2 MATERIALS AND METHODS

As outlined under “External contribution” (see 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 (2.16), histopathology (2.17) and immunohistochemistry (2.18) of renal allograft sections. These methods are described to give complete insight into the sample preparation and analysis process. Methods and results supplied by Prof. Gröne’s group are indicated in section headlines by DKFZ in brackets to unambiguously indicate the origin of contribution.

2.1 Mice

C57BL/6NCrl (C57BL/6) and BALB/cAnNCrl (BALB/c) mice were obtained from Charles River (Sulzfeld, Germany). All mice were subsequently maintained as a breeding colony in our animal facility. Mice were fed fortified rodent chow and water *ad libitum*. Mice were raised under specific pathogen-free housing conditions in individually ventilated cages lined with sawdust.

Ccr1-deficient mice ($Ccr1^{tm1Gao}$) have been generated by Gao *et al.* [179]. Ccr5-deficient mice ($Ccr5^{tm1Blck}$) have been generated in our laboratory as described elsewhere [112]. Ccr5-deficient BALB/c mice were generated by back crossing of Ccr5-deficient C57BL/6 mice against the BALB/c background for at least 10 generations. C57BL/6 mice double-deficient for Ccr1 and Ccr5 were generated by crossing of fully backcrossed Ccr1- and Ccr5-deficient mice. The distance between both loci was large enough (~ 160 kbp) to allow this approach. The theoretical recombination frequency was 1 in 625 events (0.16 cM). Heterozygous ($Ccr1^{+/-}$/$Ccr5^{+/-}$) mice obtained from these matings were crossed with $Ccr1^{+/+}$/$Ccr5^{-/-}$ mice and recombination events between Ccr1 and the Ccr5 locus were analyzed by multiplex PCR with 4 different primer pairs (see Methods section 2.14, genotyping) in the resulting offspring mice. Two female mice out of 477 live pups showed the desired $Ccr1^{+/-}$/$Ccr5^{-/-}$ genotype and were

crossed with wildtype mice to produce male $Ccr1^{+/-}/Ccr5^{+/-}$ mice for intercrossing. Mating of these mice with their $Ccr1^{+/-}/Ccr5^{-/-}$ mothers finally yielded $Ccr1^{-/-}/Ccr5^{-/-}$ mice. Deletion of Ccr1 and/or Ccr5 was confirmed by repeated genotyping using multiplex PCR analysis of genomic DNA prepared from tail snips. Transplantation experiments were performed exclusively with male mice at least 8 to 10 weeks old with a weight between 20 and 25 g. All mice used in this thesis were backcrossed for at least 10 generations to the genetic backgrounds indicated in the individual experiments. All animal experiments were performed in compliance with governmental and institutional guidelines.

2.2 Murine cell lines

J558L, plasmacytoma cell line, BALB/c	Dr. T. Kammertoens, MDC Berlin, Germany
L929s, fibrosarcoma cell line, C3H/An, male	Dr. M. Freudenberg, MPI for Immunobiology, Freiburg, Germany

2.3 Chemicals and reagents

2-Mercapthoethanol	Carl Roth, Karlsruhe, Germany
7-Amino-actionomycin D (7-AAD)	BD Biosciences, Heidelberg, Germany
Acrylamide, linear	Applied Biosystems, Darmstadt, Germany
Agarose, Ultrapure	Invitrogen, Karlsruhe, Germany
Aqua ad injectabilia	Braun, Melsungen, Germany
BD Cytofix solution	BD Biosciences, Heidelberg, Germany
Brewer's thioglycollate medium	DIFCO, Lawrence, Kansas, USA
BSA (Fraction V)	Roche Diagnostics, Mannheim, Germany
BSA, PCR grade (50x)	Fermentas, St. Leon-Rot, Germany
Diethylpyrocarbonate (DEPC)	Fluka/Sigma-Aldrich, Schnelldorf, Germany

