in $Ccr1^{-/-}$ recipients exceeded those

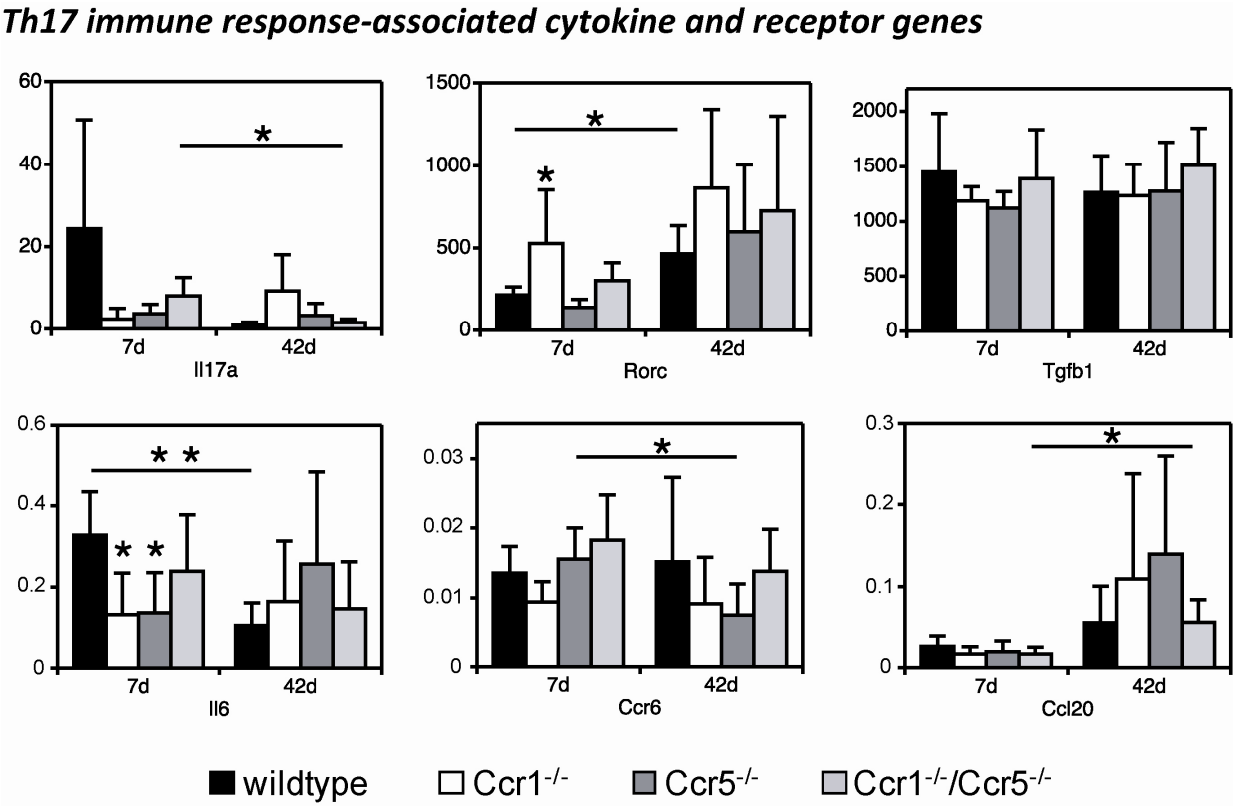

Figure 21. Effects of chemokine receptor deficiency on mRNA levels of selected Th17 (Il17a, Rorc, Tgfb1, Il6, Ccr6 and Ccl20) marker genes in renal allografts. Real-time RT-PCR was used to quantify intragraft mRNA levels for marker genes. Bar diagrams show mean values ± SD of 18S rRNA normalized expression for allografts from wildtype, $Ccr1^{-/-}$, $Ccr5^{-/-}$ and $Ccr1^{-/-}/Ccr5^{-/-}$ recipients at 7 and 42 days after transplantation. Asterisks on top of a horizontal line indicate statistically significant differences between the acute and the chronic phase for animals with the same genotype, whereas asterisks on top of an error bar indicate statistically significant differences between animals with different genotypes at the same time point (either 7d or 42d). * $p<0.05$, ** $p<0.01$.

of the other analyzed recipient groups. Interestingly, the increase of Il17a at 42d was associated with a significant elevation of Rorc in $Ccr1^{-/-}$ recipients during the acute phase of rejection. These findings suggest a Th17 skewing of immune responses in allografts of $Ccr1^{-/-}$ recipients. However, the expression profile of Tgfb1 remained constant over time and did not show changes between recipient groups. In addition, Il6 mRNA expression was relatively low, decreased in $Ccr1^{-/-}$ and $Ccr5^{-/-}$ at 7 days and potential differences among recipient groups at 42 days were washed by considerable variability. Ccr6 mRNA expression was also quite low and remained constant over time with the exception of $Ccr5^{-/-}$ recipients which showed a decrease

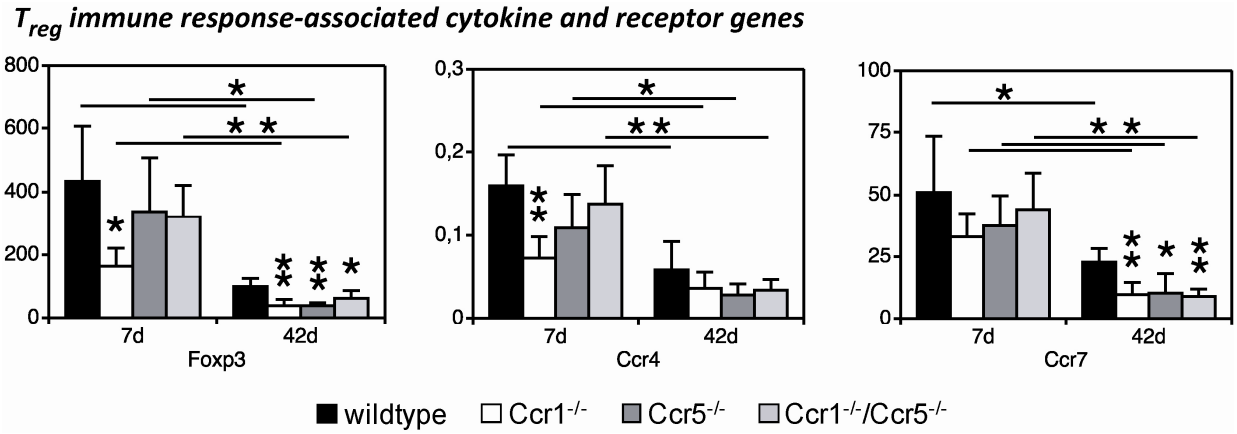

Figure 22. Effects of chemokine receptor deficiency on mRNA levels of selected Treg (Foxp3, Ccr4 and Ccr7) marker genes in renal allografts. Real-time RT-PCR was used to quantify intragraft mRNA levels for marker genes. Bar diagrams show mean values ± SD of 18S rRNA normalized expression for allografts from wildtype, $Ccr1^{-/-}$, $Ccr5^{-/-}$ and $Ccr1^{-/-}/Ccr5^{-/-}$ recipients at 7 and 42 days after transplantation. Asterisks on top of a horizontal line indicate statistically significant differences between the acute and the chronic phase for animals with the same genotype, whereas asterisks on top of an error bar indicate statistically significant differences between animals with different genotypes at the same time point (either 7d or 42d). * $p<0.05$, ** $p<0.01$.

in Ccr6 mRNA levels at 42 days compared to 7 days. Moreover, no significant differences were found for Ccr6 mRNA expression within the recipient groups. Ccl20 mRNA expression did not show differences between recipient groups at both time points, but an increase from 7 to 42 days in $Ccr1^{-/-}/Ccr5^{-/-}$ recipients. However, increased mRNA expression of the chemokine Ccl20 was not accompanied by an increase in Ccr6 mRNA levels (**Figure 21**).

Regulatory T cells exert immunosuppressive roles and were suggested to prolong allograft function [212]. Therefore, the influence of chemokine receptor Ccr1-, Ccr5- and double deficiency on expression of marker genes for regulatory T cells was analyzed (**Figure 22**). Regulatory T cells specifically express the transcription factor Foxp3 and Treg precursors express the chemokine receptor Ccr7 which is involved in homing of naive and regulatory T cells to secondary lymphoid organs as well as migration of dendritic cells into lymph nodes via afferent lymphatic vessels [213]. Recently, it has been shown by Lee *et al.* that the chemokine receptor Ccr4 is required for recruitment of tolerance mediating regulatory T cells to cardiac allografts [214]. Expression levels of these markers decreased over time in all recipient groups

and Foxp3 as well as Ccr4 mRNA levels were considerably lower in $Ccr1^{-/-}$ recipients at 7 days (**Figure 22**). Additionally, expression of Ccr7 and Foxp3 was significantly reduced in allografts from $Ccr1^{-/-}$, $Ccr5^{-/-}$ and $Ccr1^{-/-}/Ccr5^{-/-}$ recipients at day 42. These results indicate reduced suppression of immune responses in the analyzed chemokine receptor deficient recipients during chronic phase of rejection and in $Ccr1^{-/-}$ recipients already during acute phase. Reduced suppression could lead to enhanced effects of Th1- or Th2-skewed immune responses.

Besides T cells, macrophages constitute a major fraction of infiltrating leukocytes within the allograft. For that reason, the effect of chemokine receptor deficiency on the expression of certain macrophage marker genes in renal allografts was tested. Numbers of $F4/80^{+}$ cells (mostly macrophages) remained constant in single-deficient recipients compared to wildtype recipients. Therefore, mRNA expression of macrophage phenotype associated marker genes was analyzed to determine, whether loss of Ccr1 and/or Ccr5 influences macrophage polarization.

Genes induced in classically activated macrophages (CAMs) are Tnf, Ifng and the chemokines Ccl3, Ccl4, Ccl5 along with Cxcl10 (**Figure 20**). Interestingly, all of these genes were significantly reduced in renal allografts from $Ccr1^{-/-}$ as well as $Ccr5^{-/-}$ recipients at 7 days and showed lower expression at 42 days than at 7 days while Nos2, another marker for CAMs, remained constant over time showing no differences between recipient groups (**Figure 23**).

A strong increase for IL-4 and Il13 was observed between 7 and 42 days, which was paralleled by a decrease of marker expression for Th1-type immune responses and classical macrophage activation, respectively. Since IL-4 and IL-13 are known to induce alternatively activated macrophages (AAM) it was tempting to speculate whether intragraft macrophages might be skewed towards such a phenotype. Therefore, the intragraft mRNA expression of several signature genes for AAM including Arg1, Chi3l3, Retnla, Mrc1, Mrc2 and Fcer2a was tested.

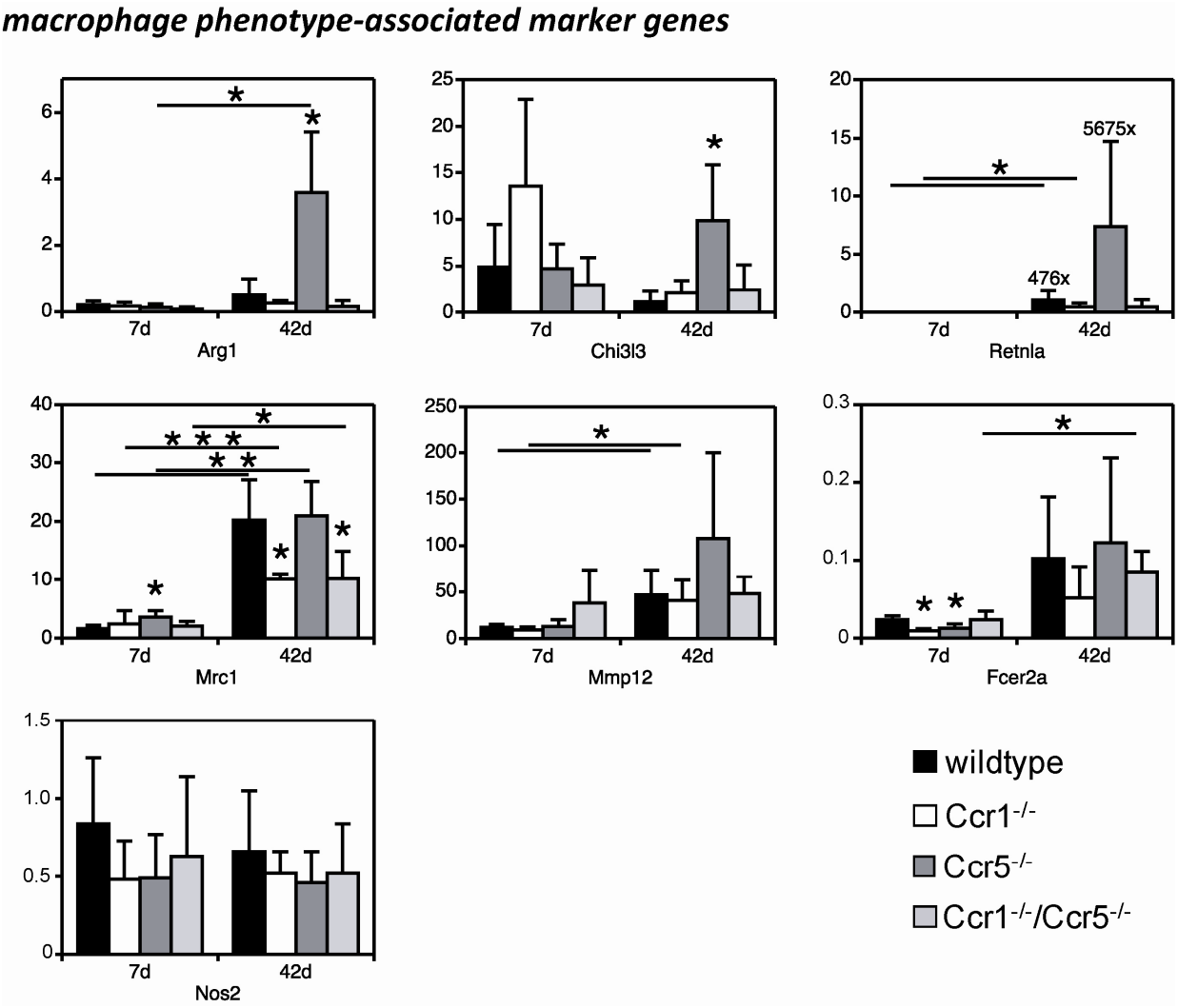

Figure 23. Effects of chemokine receptor deficiency on mRNA expression of macrophage phenotype-associated marker genes (Arg1, Chi3l3, Retnla, Mrc1, Mmp12, Fcer2a and Nos2) in renal allografts. Real-time RT-PCR was used to quantify intragraft mRNA levels for marker genes. Bar diagrams show mean values ± SD of 18S rRNA normalized expression for allografts from wildtype, $Ccr1^{-/-}$, $Ccr5^{-/-}$ and $Ccr1^{-/-}/Ccr5^{-/-}$ recipients at 7 and 42 days after transplantation. Asterisks on top of a horizontal line indicate statistically significant differences between the acute and the chronic phase for animals with the same genotype, whereas asterisks on top of an error bar indicate statistically significant differences between animals with different genotypes at the same time point (either 7d or 42d). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Surprisingly, allografts from $Ccr5^{-/-}$ recipients displayed considerably increased expression of Arg1, Chi3l3 and Retnla at 42 days while no statistically significant differences were observed at 7 days between the groups (**Figure 23**). Mannose receptor C type 1 (Mrc1) mRNA levels were

significantly increased in Ccr5$^{-/-}$ recipients at 7 days. During the chronic phase, however, Mrc1 mRNA levels in Ccr5$^{-/-}$ recipients remained at wildtype niveau, while significantly less Mrc1 expression was observed in Ccr1$^{-/-}$ and double-deficient recipients.

The mRNA expression profiles observed for Mmp12, a matrix metalloproteinase associated with alternatively activated macrophages [215], supported the findings for Arg1, Chi3l3 and Retnla in Ccr5$^{-/-}$ recipients (**Figure 23**). The levels of Mmp12 mRNA were low during acute phase and increased over time in all groups. Mmp12 mRNA expression was highest in the group of Ccr5-deficient recipients but did not reach statistical significance due to large intragroup variation.

The next gene analyzed was Fcer2a (CD23), which encodes the Fcε receptor 2a. Mantovani *et al.* [216] proposed Fcer2a to be a signature gene for macrophages with an alternative activation phenotype. The mRNA expression of Fcer2a was determined in our renal allograft samples. Ccr1 and Ccr5-deficient recipients demonstrated reduced Fcer2a mRNA levels at 7d post transplantation (**Figure 23**). Furthermore, Fcer2a mRNA expression was diminished in Ccr1- and Ccr1-/Ccr5-deficient recipients, but allografts from Ccr5$^{-/-}$ mice had Fcer2a levels comparable to animals of the wildtype group.

In summary, macrophages in renal allografts from Ccr5$^{-/-}$ recipients appear to be skewed towards an alternative activation phenotype – a finding which might explain the beneficial effects of Ccr5 deficiency on allograft rejection.

3.1.6 Protein expression of alternatively activated macrophage (AAM) markers in renal allografts

To confirm the mRNA data on alternative macrophage activation at the protein level immunohistochemical stainings for Arg1, Chi3l3 and Mrc1 were performed in allograft sections taken from wildtype and $Ccr5^{-/-}$ recipients at 42 days post transplantation. Representative micrographs are shown in **Figure 24A**. The quantitative evaluation of all 3 stainings is shown in **Figure 24B**. Significantly increased numbers of $Arg1^{+}$ and $Chi3l3^{+}$ cells were observed in the tubulointerstitium as well as an increase of $Mrc1^{+}$ and $Chi3l3^{+}$ cells in the glomerular

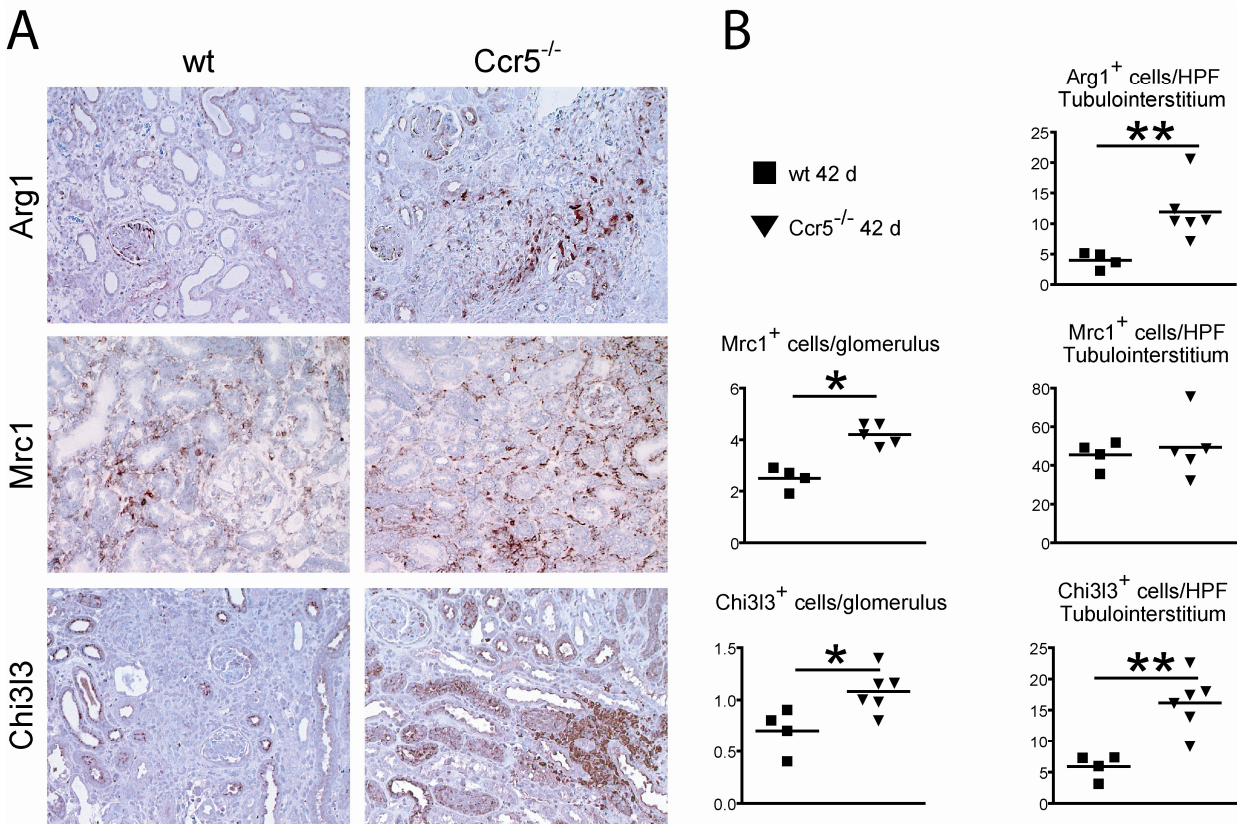

Figure 24A-B. Effect of Ccr5 deficiency on the expression of signature genes for alternatively activated macrophages in chronically rejecting renal allografts. Renal allografts from wildtype (wt) and $Ccr5^{-/-}$ recipients were collected 42 days after transplantation and sections were stained by immunohistochemistry for arginase 1 (Arg1), mannose receptor C type 1 (Mrc1) and chitinase 3-like 3 (Chi3l3). All 3 markers are associated with AAM. **A)** Representative micrographs of the immunohistochemical stainings. **B)** Quantitative evaluation of the immunohistochemical staining exemplified in A). Asterisks indicate significance between wildtype and $Ccr5^{-/-}$ groups (* $p<0.05$, ** $p<0.01$).