DMSO	Merck, Darmstadt, Germany
dNTP set (ATP, CTP, GTP and TTP; each 100 mM)	GE Healthcare, München, Germany
EDTA (EDTA-Na_2, Titriplex III, molecular biology)	Merck, Darmstadt, Germany
Ethidium bromide, aqueous solution, 1.0%	Merck, Darmstadt, Germany
Formaldehyde, p.a., ≥37%	Merck, Darmstadt, Germany
Formamide, p.a.	Fluka/Sigma-Aldrich, Schnelldorf, Germany
Gelatin	Sigma-Aldrich, Schnelldorf, Germany
Griess' reagent for nitrite	Fluka/Sigma-Aldrich, Schnelldorf, Germany
Hydrochloric acid, 37%	Merck, Darmstadt, Germany
$MgCl_2$ (25 mM)	Fermentas, St. Leon-Rot, Germany
MOPS PUFFERAN	Carl Roth, Karlsruhe, Germany
Sodium acetate	Merck, Darmstadt, Germany
Sodium hydroxide pellets, p.a.	Merck, Darmstadt, Germany
Nonidet P40 (Igepal CA 630)	Fluka/Sigma-Aldrich, Schnelldorf, Germany
PCR Optimizer (2.5x)	Bitop, Witten, Germany
Rox reference dye (25x)	Invitrogen, Karlsruhe, Germany
SYBRgreen I (250x)	Fluka/Sigma-Aldrich, Schnelldorf, Germany
Taq buffer without detergent (10x)	Fermentas, St. Leon-Rot, Germany
Tris base (PUFFERAN), p.a.	Carl Roth, Karlsruhe, Germany
Triton X-100	Fluka/Sigma-Aldrich, Schnelldorf, Germany
Tween 20	Fluka/Sigma-Aldrich, Schnelldorf, Germany

2.4 Antibodies

Table 1 summarizes all antibodies used in this study. Antibodies were purchased from Sigma (Taufkirchen, Germany), BD Biosciences (Heidelberg, Germany), Abd Serotec (Düsseldorf, Germany), Biogenesis (Poole, UK), eBiosciences (San Diego, California, USA) and R&D Systems (Minneapolis, Minnesota, USA). The anti-Chi3l3 antibody was a kind gift of Dr. Kimura (NCI, Bethesda, Maryland, USA).

Table 1. Monoclonal and polyclonal antibodies used in this study. (continued)

Antibodies for immunohistochemistry

murine antigen	isotype	clone	µg/µl	final dilution	label	manufacturer
α-smooth muscle actin (α-SMA)	mouse IgG2a, mouse ascites fluid	1A4	unknown	1:200	none (*	Sigma
Arginase 1	mouse IgG1, crossreacts with mouse	19	0.25	1:20	none (*	BD Biosiences
CD4	rat IgG2a, kappa	H129.19	unknown	1:20	none (*	BD Biosiences
CD8a	rat IgG2a, kappa	53-6.7	unknown	1:50	none (*	BD Biosiences
CD11c	hamster IgG1, lambda2	HL3	0.125	1:20	none (*	BD Biosiences
CD206 (MRC1)	rat IgG2a	MR5D3	1.00	1:100	none (*	Abd Serotec
Chi3l3 (Ym1/2)	rabbit IgG	polyclonal	unknown	1:60	none (*	Dr. Kimura
Collagen I/III	rabbit IgG	polyclonal	0.10	1:20	none (*	Biogenesis
F4/80	rat IgG2b, kappa	CI:A3-1	1.00	1:2000	none (*	Abd Serotec

*) Antibody binding was detected using the ABC detection system (Vector Laboratories).

Antibodies for determination of alloreactive antibody levels

murine antigen	isotype	clone	µg/µl	final dilution	label	manufacturer
IgG + IgM	goat anti-mouse Ig	polyclonal	0.50	1:20	FITC	BD Biosciences