compartment of renal allografts from $Ccr5^{-/-}$ recipients at 42d. Due to the fact that podocytes residing within the glomerular compartment stained positive for Arg1 made it impossible to determine the number of $Arg1^{+}$ macrophages within this compartment. These protein data strongly support the hypothesis that Ccr5 deficiency leads to a change of macrophage phenotype during the chronic phase of renal allograft rejection.

3.2 Effects of Ccr5 deficiency on macrophage polarization

Loetscher *et al.* showed that the chemokine receptor CCR5 is highly abundant on $CD4^{+}$ Th1 cell clones [61]. Therefore a lack of CCR5 could lead to diminished Th1 immune responses by reduced chemotaxis or signaling in Th1 cells. This finding and the marked increase of signature gene expression indicative for alternatively activated macrophages (Arg1, Chi3l3, Retnla and Mrc1) in renal allografts from $Ccr5^{-/-}$ recipients at day 42 led to the hypothesis that Ccr5 deficiency might induce a shift in macrophage polarization already under basal conditions. To test this hypothesis, the expression of specific markers for the alternative activation pathway was tested by real-time RT-PCR and/or flow cytometry in wildtype and $Ccr5^{-/-}$ macrophages of different origins: spleen and cultivated splenocytes, elicited peritoneal macrophages and bone marrow-derived macrophages (BMDM). These cellular systems provide macrophage populations with different activation states and degrees of purity – allowing the study of effects due to other cell populations and inflammatory stimuli on macrophage polarization.

3.2.1 Spleens from $Ccr5^{-/-}$ mice show increased expression of AAM marker genes

Total RNA was isolated from spleens of C57BL/6 wildtype and $Ccr5^{-/-}$ mice ($n \geq 5$) and subjected to reverse transcription. The cDNA obtained by this procedure was then used as a template for real-time RT-PCR. Loss of Ccr5 was accompanied by significantly increased mRNA expression of the AAM signature genes Arg1, Mmp12, Mrc1 and Mrc2 (**Table 6**). In addition, mRNAs for the

Table 6. Effect of Ccr5 deficiency on AAM and Th2 marker gene expression in spleens of unchallenged mice. Real-time RT-PCR was used to quantify mRNA expression in spleens from wildtype and $Ccr5^{-/-}$ C57BL/6 mice (n≥5 per group, all female, all 20 weeks of age). Shown are means and corresponding standard deviations. Ratios are fold changes of $Ccr5^{-/-}$ to wt means. Significance values (p) were calculated using Student's t test and values reaching significance levels ($p < 0.05$) are highlighted in bold letters.

gene	marker type	wildtype		Ccr5-/-		ratio Ccr5-/- vs wt	p value
		mean relative expression	SD	mean relative expression	SD		
Arg1	AAM	0.0005	0.0002	0.0019	0.0011	**3.92**	**0.0041**
Chi3l3	AAM	0.64	0.20	1.43	1.19	2.25	0.2109
Retnla	AAM	0.44	0.16	0.72	0.45	1.64	0.2504
Mmp12	AAM	0.20	0.06	0.61	0.42	**3.02**	**0.0186**
Mrc1	AAM	11.30	1.09	36.57	16.37	**3.24**	**0.0161**
Mrc2	AAM	0.15	0.03	0.30	0.16	**1.97**	**0.0407**
Msr1	AAM	0.23	0.07	0.44	0.23	1.89	0.0772
Tgfb1	AAM / Th2	1.47	0.08	4.02	1.63	**2.73**	**0.0015**
Il10	AAM / Th2	0.010	0.004	0.067	0.068	**6.57**	**0.0354**
Gata3	Th2	0.11	0.04	0.25	0.08	**2.27**	**0.0010**
Stat6	Th2	12.37	1.57	34.64	18.44	**2.80**	**0.0067**
Itgam	macrophages	4.28	0.83	10.97	8.51	2.56	0.0608

cytokines Tgfb1 and Il10, which have been associated with regulatory macrophages [2, 155], were also markedly up-regulated (2.7 and 6.6 fold, respectively) in spleens from mice lacking Ccr5. mRNAs for the AAM marker genes Chi3l3, Retnla and Msr1 were increased in $Ccr5^{-/-}$ spleens but the differences did not reach statistical significance. To assess the effect of Ccr5 deficiency on Th2-polarization mRNA expression of the classical Th2 cytokine Il4 and the Th2-associated transcription factors Gata3 and Stat6 was analyzed. Expression of Il4 mRNA was below detection limit (data not shown) but both transcription factors were significantly increased in spleens of $Ccr5^{-/-}$ mice indicating Th2 skewing already under basal conditions in mice lacking Ccr5. Taken together, these findings suggest that macrophages are already polarized towards an alternative activation phenotype in spleens from unchallenged Ccr5-deficient mice.

3.2.2 In vitro cultivated Ccr5$^{-/-}$ splenocytes show enhanced expression of AAM marker genes

Due to the findings in spleens from unchallenged mice, splenocytes were used in a first attempt to obtain an *in vitro* system useable for the analysis of the mechanisms underlying alternative macrophage activation in Ccr5$^{-/-}$ mice. In a first pilot experiment, splenocytes from one wildtype and two Ccr5$^{-/-}$ mice were cultured in tissue culture 6 well plates for 24h at a density of 5x10^6 per well and AAM signature gene expression was determined using real-time RT-PCR (**Table 7**). The mRNA expression of Chi3l3 and Retnla, two widely accepted markers for alternatively activated macrophages, showed a marked increase (23.1 and 29.9 fold, respectively) in cultivated Ccr5$^{-/-}$ compared to wildtype splenocytes. This increase was even more pronounced than in spleens from unchallenged mice (compare **Table 6**). Furthermore, Arg1 and Msr1 mRNAs showed up-regulation in Ccr5$^{-/-}$ splenocytes on a comparable level to direct lysates from

Table 7. Effect of Ccr5 deficiency on AAM marker gene expression in cultivated splenocytes. Real-time RT-PCR was used to quantify mRNA expression of splenocytes from wildtype (n=1) and Ccr5$^{-/-}$ (n=2) C57BL/6 (male, 16-19w) mice cultivated for 24h in 6 well plates with 3 ml SSB medium per well. Shown are means and ratios are fold changes of Ccr5$^{-/-}$ to wildtype means. Compare Table 6 for expression data in direct lysates of total spleens.

probe	marker type	wildtype mean relative expression	Ccr5-/- mean relative expression	ratio
Arg1	AAM	0.006	0.021	3.4
Chi3l3	AAM	0.06	1.49	23.2
Retnla	AAM	0.67	20.1	29.9
Mmp12	AAM	26.0	23.4	-1.1
Mrc1	AAM	1.04	1.37	1.3
Msr1	AAM	1.78	5.94	3.3
Il10	AAM / Th2	0.20	0.41	2.0
Stat6	Th2	6.71	7.03	1.0
Gata3	Th2	0.29	0.30	1.0
Itgam	macrophages	18.76	116.94	6.23

spleens while other AAM markers which showed up-regulation in Ccr5$^{-/-}$ direct lysates were not differentially regulated upon *in vitro* cultivation (Mmp12 and Mrc1). The difference in wildtype and Ccr5$^{-/-}$ splenocytes for Il10 mRNA expression was also less pronounced and the difference observed for mRNA expression of the Th2-associated transcription factors Gata3 and Stat6 was lost upon *in vitro* cultivation. Interestingly, mRNA for Itgam (protein name CD11b) showed an increase (6.2 fold) in Ccr5$^{-/-}$ spleen cells compared to wildtype splenocytes after cultivation.

3.2.3 In vitro cultivated Ccr5$^{-/-}$ splenocytes show increased frequencies of CD206^{+} cells

To confirm these findings on protein level, splenocytes were cultured for 24h in Teflon bags to allow easy detachment of adherent cells for subsequent flow cytometric analysis of AAM marker expression. In this experiment, antibodies directed against CD204 and CD206 were used to quantify the effect of Ccr5 deficiency on alternative macrophage activation. Both proteins are known to be up-regulated in alternatively activated macrophages and have a function as phagocytic receptors [160]. The antibody directed against CD204 recognizes an epitope present on macrophage scavenger receptors 1 and 2 (gene symbols Msr1 and Msr2) and the antibody directed against CD206 recognizes the mannose receptor C type 1 (gene symbol Mrc1).

Analysis of flow cytometry data revealed that Ccr5$^{-/-}$ splenocytes have higher frequencies of CD11b^{+} (4.2% *vs.* 2.6%) and CD206^{+} (5.9% *vs.* 3.8%) cells than splenocytes from wildtype mice (**Figure 25**, lower panel). Frequencies of CD204^{+} cells did not differ between wildtype and Ccr5$^{-/-}$ splenocytes (**Figure 25**, upper panel). Surprisingly, subpopulations with double-positive (CD11b^{+}/CD204^{+} or CD11b^{+}/CD206^{+}) staining could not be identified. This finding suggests that a subpopulation other than CD11b^{+} macrophages in the spleen expresses the alternative activation markers CD204 (Msr1/Msr2) and CD206 (Mrc1).

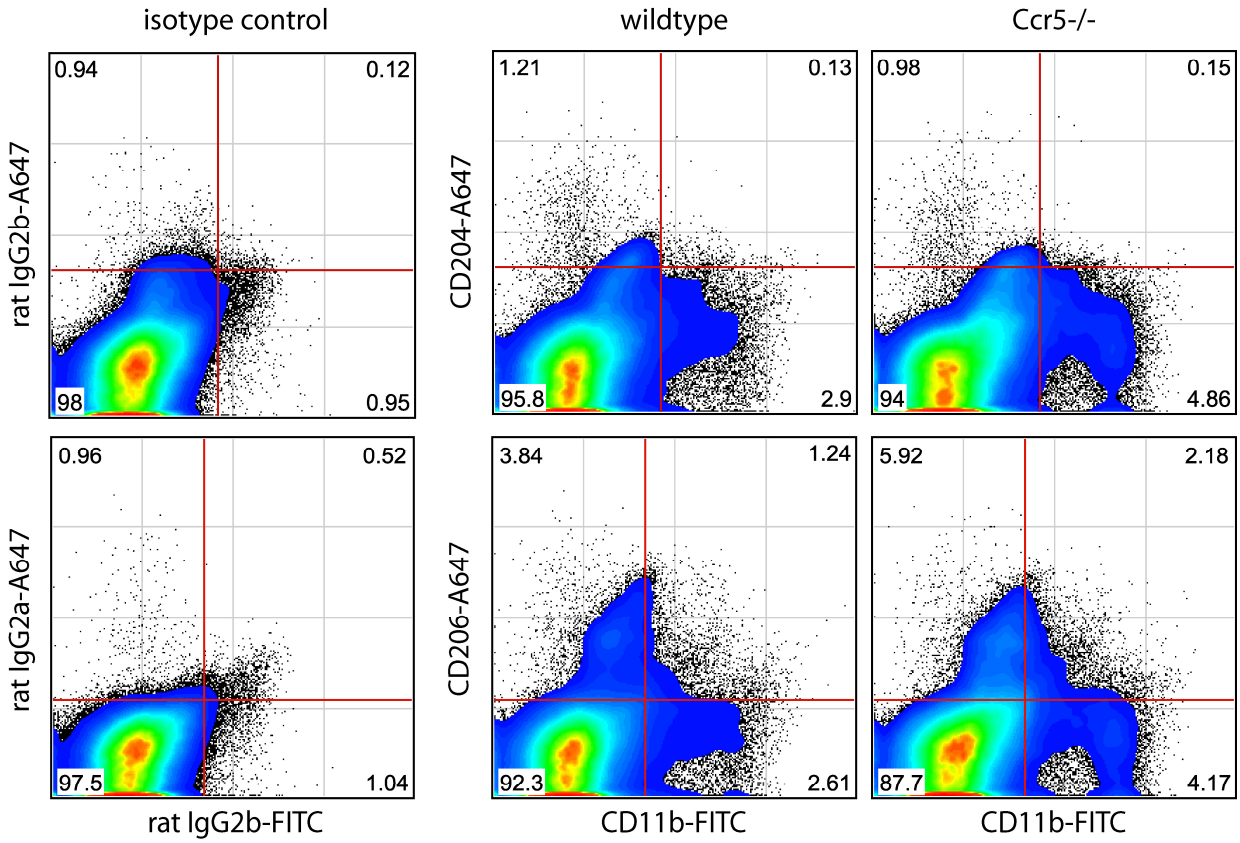

Figure 25. Effect of Ccr5-deficiency on surface expression of AAM markers CD204 (macrophage scavenger receptors 1 and 2) and CD206 (mannose receptor C type 1) on splenocytes from unchallenged mice. Splenocytes from unchallenged mice (C57BL/6, male, 18w, n=1) were harvested and cultivated in Teflon bags at a density of $1.0x10^6$ cells/ml in SSB medium. After resting for 24h at 37°C and 10% CO_2 the cells were harvested, remaining erythrocytes were lysed and surface staining of CD11b and CD204 (upper panel) or CD206 (lower panel) was performed using fluorochrome-conjugated antibodies as described in materials and methods. Isotype-matched control antibodies were used to check unspecific binding (plots on the left). 300,000 events were counted in every sample. For analysis a gate was set in an FSC-SSC plot to exclude debris and remaining erythrocytes and quadrant gates were set according to isotype control antibodies.

3.2.4 Polarization of cultivated peritoneal macrophages

Observations made in renal allograft rejection and in the spleen under basal conditions show that Ccr5 deficiency results in a shift towards alternative macrophage activation. To further explore the possibility that this effect might be a general phenomenon of Ccr5 deficiency, mRNA expression of macrophage activation marker genes was analyzed in an inflammatory

model of experimental peritonitis. Intraperitoneal injection of sterile thioglycollate solution causes the rapid influx of granulocytes, consisting primarily of neutrophils, in the first few hours after injection. Between day 3 and 5 after injection, peritoneal cells mainly consist of macrophages. Thus, harvesting peritoneal cells by lavage at this time point yields cell populations consisting predominantly of macrophages [217, 218].

In a first experiment, peritoneal lavage cells (PLCs) from 4 mice per genotype were harvested 4 days after thioglycollate-injection, pooled and cultured for 2 days. Non-adherent cells were removed 24h after plating by washing, while adherent cells (mostly macrophages) were further incubated. Subsequently, peritoneal macrophages were incubated for 24h with or without IL-4, which is the typical inductor of alternative macrophage activation. Already under unstimulated conditions, considerably increased AAM marker mRNA expression (Arg1) was observed in $Ccr5^{-/-}$ peritoneal macrophages compared to wildtype cells (**Figure 26A**). Upon IL-4 stimulation Arg1 mRNA levels increased dramatically, but the differences between wildtype and

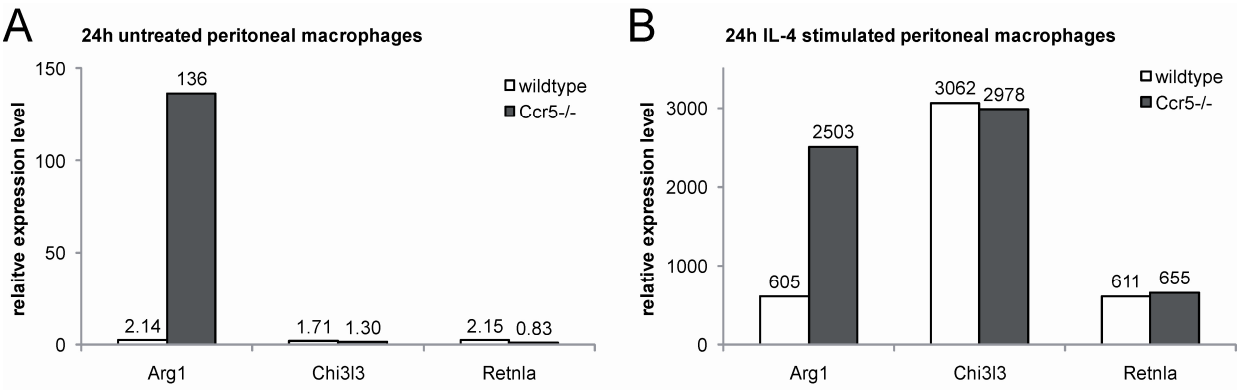

Figure 26A-B. Effect of Ccr5 deficiency on gene expression of macrophage polarization markers in thioglycollate-elicited peritoneal lavage cells (PLCs) after *in vitro* cultivation. PLCs were harvested from the peritoneal cavity 4 days after i.p. injection of 2.5 ml 4% thioglycollate solution. Cells from 4 mice (C57BL/6) were pooled, plated into the wells of 6 well plates ($10x10^6$ cells/well) and incubated in 3 ml PM medium/well for 24h at 37°C and 5% CO_2. After this time, non-adherent cells were removed, while adherent PLCs (mostly macrophages) were further incubated for 24h. Following this initial *in vitro* cultivation period, medium was replaced and cells were either left untreated (**A**) or stimulated with 100 ng/ml IL-4 (**B**) for 24h. Afterwards, total RNA was prepared and mRNA expression of marker genes for AAMs (Arg1, Chi3l3 and Retnla) was analyzed by real-time RT-PCR.

Ccr5-deficient cells were less pronounced as compared to the unstimulated situation (**Figure 26B**). Although, mRNA expression of the AAM marker genes Chi3l3 and Retnla increased dramatically upon IL-4 stimulation, no differential expression was observed between wildtype and $Ccr5^{-/-}$ PLCs.

Next, experiments were made to verify these findings on a single animal basis, but due to large intragroup variability – especially when using female mice – these results could not be replicated yet (data not shown).