Antibodies for flow cytometry

murine antigen	isotype	clone	µg/µl	final dilution for		label	manufacturer
				splenocytes	BMDM		
CCR6	rat IgG2a	140706	0.10	1:50	not used	FITC	R&D Systems
CD3ε	hamster IgG1, kappa	145-2C11	0.20	1:400	1:400	Alexa488	BD Biosciences
CD11b (Integrin αM, Mac-1)	rat IgG2b, kappa	M1/70.15	0.10	1:200	1:100	FITC	Abd Serotec
CD11b (Integrin αM, Mac-1)	rat IgG2b, kappa	M1/70	0.20	1:400	1:200	PE	BD Biosciences
CD11c (Integrin αX)	hamster IgG1, lambda2	HL3	0.20	1:400	1:200	PE	BD Biosciences
CD19	rat IgG2a, kappa	1D3	0.20	1:100	1:100	Alexa647	BD Biosciences
CD45	rat IgG2b, kappa	30-F11	0.50	1:100	1:100	FITC	BD Biosciences
CD115 (CSF1-R1, c-fms)	rat IgG1	604B5 2E11	0.01	1:600	1:100	PE	Abd Serotec
CD124 (IL4Ra)	rat IgG2a, kappa	mIL4R-M1	0.20	1:400	1:100	PE	BD Biosciences
CD169 (MOMA-1, Siglec1)	rat IgG2a	Moma-1	0.10	1:500	1:100	FITC	Abd Serotec
CD204 (MSR1/2)	rat IgG2b	2F8	0.05	1:1000	1:400	Alexa647	Abd Serotec
CD206 (MRC1)	rat IgG2a	MR5D3	0.05	1:400	1:100	Alexa647	Abd Serotec
CD206 (MRC1)	rat IgG2a	MR5D3	0.10	1:400	1:100	PE	Abd Serotec
CD206 (MRC1), intracellular	rat IgG2a	MR5D3	0.05	1:1000	1:100	Alexa647	Abd Serotec
CD206 (MRC1), intracellular	rat IgG2a	MR5D3	0.10	1:1000	1:100	PE	Abd Serotec
CD209b (SIGN-R1, Er-tr9)	rat IgM	Er-tr9	0.05	1:400	1:100	Alexa488	Abd Serotec
F4/80	rat IgG2a, kappa	BM8	0.20	1:800	1:400	PE	eBioscience
F4/80	rat IgG2b, kappa	CI:A3-1	0.01	1:200	1:100	APC	Abd Serotec
Gr-1 (Ly-6C/6G)	rat IgG2b, kappa	RB6-8C5	0.20	1:500	1:100	PE	BD Biosciences
MARCO	rat IgG1	ED31	0.10	1:600	1:100	PE	Abd Serotec
MOMA-2	rat IgG2b	Moma-2	0.01	1:800	1:200	PE	Abd Serotec
MOMA-2, intracellular	rat IgG2b	Moma-2	0.01	1:1000	1:200	PE	Abd Serotec
NK-1.1 (NK cells)	rat IgG2a, kappa	PK136	0.20	1:100	1:100	PE	BD Biosciences
CD16/32 (FcR Seroblock)	rat IgG2b	FCR4G8	1.00	1:200	1:100	none	Abd Serotec

Table 1 (continued)**. Monoclonal and polyclonal antibodies used in this study.**

Isotype-matched control antibodies for flow cytometry

murine antigen	isotype	clone	µg/µl	final dilution	label	manufacturer
TNP-KHL	hamster IgG1, kappa	A19-3	0.20	matched with test Ab	Alexa488	BD Biosciences
TNP-KHL	hamster IgG1, lambda1	G235-2356	0.20	matched with test Ab	PE	BD Biosciences
mouse Ig	rat IgG1, kappa	R3-34	0.20	matched with test Ab	PE	BD Biosciences
unspecific	rat IgG2a, kappa	R35-95	0.20	matched with test Ab	Alexa647	BD Biosciences
human lymphocytes	rat IgG2a	YTH71.3	0.10	matched with test Ab	FITC	Abd Serotec
human lymphocytes	rat IgG2a	YTH71.3	0.10	matched with test Ab	PE	Abd Serotec
TNP-KHL	rat IgG2b, kappa	A95-1	0.50	matched with test Ab	FITC	BD Biosciences
TNP-KHL	rat IgG2b, kappa	A95-1	0.20	matched with test Ab	PE	BD Biosciences
DNP	rat IgG2b	?	0.05	matched with test Ab	Alexa647	Abd Serotec
TNP-KHL	rat IgG2b, kappa	A95-1	0.20	matched with test Ab	APC	BD Biosciences

2.5 Recombinant proteins and reagents used for *in vitro* cell stimulation

Interferon gamma (IFNg), murine recombinant	Peprotech, Rocky Hill, USA
Interleukin-4 (IL-4), murine recombinant	Peprotech, Rocky Hill, USA
LPS from *E. coli* 0111:B4, cell culture tested, γ-irradiated	Sigma-Aldrich, Schnelldorf, Germany
M-CSF (CSF1), murine recombinant	Peprotech, Rocky Hill, USA
RNasin, recombinant Ribonuclease inhibitor, 40 u/µl	Promega, Mannheim, Germany