3.2.5 Generation and phenotypic evaluation of BMDM

3.2.5.1 Phenotype of BMDM

Splenocytes and peritoneal lavage cells as well represent highly heterogeneous cell populations making it difficult to attribute specific effects to a certain cell population. Therefore, a system was established to generate large quantities of highly pure macrophages. Bone marrow cells were cultivated in petri dishes using medium with recombinant murine M-CSF (macrophage colony stimulating factor) to drive macrophage differentiation. The cells were differentiated for 8d and then incubated for 48h in medium without M-CSF to obtain a non-proliferative state receptive to stimulation. To assess the polarization state of these BMDM mRNA levels of AAM and CAM signature genes as well as Th1 and Th2 associated gene markers were determined (**Table 8**). The mRNAs for Chi3l3, Msr2 and Il4ra were moderately increased (1.6x) and Retnla mRNA showed a 5.9x increase in $Ccr5^{-/-}$ BMDM. The remainder of the investigated AAM signature genes (Arg1, Mrc1, Mrc2, Mmp12, Trem2, Il10 and Stat6) was not differentially expressed in $Ccr5^{-/-}$ BMDM compared to wildtype BMDM. Il4 mRNA expression was below detection limit (data not shown). On the other hand, the Th1-associated marker gene Ccl5 showed a moderate 1.7x fold decrease in $Ccr5^{-/-}$ BMDM (**Table 8**). The Th1-associated marker genes Tnf and Ccl3 did not show differential expression between wildtype and $Ccr5^{-/-}$ BMDM

Table 8. Effects of Ccr5 deficiency on mRNA expression in unstimulated BMDM. BMDM were generated from male C57BL/6 mice (wildtype: n=3, $Ccr5^{-/-}$: n=2) by plating bone marrow cells on tissue culture dishes and cultivation for 8 days in BBM medium in the presence of recombinant murine M-CSF (20 ng/ml). After this time BMDM were harvested and cultivated for 48h in tissue culture 6 well plates with BBM medium without M-CSF (3 ml/well). Total RNA was prepared and subjected to real-time RT-PCR analysis.

probe	marker type	wildtype mean relative expression	Ccr5-/- mean relative expression	ratio Ccr5-/- vs wt
Arg1	AAM	0.17	0.18	1.07
Chi3l3	AAM	0.81	1.32	1.63
Retnla	AAM	5.72	33.8	**5.92**
Mrc1	AAM	206	169	-1.22
Mrc2	AAM	39.3	49.2	1.25
Mmp12	AAM	881	788	-1.12
Msr2	AAM	57.3	92.4	1.61
Trem2	AAM	265.3	259	-1.03
Il10	Th2, AAM	14.0	13.9	-1.01
Il4ra	Th2, AAM	160	271	1.69
Stat6	Th2, AAM	86.0	80.4	-1.07
Tnf	Th1	26.1	21.8	-1.20
Ccl3	pro-inflammatory, Th1	2.48	2.36	-1.05
Ccl4	pro-inflammatory	51.9	59.4	1.14
Ccl5	pro-inflammatory, Th1	14.5	8.41	-1.72
Itgam	macrophages	46.8	47.5	1.02
Emr1	macrophages	61.7	68.6	1.11
Csf1	MΦ proliferation	389	364	-1.07
Csf1r	MΦ proliferation	2132	2637	1.24

and the Th1/CAM signature genes Ifng, Il12a and Nos2 were expressed below detection limit (data not shown). In summary, mRNA expression of AAM and Th2 signature genes is only moderately increased in unstimulated $Ccr5^{-/-}$ BMDM compared to wildtype BMDM. However, mRNA expression levels of CAM- and Th1-associated markers were either very weak or tended to decrease in $Ccr5^{-/-}$ BMDM compared to wildtype BMDM in most cases. In addition, BMDM generated in this way showed marked mRNA expression of macrophage markers Itgam (protein name CD11b) and Emr1 (protein name F4/80) (**Table 8**). Nevertheless, no differential expression was found for the investigated macrophage markers between wildtype and $Ccr5^{-/-}$ BMDM. Interestingly, the highest mRNA expression levels were observed for the M-CSF gene

(Csf1) and its associated receptor Csf1r (**Table 8**). The high abundance of Csf1r, Emr1 and Itgam mRNAs suggests that the generated cell population consists predominantly of macrophages.

Since BMDM showed no genotype-specific differences for AAM marker expression under unstimulated conditions macrophage polarization was analyzed after stimulation with IL-4, which is the classical inductor of AAMs. Arginase converts L-arginine to urea and ornithine whereas the competing enzyme nitric oxide synthase generates nitrite and citrulline from the same substrate [219]. Therefore, arginase enzyme activity and NO production assays were established to determine macrophage polarization on a functional level. Urea production was determined in protein lysates and nitrite concentrations in supernatants of wildtype and $Ccr5^{-/-}$ BMDM under vehicle- and IL-4 stimulated conditions (**Figure 27A-C**).

Under unstimulated conditions no significant differences were observed for mRNA expression of the AAM signature genes (**Figure 27A)** and for NO production between wildtype and $Ccr5^{-/-}$ BMDM. However, arginase enzyme activity showed a slight but significant increase in $Ccr5^{-/-}$ BMDM without IL-4 stimulation (**Figure 27B**). Upon IL-4 stimulation mRNA expression of the AAM signature genes Arg1, Chi3l3 and Retnla increased dramatically in both groups and surprisingly, mRNA expression of Chi3l3 and Retnla was significantly reduced in $Ccr5^{-/-}$ compared to wildtype BMDM (**Figure 27A**). On a functional level, the presence of IL-4 markedly stimulated arginase enzyme activity and reduced NO production in comparison to unstimulated samples but differences between wildtype and $Ccr5^{-/-}$ BMDM were not observed (**Figure 27B+C**). In summary, the results on the functional level reflect the findings on mRNA level showing only a mild trend towards an alternative activation phenotype of $Ccr5^{-/-}$ BMDM.

These findings fostered the idea, that BMDM might need an initial maturation or priming step in order to obtain an alternative activation phenotype. For that reason cells were primed with different stimuli before addition of IL-4 to induce alternative activation. Bacterial lipopolysaccharide (LPS) was used to trigger macrophage activation. LPS is a component of the

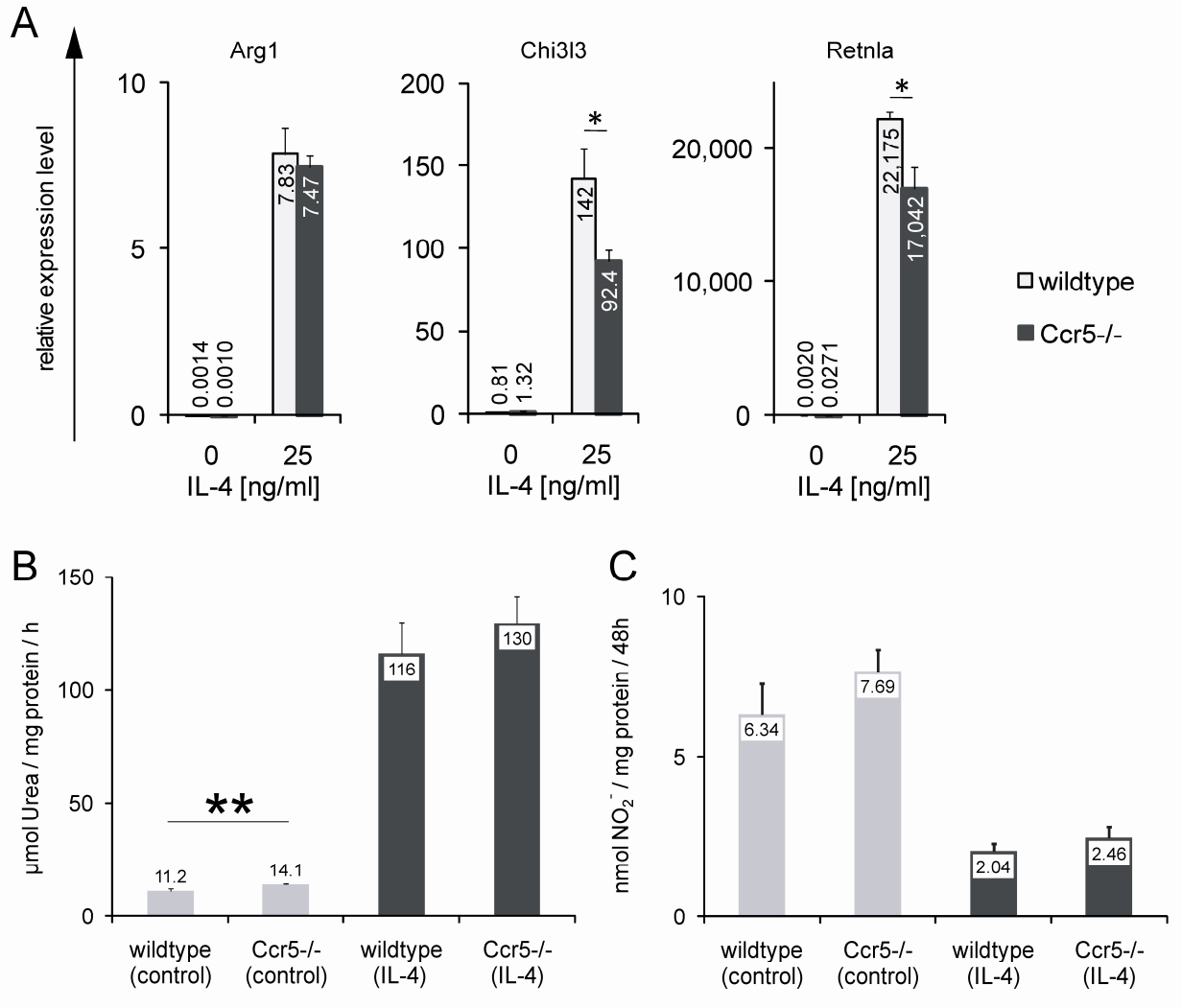

Figure 27A-C. Effect of Ccr5 deficiency on AAM marker expression, urea and NO production in BMDM. BMDM (C57BL/6, n=3, all male) were plated into the wells of 6 well plates for expression analysis **(A)** or 12 well plates for functional assays **(B+C)** at a density of $0.5x10^6$ cells/ml and cultivated with BBM medium alone or with murine recombinant IL-4 (25 ng/ml) for 48h. **(A)** Total RNA was subjected to real-time RT-PCR to determine mRNA levels of AAM signature genes Arg1, Chi3l3 and Retnla. Protein lysates were used to determine arginase enzyme activity **(B)** and supernatants were used to determine NO production **(C)**. *: $p < 0.05$.

cell wall of Gram-negative bacteria and induces classical activation of macrophages. Furthermore, interferon gamma (IFN-γ) was applied as a non-pathogen derived stimulus for macrophages. However, in this experiment no differences for the mRNA expression of AAM markers Arg1, Chi3l3 or Retnla were observed besides a slight increase of Arg1 mRNA after LPS/IL-4 stimulation (**Figure 28**). Furthermore, treatment with D-PBS, LPS or IFN-γ alone did not