2.6 Enzymes

Taq DNA polymerase (recombinant, 5u/µl)	New England Biolabs, Frankfurt, Germany
AmpliTaq DNA polymerase (recombinant, 5u/µl)	Applied Biosystems, Foster City, CA, USA
Reverse transcriptase, SuperScript I	Invitrogen, Karlsruhe, Germany
Reverse transcriptase, SuperScript II	Invitrogen, Karlsruhe, Germany

2.7 Oligonucleotides

Pre-designed assays as well as custom-made primer/probes sets for TaqMan-PCR were ordered from Applied Biosystems (ABI, Foster City, California, USA). Custom primer pairs for SYBRgreen qPCR assays or mouse genotyping were obtained desalted in a 10-100 nmol scale from Invitrogen (Karlsruhe, Germany). Primers (**Table 2 and 3**) were designed using either the computer program 'Primer' provided by the software package HUSAR [180], the online portal of Primer3 [181] or the online database qPrimerDepot (http://mouseprimerdepot.nci.nih.gov/, [182]). To exclude amplification of unspecific products, custom-made primer pairs were subjected to melting curve analysis using the ABI PRISM 7000 Sequence Detection System. Random hexanucleotides for reverse transcription reactions were ordered from Roche Diagnostics (Mannheim, Germany). Oligonucleotides for mouse genotyping are summarized in **Table 2**. Pre- and self-designed oligonucleotides for real-time qPCR are listed in **Tables 3A** and **3B**, respectively.

Table 2. Oligonucleotides used for mouse genotyping by PCR.

allele	assay design	primer sequence	amplicon sizes [bp]
Ccr1 wildtype	HUSAR	forward: 5'-GAGTTCACTCACCGTACCTGTAGC-3' reverse: 5'-TGACCTTCTTCTCACTGGGTCTTC-3'	180
Ccr1 knock-out	HUSAR	forward: 5'-GCTGTCTCTGATCTGGTCTTCCTT-3' reverse: 5'-TGGGTGGAGAGGCTTTTTGCTTCCTCTTGC-3'	155
Ccr5 wildtype	HUSAR	forward: 5'-CGCTTCTTGCTGTCTATGGATG-3' reverse: 5'-CGGTGTGGTAGGATTTAGGTCTG-3'	277
Ccr5 knock-out	HUSAR	forward: 5'-TGGATTTTCAAGGGTCAGTTCC-3' reverse: 5'-TGTGCTGCAAGGCGATTAAG-3'	223

Table 3A. Oligonucleotides used for real-time quantitative RT-PCR.

gene	oligonucleotide supplier	assay type	assay order number
18S	Applied Biosystems (ABI)	pre-designed	4310893E (eukaryotic 18S rRNA, VIC-labeled probe)
Arg1	Applied Biosystems (ABI)	pre-designed	Mm00475988_m1 (FAM-labeled probe)
Ccl2	Applied Biosystems (ABI)	pre-designed	Mm00441242_m1 (FAM-labeled probe)
Ccl3	Applied Biosystems (ABI)	pre-designed	Mm00441258_m1 (FAM-labeled probe)
Ccl4	Applied Biosystems (ABI)	pre-designed	Mm00443111_m1 (FAM-labeled probe)
Ccl5	Applied Biosystems (ABI)	pre-designed	Mm01302428_m1 (FAM-labeled probe)
Ccl20	Applied Biosystems (ABI)	pre-designed	Mm00444228_m1 (FAM-labeled probe)
Ccr2	Applied Biosystems (ABI)	pre-designed	Mm00445551_m1 (FAM-labeled probe)
Ccr4	Applied Biosystems (ABI)	pre-designed	Mm00438271_m1 (FAM-labeled probe)
Ccr6	Applied Biosystems (ABI)	pre-designed	Mm01700299_m1 (FAM-labeled probe)
Chi3l3	Applied Biosystems (ABI)	pre-designed	Mm00657889_mH (FAM-labeled probe)
Cxcl10	Applied Biosystems (ABI)	pre-designed	Mm00445235_m1 (FAM-labeled probe)
Fcer2a	Applied Biosystems (ABI)	pre-designed	Mm00442792_m1 (FAM-labeled probe)
Foxp3	Applied Biosystems (ABI)	pre-designed	Mm00475156_m1 (FAM-labeled probe)
Gata3	Applied Biosystems (ABI)	pre-designed	Mm00484683_m1 (FAM-labeled probe)
Ifng	Applied Biosystems (ABI)	pre-designed	Mm00801778_m1 (FAM-labeled probe)
Il4	Applied Biosystems (ABI)	pre-designed	Mm00445259_m1 (FAM-labeled probe)
Il6	Applied Biosystems (ABI)	pre-designed	Mm00446190_m1 (FAM-labeled probe)
Il10	Applied Biosystems (ABI)	pre-designed	Mm00439616_m1 (FAM-labeled probe)
Il12a	Applied Biosystems (ABI)	pre-designed	Mm00434165_m1 (FAM-labeled probe)
Il13	Applied Biosystems (ABI)	pre-designed	Mm00434204_m1 (FAM-labeled probe)
Il17a	Applied Biosystems (ABI)	pre-designed	Mm00439619_m1 (FAM-labeled probe)
Mmp12	Applied Biosystems (ABI)	pre-designed	Mm00500554_m1 (FAM-labeled probe)
Mrc1	Applied Biosystems (ABI)	pre-designed	Mm00485148_m1 (FAM-labeled probe)
Nos2	Applied Biosystems (ABI)	pre-designed	Mm00440485_m1 (FAM-labeled probe)
Retnla	Applied Biosystems (ABI)	pre-designed	Mm00445109_m1 (FAM-labeled probe)
Rorc	Applied Biosystems (ABI)	pre-designed	Mm00441139_m1 (FAM-labeled probe)
Stat6	Applied Biosystems (ABI)	pre-designed	Mm01160477_m1 (FAM-labeled probe)
Tbx21	Applied Biosystems (ABI)	pre-designed	Mm00450960_m1 (FAM-labeled probe)
Tnf	Applied Biosystems (ABI)	pre-designed	Mm00443258_m1 (FAM-labeled probe)
gene	**oligonucleotide supplier**	**assay design**	**primer and probe (FAM-labeled) sequences**
Ccr7	Applied Biosystems (ABI)	HUSAR	FP: 5'-TCCTTGTCATTTTCCAGGTGTG-3' RP: 5'-CGTGGTATTCTCGCCGATGTA-3' probe: 5-'TTCTGCCAAGATGAGGTCACCGATGA-3'
Il2	Applied Biosystems (ABI)	HUSAR	FP: 5'-GTTGTAAAACTAAAGGGCTCTGACAA-3' RP: 5'-TGTTGAGATGATGCTTTGACAGAAG-3' probe: 5-'TGAGTGCCAATTCGATGATGAGTCAGC-3'
Tfgb1	Applied Biosystems (ABI)	HUSAR	FP: 5'-CACAGTACAGCAAGGTCCTTGC-3' RP: 5'-AGTAGACGATGGGCAGTGGCT-3' probe: 5-'CGCTTCGGCGTCACCGTGCT-3'

Table 3B. Oligonucleotides used for real-time quantitative RT-PCR.