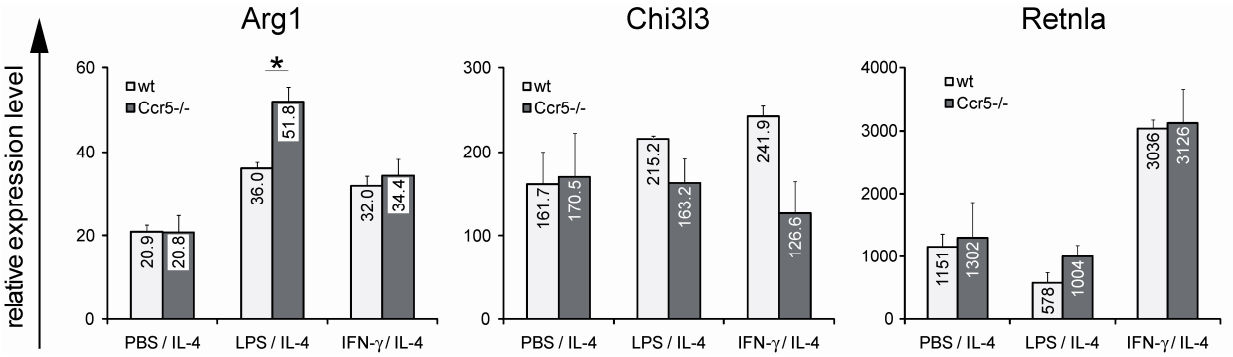

Figure 28. Effect of Ccr5 deficiency on AAM marker mRNA expression in pre-stimulated BMDM. $1.5x10^6$ BMDM (C57BL/6, n=2) were plated into each well of a 6 well plate with 3 ml BBM medium/well and allowed to adhere for 6h. After this time LPS (100 ng/ml), murine recombinant IFN-γ (10 ng/ml) or D-PBS (vehicle control) were added and the cells were incubated at 37°C and 5% CO_2. After 18h medium was replaced by IL-4 containing medium (25 ng/ml) and cells were incubated for 48h. Total RNA was prepared from these cells and subjected to real-time RT-PCR analysis. *: $p<0.05$.

induce any significant differences in Arg1, Chi3l3 and Retnla mRNA expression between wildtype and $Ccr5^{-/-}$ BMDM (data not shown).

The mRNA expression of AAM (Arg1, Chi3l3 and Retnla) and CAM (Nos2) signature genes might show time-dependent differences. Therefore, a time-course experiment was performed. Wildtype BMDM were stimulated with IL-4 and cells were harvested after 24, 48, 72 and 120h. While Arg1 mRNA decreased steadily over time, expression of Retnla mRNA peaked between 48 and 72h (**Figure 29**). Interestingly, expression levels of Chi3l3 mRNA were rather low from 24 to 72 h but increased drastically after 72h. In comparison to the AAM markers, expression of Nos2 mRNA was extremely low between 24 and 72h, but increased noticeably after 72h suggesting that IL-4 availability starts to decline at this time resulting in the release of Nos2 suppression. These results clearly show that the time courses of individual AAM signature genes are different.

It was unclear, whether the AAM observed in renal allografts from Ccr5-deficient recipients originated from the allograft (= BALB/c) or from the recipient (= C57BL/6). Therefore, an IL-4 dose-response experiment was performed with wildtype and Ccr5-/- BMDM generated from BALB/c mice (**Figure 30A-D**). In wildtype as well as $Ccr5^{-/-}$ BMDM from BALB/c mice expression

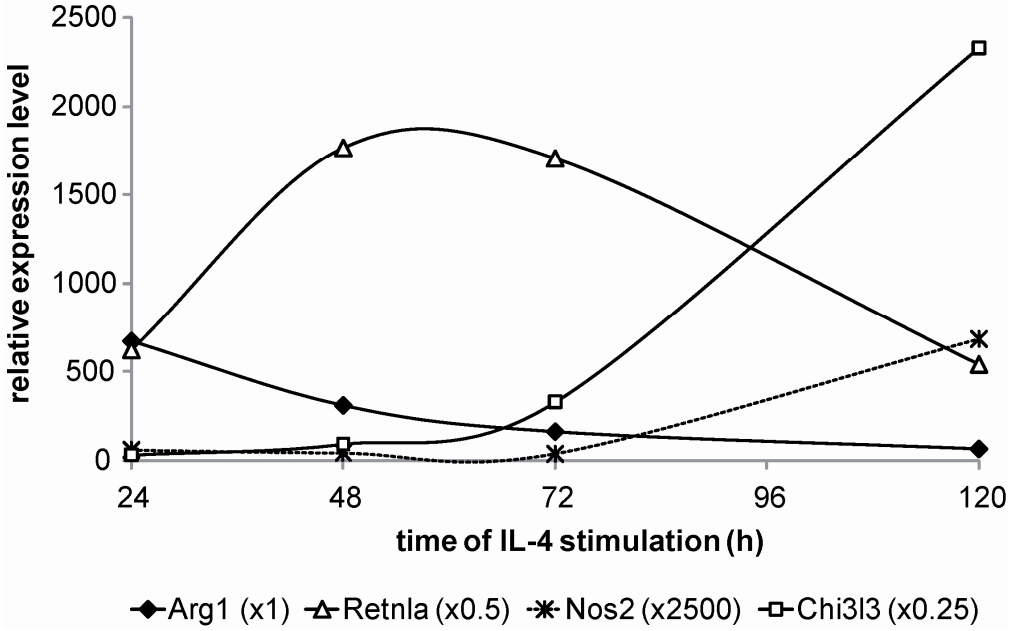

Figure 29. Time course of marker gene expression in IL-4 stimulated wildtype BMDM. Wildtype C57BL/6 BMDM were generated in Petri dishes and $1.5x10^6$ cells were plated into each well of a 6 well plate with 3 ml BBM medium per well and stimulated with murine recombinant IL-4 (10 ng/ml). Cells were lysed at the indicated time points and total RNA was prepared. Expression levels for each gene were adjusted with the indicated factors in brackets to allow side by side comparison on the same axis of ordinates.

of the AAM signature genes Arg1, Chi3l3 and Retnla increased in a dose-dependent manner. Without IL-4 (0 ng/ml), Arg1, Chi3l3 and Retnla mRNA was undetectable in wildtype and $Ccr5^{-/-}$ BALB/c BMDM (**Figure 30A**). At 0.4 ng/ml IL-4, mRNA expression of all three markers was slightly increased in wildtype BABL/c BMDM compared to $Ccr5^{-/-}$ BALB/c BMDM. Interestingly, at concentrations from 2 to 50 ng/ml IL-4 $Ccr5^{-/-}$ BALB/c BMDM showed consistently increased expression of Arg1 and Chi3l3 mRNA compared to wildtype BALB/c BMDM with the strongest differences at a concentration of 10 ng/ml IL-4. However, Retnla mRNA levels did not differ significantly in wildtype and $Ccr5^{-/-}$ BALB/c BMDM at any concentration of IL-4. The mRNA expression of the CAM marker gene Nos2 decreased in an IL-4-dependent manner in wildtype as well as $Ccr5^{-/-}$ BALB/c BMDM. However, at low concentrations of IL-4 (0 and 0.4 ng/ml) Ccr5-deficient BMDM from BALB/c mice showed considerably less Nos2 mRNA expression as wildtype BALB/c BMDM (**Figure 30A**). In contrast, BMDM from C57BL/6 mice showed mRNA

expression of Arg1, Chi3l3 and Retnla already under unstimulated conditions and the Retnla mRNA levels were considerably increased in Ccr5$^{-/-}$ compared to wildtype C57BL/6 BMDM but the differences did not reach statistical significance due to large inter-animal variability (**Figure 27A**). To further corroborate these findings on a functional level arginase enzyme activity and nitrite production were determined in BALB/c BMDM stimulated for 48h with different concentrations of IL-4 (**Figure 30B-D**). Nitrite production decreased already under unstimulated conditions in Ccr5$^{-/-}$ BMDM as compared to wildtype BMDM (**Figure 30B**). With increasing amounts of IL-4, NO production diminished in BMDM of both genotypes. However, at 25 ng/ml IL-4 nitrite production in Ccr5$^{-/-}$ BMDM slightly increased, resulting in comparable NO

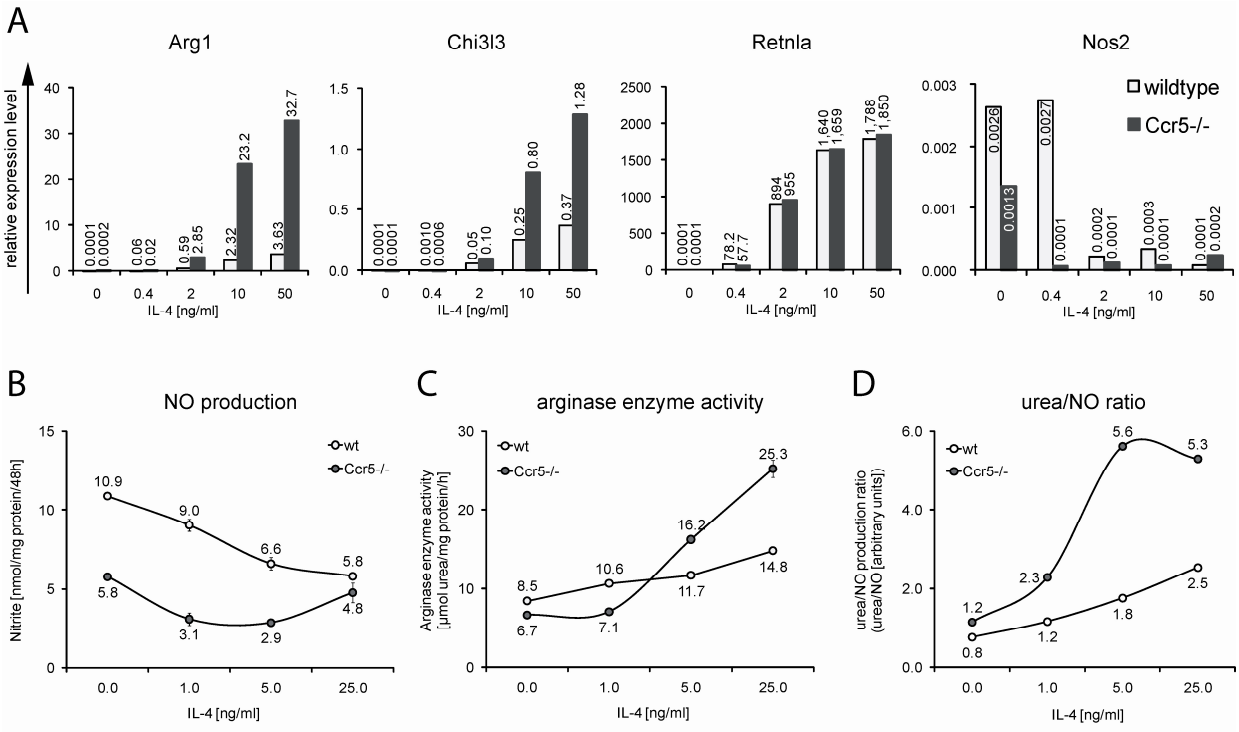

Figure 30A-D. Mouse strain contribution to macrophage polarization in wildtype and Ccr5$^{-/-}$ BMDM. **(A)** BMDM from wildtype and Ccr5$^{-/-}$ BALB/c mice (n=1) were plated into the wells of 6 well plates for expression analysis **(A)** or 24 well plates for functional assays **(B-D)** at a density of 0.5x10^6 cells/ml (BBM medium). The cells were cultivated for 24h before incubation with BBM medium alone or with varying concentrations of murine recombinant IL-4 for 48h. **(A)** Total RNA was subjected to real-time RT-PCR to determine mRNA levels of AAM signature genes (Arg1, Chi3l3 and Retnla) and the CAM marker gene Nos2. Protein lysates were used to determine arginase enzyme activity **(B)** and supernatants were used to determine NO production **(C)**. **(D)** shows urea/NO production ratios to indicate the general macrophage polarization status.