gene	assay design	forward primer sequence	reverse primer sequence
18S rRNA	Primer3	5'-GCAATTATTCCCCATGAACG-3'	5'-AGGGCCTCACTAAACCATCC-3'
Arg1	Primer depot	5'-AGAGATTATCGGAGCGCCTT-3'	5'-TTTTTCCAGCAGACCAGCTT-3'
Ccl3	Primer depot	5'-ACCATGACACTCTGCAACCA-3'	5'-GTGGAATCTTCCGGCTGTAG-3'
Ccl4	Primer depot	5'-CATGAAGCTCTGCGTGTCTG-3'	5'-GAAACAGCAGGAAGTGGGAG-3'
Ccl5	Primer depot	5'-GTGCCCACGTCAAGGAGTAT-3'	5'-CCACTTCTTCTCTGGGTTGG-3'
Ccl20	Primer depot	5'-CTTGCTTTGGCATGGGTACT-3'	5'-TGTACGAGAGGCAACAGTCG-3'
Ccr6	Primer depot	5'-GGAGCCTGGATAACCACTGA-3'	5'-TTGAATGGCAGACACTCACAG-3'
Cd68	Primer depot	5'-ATCCCCACCTGTCTCTCTCA-3'	5'-ACCGCCATGTAGTCCAGGTA-3'
Chi3l3	Primer depot	5'-TCTGGGTACAAGATCCCTGAA-3'	5'-TTTCTCCAGTGTAGCCATCCTT-3'
Csf1	Primer3	5'-AAGGTCCTGCAGCAGTTGAT-3'	5'-CATCCAGCTGTTCCTGGTCTA-3'
Csf1r	Primer depot	5'-CTCTGCTGGTGCTACTGCTG-3'	5'-TTGCCTTCGTATCTCTCGATG-3'
Emr1	Primer depot	5'-GGATGTACAGATGGGGGATG-3'	5'-CATAAGCTGGGCAAGTGGTA-3'
Fcer2a	Primer3	5'-ATCCCTGGGCTTGAATGAG-3'	5'-TGCAGTTCCCTTTGAAATCAG-3'
Gata3	Primer depot	5'-GCCTGCGGACTCTACCATAA-3'	5'-AGGATGTCCCTGCTCTCCTT-3'
Ifng	Primer depot	5'-ACAGCAAGGCGAAAAAGGAT-3'	5'-TGAGCTCATTGAATGCTTGG-3'
Il4	Primer depot	5'-TGAACGAGGTCACAGGAGAA-3'	5'-CGAGCTCACTCTCTGTGGTG-3'
Il4ra	Primer3	5'-TGGATCTGGGAGCATCAAG-3'	5'-GGGATGCATGTGAGGTTTTC-3'
Il10	Primer depot	5'-ATCGATTTCTCCCCTGTGAA-3'	5'-TGTCAAATTCATTCATGGCCT-3'
Il12a	Primer3	5'-CTATGGTCAGCGTTCCAACA-3'	5'-GGCCAAAAAGAGGAGGTAGC-3'
Itgam	Primer depot	5'-ATTCGGTGATCCCTTGGATT-3'	5'-GTTTGTTGAAGGCATTTCCC-3'
Itgax	Primer3	5'-GAGAAGACCAGTGTGGTCGAA-3'	5'-ATTGGGTGAGTGGGTTCTGA-3'
Lamp1	Primer depot	5'-GTGGCAACTTCAGCAAGGA-3'	5'-GATACAGTGGGGTTTGTGGG-3'
Marco	Primer depot	5'-GAAGACTTCTTGGGCAGCAC-3'	5'-CCATTTCTCTTCTTGGGCAC-3'
Mmp12	Primer3	5'-GCACATTTTGATGAGGCAGA-3'	5'-TGAACAGCAACAAGGAAGAGG-3'
Mrc1	Primer3	5'-ATATATAAACAAGAATGGTGGGCAGT-3'	5'-TCCATCCAAATGAATTTCTTATCC-3'
Mrc2	Primer3	5'-GCAAAACCTGCAGAAGCTGT-3'	5'-ACCATCTGTCCACCTGAAGC-3'
Ms4a2	Primer3	5'-AATCCTCCAGTGCACCTGAC-3'	5'-TTTGTGTTGCTCCCAGGAA-3'
Msr1	Primer3	5'-TGACAAAAGAGATGACAGAGAATCA-3'	5'-TAGTGCTGTGAGGAAGGGATG-3'
Msr2	Primer3	5'-GCTCCTATTCCTGTAAGGCAGAT-3'	5'-TGGAAACAGCTCTTGGACATT-3'
Nos2	Primer depot	5'-TGAAGAAAACCCCTTGTGCT-3'	5'-TTCTGTGCTGTCCCAGTGAG-3'
Retnla	Primer depot	5'-CCCTTCTCATCTGCATCTCC-3'	5'-CTGGATTGGCAAGAAGTTCC-3'
Siglec1	Primer3	5'-AGCAACCGCTGGTTAGATGT-3'	5'-AGTTCCTCTCCATGCCTTCA-3'
Stat6	Primer3	5'-AACTCAGCTCAGATATGGGGTATC-3'	5'-CAGGTGAGGCTCCTGAAAAG-3'
Tnf	Primer depot	5'-CCACCACGCTCTTCTGTCTAC-3'	5'-AGGGTCTGGGCCATAGAACT-3'

2.8 Buffers and solutions

Unless noted otherwise in the text, all buffers and solutions were prepared using ultrapure water generated by a Millipore water purification system.