production levels in BMDM of both genotypes. Arginase enzyme activity increased in BMDM of both genotypes in a dose-dependent manner. While arginase enzyme activity was comparable in BMDM of both genotypes at 0 and 1.0 ng/ml IL-4, arginase enzyme activity was elevated in $Ccr5^{-/-}$ BMDM at IL-4 concentrations of 5.0 and 25.0 ng/ml compared to wildtype BMDM (**Figure 30C**).

Macrophage polarization is a continuous process showing also intermediate phenotypes between the (extreme) endpoints of alternative and classical activation [163]. Thus, the ratio of arginase enzyme activity to nitrite production was calculated to determine the general effect of Ccr5 deficiency on macrophage polarization (**Figure 30D**). This ratio showed a dose-dependent increase in BMDM of both genotypes, but ratios were higher at all tested concentrations in $Ccr5^{-/-}$ BMDM compared to wildtype BMDM. This result suggests that L-arginine is predominantly degraded by arginase and might be limited to nitric oxide synthase in Ccr5-deficient BMDM.

In summary, these findings suggest that BMDM from BALB/c mice are more easily skewed towards an alternative activation phenotype in the absence of Ccr5 than BMDM derived from C57BL/6 mice.

3.2.5.2 Phenotype of BMDM generated in Teflon bags

Teflon is the brand name coined by DuPont for the synthetic fluorocarbon polymer polytetrafluoroethylene (PTFE). Teflon foil is a transparent, biologically inert material that is gas permeable but impermeable to water and electrolytes. Consequently, for cells grown directly on Teflon foil the medium does not act as a diffusion barrier allowing optimal growth conditions. Due to the high electronegativity of fluorine and the strength of the carbon-fluorine bonds PTFE is not wetted by water- or oil-containing substances and is chemically non-reactive. The use of heat-sealed Teflon bags to generate highly pure populations of macrophages from

bone marrow precursor cells has already been described in the early 1970s [220-222]. Macrophages generated in Teflon bags offer several advantages over conventional BMDM generated in Petri dish plates. On the one hand, macrophages adhere only weakly to the hydrophobic Teflon bags allowing easy detachment of cells thereby minimizing cellular damage which would be induced by the use of proteases such as Trypsin. The Teflon bags are simply put on ice for 30 minutes resulting in quantitative detachment of the cells from the hydrophobic surface. On the other hand, Teflon bags provide means of generating high amounts of ≥95% pure macrophages (80-100x10^6 from a single mouse). The fact that no medium changes are required during the differentiation period reduces growth factor consumption and the risk of contamination. Additionally, macrophages obtained from Teflon bags can be passaged for several weeks allowing a constant source of cells for experiments.

Characterization of BMDM generated in Teflon bags

To investigate the changes in the composition of the bone marrow cells during the differentiation period DiffQuick stained cytospins were prepared from fresh bone marrow cells and from bone marrow cells that were cultivated in Teflon bags in the presence of L929s supernatant for 10 days (**Figure 31**). Fresh bone marrow was mainly composed of cells resembling polymorphonuclear leukocytes and to a lesser extent of cells with lymphocyte or monocyte morphology (**Figure 31A**). In striking contrast, cells generated by 10 days incubation of bone marrow in Teflon bags showed a highly uniform macrophage-like morphology (compare [223] and [195]) with extended and vesicle-rich cytoplasm (**Figure 31B**).

In the next step, expression analysis of certain cell-specific markers by flow cytometry was performed to confirm the impression that Teflon bag cultured bone marrow cells consist mainly of macrophages (**Figure 32**). To this end, fluorochrome-labeled antibodies directed against the typical macrophage markers CD11b and F4/80 were used. Antibodies directed against CD11c

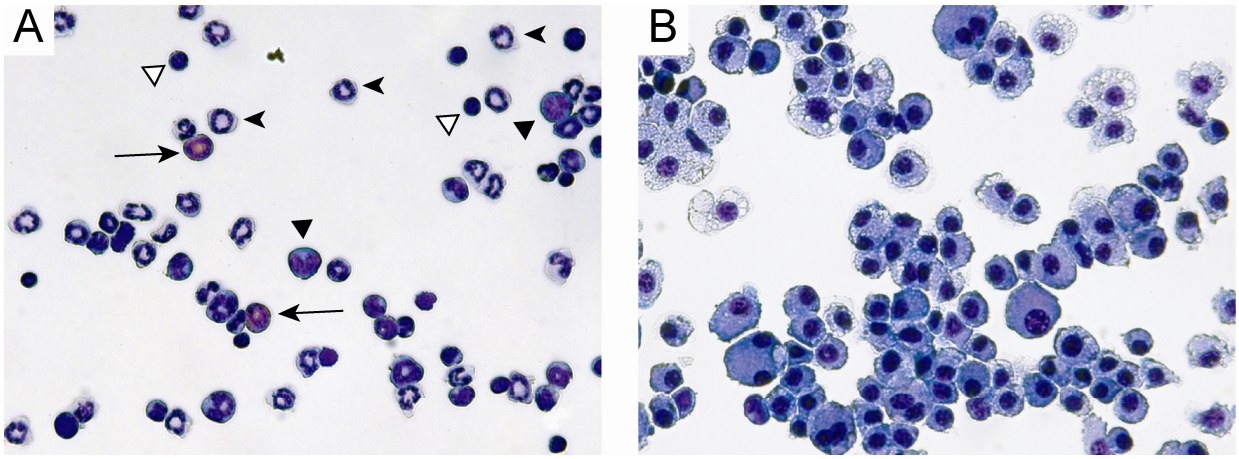

Figure 31. DiffQuick stained cytospins of fresh bone marrow cells (A) and BMDM generated by culture of unfractioned bone marrow in Teflon bags for 10 days (B). Arrows: eosinophils, arrowheads: neutrophils, white triangles: lymphocytes and black triangles: monocytes. Note the large vesicle-rich cytoplasm of cells in shown **(B)**. Both photomicrographs 400x.

(dendritic cells), Gr-1 (granulocytes), CD3ε (T cells), CD19 (B cells) and NK1.1 (natural killer cells) were used to exclude the presence of other cell types. Furthermore, an antibody against CD45 was applied to determine the percentage of leukocytes within the generated cell population. Isotype-matched antibodies without a relevant specificity were used as controls for unspecific binding (isotype controls). Non-viable cells were excluded from the analysis by staining with 7-AAD (7-amino-actinomycin D), a fluorescent chemical compound that intercalates with nucleic acids of dead and dying cells but is efficiently excluded from viable cells [197]. We speculated that Ccr5 deficiency might already have an effect at the level of macrophage differentiation and therefore included cells from Ccr5-deficient mice in this analysis. Nearly all BMDM were positive for CD11b and F4/80 (≥98.5%). No differences were observed for CD11b and F4/80 expression between wildtype and $Ccr5^{-/-}$ BMDM. BMDM were also highly positive for the common leukocyte antigen CD45 (wildtype: 79.2%, $Ccr5^{-/-}$: 87.9%) whereas expression of CD3ε (T cells), NK1.1 (NK cells) or Gr-1 (granulocytes) did not exceed isotype control antibody levels. Interestingly, BMDM showed moderate expression of CD19 and CD11c. Both of these markers were differentially regulated between wildtype and $Ccr5^{-/-}$ BMDM ($CD19^{+}$: wt: 20.7%, $Ccr5^{-/-}$: 12.3%; $CD11c^{+}$: wt: 8.20%, $Ccr5^{-/-}$: 20.6%) (**Figure 32**). However, additional experiments

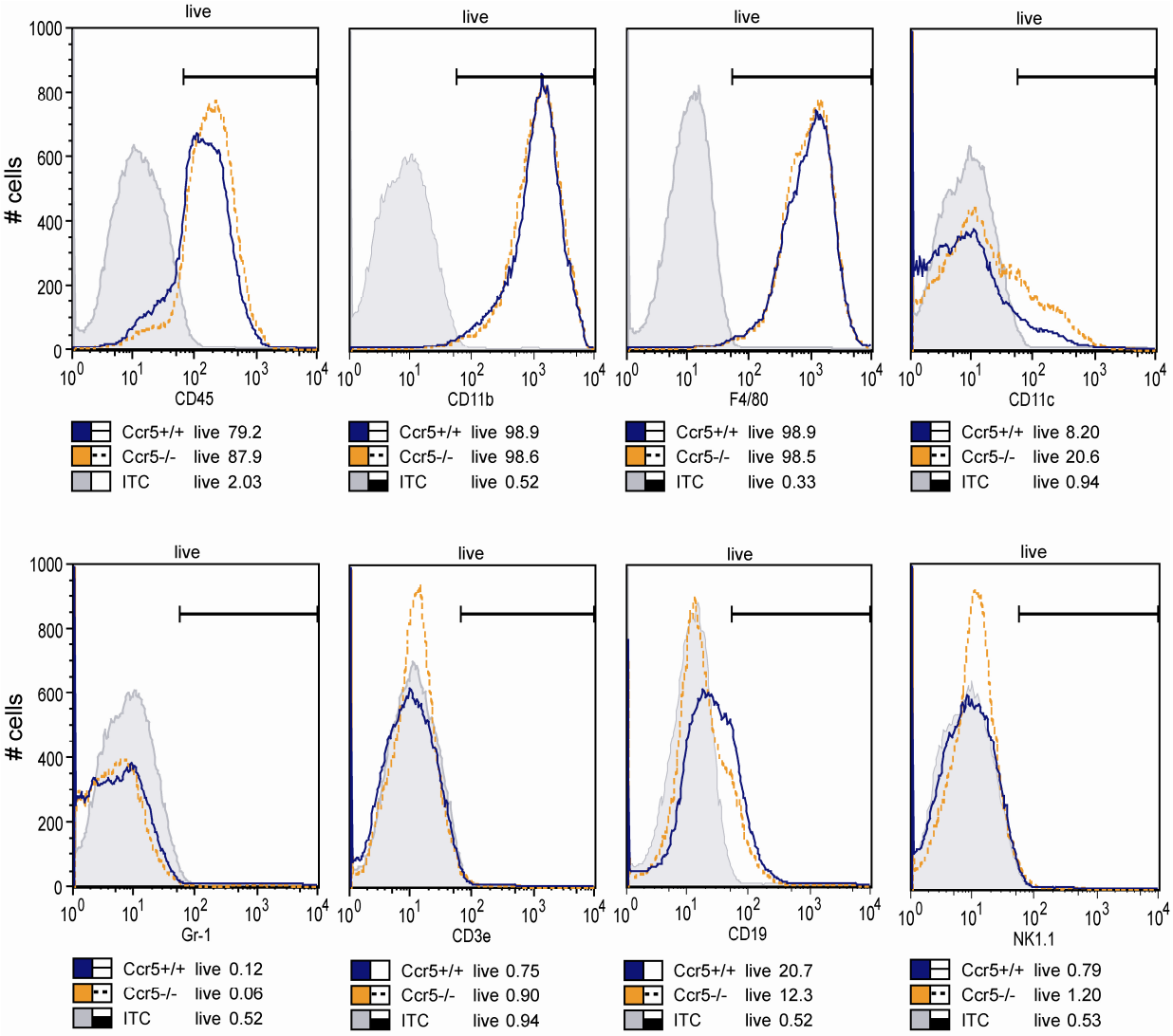

Figure 32. Flow cytometric characterization of wildtype and Ccr5$^{-/-}$ BMDM generated in Teflon bags. Bone marrow cells (n=1) were seeded into Teflon bags containing 50 ml differentiation DBM medium at a density of 10x10^6 per bag before flow cytometric analysis. BMDM were harvested after 10 days incubation at 37°C and 10%CO_2 and stained for the indicated surface markers. Live/dead cell discrimination was carried out using 7-AAD and an appropriate live cell gate was set (FL3 (7-AAD) *vs.* FSC). 50,000 events were counted for each sample. Results of analysis are shown as histograms: isotype control antibody (ITC, shaded areas), wildtype (Ccr5$^{+/+}$, blue lines) and Ccr5$^{-/-}$ BMDM (orange dotted lines). Markers used are: CD45 (common leukocyte antigen), CD11b (macrophages), F4/80 (macrophages), CD11c (dendritic cells), Gr-1 (granulocytes), CD3ε (T cells), CD19 (B cells) and NK1.1 (NK cells). Numbers below each histogram indicate percentages of positive live cells in plotted regions which were set according to isotype control antibodies.

with increased numbers of animals are required to confirm these differences. In conclusion, these data show that cell populations are highly pure macrophages on the basis of morphology and expression of the macrophage markers CD11b and F4/80.

Effect of Ccr5 deficiency on macrophage polarization of BMDM generated in Teflon bags

To study the effect of Ccr5 deficiency on macrophage polarization Teflon bag BMDM (C57BL/6) were stimulated with IFN-γ+LPS to induce classical activation and the combination of IL-4+IL13 was applied to provoke alternative activation. Unstimulated samples were carried along to investigate macrophage polarization under basal conditions (**Figure 33**).

In vehicle-treated BMDM from $Ccr5^{-/-}$ mice a slight and statistically not significant increase for Chi3l3 (5.6x) and Retnla (2.1x) mRNA expression was observed compared to wildtype BMDM, while Arg1 and Mmp12 mRNA levels were not influenced by loss of Ccr5. Combined stimulation of BMDM with IL-4+IL-13 (each 20 ng/ml, for 24h) induced a dramatic up-regulation of Arg1 (~140x), Chi3l3 (~680x) and Retnla (~7100x) and a moderate increase of Mmp12 (~1.8x) mRNA expression compared to vehicle-treated BMDM. However, these AAM marker genes were not differentially regulated in $Ccr5^{-/-}$ compared to wildtype BMDM upon IL-4+IL-13 stimulation. Interestingly, combined stimulation with IFN-γ+LPS – stimuli typically inducing classical macrophage activation – resulted in significantly increased mRNA expression of AAM markers Arg1 (1.9x) and Mmp12 (1.6x) in $Ccr5^{-/-}$ compared to wildtype BMDM (**Figure 33**). Furthermore, $Ccr5^{-/-}$ BMDM showed 5.1x up-regulation of Retnla mRNA in response to IFN-γ+LPS compared to wildtype BMDM. However, this difference did not reach statistical significance due to large inter-animal variability. The increased expression of AAM marker genes in $Ccr5^{-/-}$ BMDM upon IFN-γ+LPS stimulation suggests that CCR5 exerts a suppressive effect on alternative macrophage activation under conditions that normally lead to classical macrophage activation.

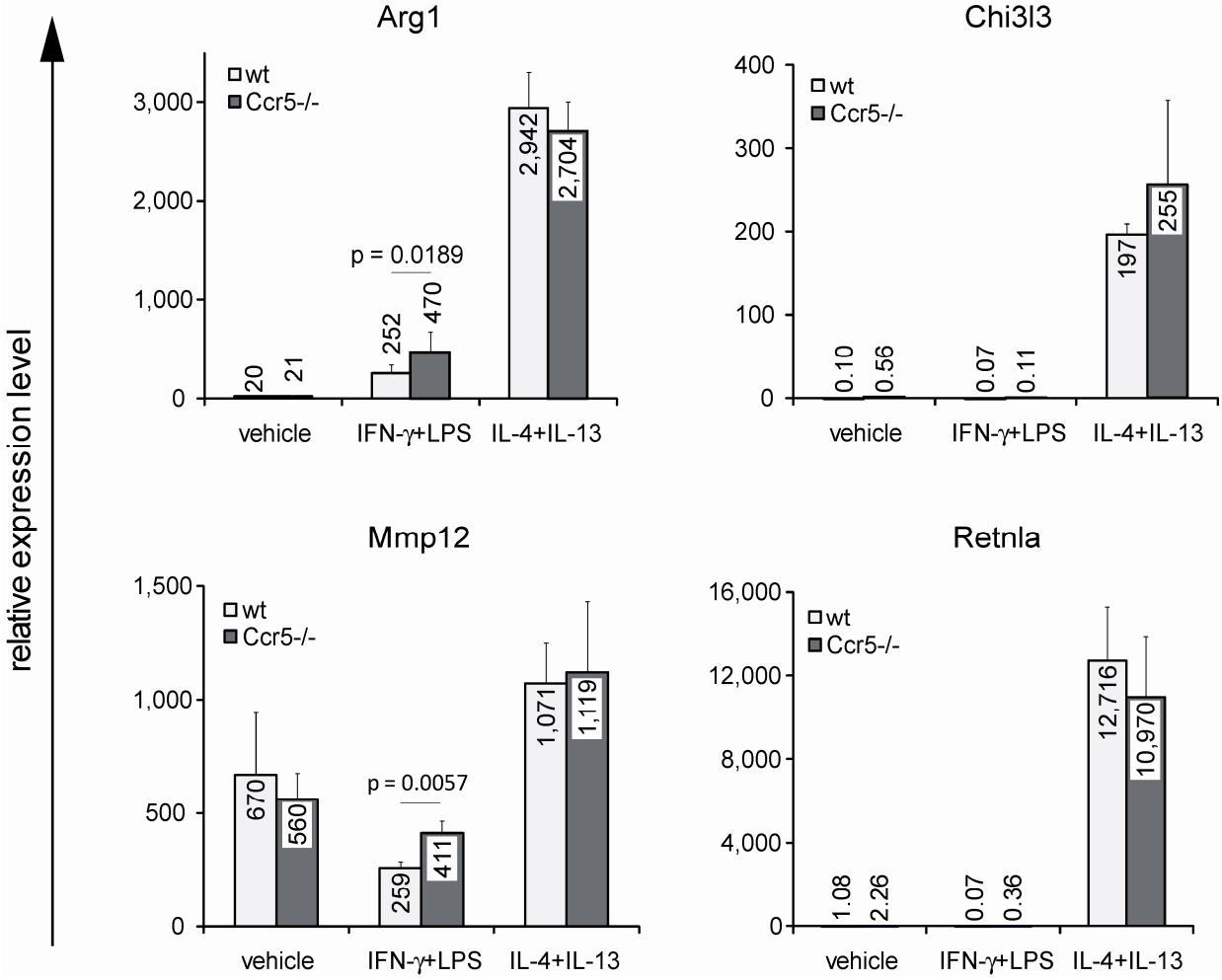

Figure 33. Effect of Ccr5 deficiency on mRNA expression AAM marker genes (Arg1, Chi3l3, Mmp12 and Retnla in stimulated BMDM generated in Teflon bags. Bone marrow cells (C57BL/6, male, 22-24w, n=4) were differentiated to BMDM in Teflon bags and plated in the wells of 6 well plates at a density of $2.5x10^6$ cells/well in 3 ml SSB medium/well. 3h after seeding (adherence period) stimuli were added in final concentrations of 20 ng/ml (IFN-γ, IL-4 and IL-13) or 100 ng/ml (LPS) and cells were further incubated for 24h at 37°C and 10% CO_2. After this time total RNA was prepared from the stimulated BMDM and subjected to real-time RT-PCR. Depicted are means ± SD of relative expression levels. Statistical significance (p) was calculated using Student's t test.