Acid mix for arginase enzyme assays	70 ml H_2O, 30 ml H_3PO_4 (85%) and 10 ml H_2SO_4 (96%) were mixed (7/3/1, v/v/v) and used immediately.
Brewer's thioglycollate solution, 4%	4.0 g Brewer's thioglycollate were dissolved in 100 ml H_2O and autoclaved.
BSA stock solution, 10 mg/ml	100 mg BSA (Fraction V) were dissolved in 10 ml protein lysis buffer and used immediately for arginase enzyme activity assays.
DEPC-H_2O	1.0 ml DEPC was added to 1.0 l of H_2O, stirred over night and autoclaved.
EDTA, 0.5 M, pH7.5, 100 ml	18.61 g EDTA-Na_2 (Triplex III) were dissolved in H_2O, pH was adjusted to 7.5 using sodium hydroxide pellets (~4 g) and volume was adjusted to 100 ml using H_2O. EDTA solution was autoclaved before use.
(α)-Isonitrosopropiofenon, 6%	600 mg α-ISPF were dissolved in 10 ml 100% EtOH and used immediately.
L-arginine, 0.5 M, pH 9.7, 50 ml	4.36 g L-arginine were dissolved in 40 ml H_2O, adjusted to pH 9.7 using 6 N HCl and filled up to 50 ml using H_2O.
$MnCl_2$, 1 M, 10 ml	1.26 g $MnCl_2$ were dissolved in 10 ml H_2O and autoclaved.
$MnCl_2$ solution, 10 mM, 100 ml	1 ml 1 M $MnCl_2$ was added to 99 ml 50 mM Tris-HCl (pH 7.5) and autoclaved.
MOPS buffer pH 7.0, 10x	200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA in H_2O. pH was adjusted to 7.0 using 1 M sodium hydroxide. MOPS buffer was protected from light and stored at RT.
$NaNO_2$ stock solution, 25 µg/ml	125 mg $NaNO_2$ were dissolved in 5.0 ml medium, diluted 1:1000 in medium and used immediately for nitrite determination.

PBND buffer, 1x	50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM MgCl2, 0.1 mg/ml gelatin, 0.45% (v/v) Nonidet P40, 0.45% (v/v) Tween 20, mixed in H_2O and sterilized by filtration.
Protease inhibitor cocktail, 25x	1 tablet (Roche Complete without EDTA) was dissolved in 2 ml D-PBS and stored in 200 µl aliquots at -20°C.
Protein lysis buffer	50 mM Tris-HCl pH 7.5, 0.5% Triton X-100, 40 µl/ml 25x protease inhibitor cocktail (Roche Complete without EDTA) were added just before use.
Proteinase K solution	20 mg/ml Proteinase K (lyophilized powder; Merck, Darmstadt, Germany) dissolved in millipore H_2O.
RNA isolation lysis buffer	Lysis buffer from respective RNA isolation kit containing 10 µl of 2-ME per milliliter.
RNA loading buffer	5 ml contain: 2950 µl Formamide 1180 µl Formaldehyde (≥37%) 855.5 µl 10x MOPS pH 7.0 29.5 µl Ethidium bromide (10mg/ml) RNA loading buffer was stored in 1 ml aliquots at -20°C.
Staining buffer for flow cytometry	2% heat-inactivated FCS in D-PBS.
SYBRgreen buffer for TaqMan, 2x	5 ml contain: 1000 µl 10x Taq Buffer (w/o deterg.) 75 µl 25 mM dNTPs 200 µl Rox reference dye (25x) 2000 µl PCR optimizer (2.5x) 100 µl BSA, PCR grade (50x) 20 µl SYBRgreen I (250x) 1200 µl 25 mM $MgCl_2$ 405 µl Aqua ad injectabilia SYBRgreen buffer was stored in 1.20 ml aliquots at 4°C.
TAC erythrocyte lysis buffer, 1x	TAC buffer was freshly prepared before use by mixing 90% solution A and 10% solution B. Solution A (170 mM NH_4Cl in H_2O) and B (240 mM Tris-HCl pH 7.5 in H_2O) were filter-sterilized and stored at 4°C until use.
TBE buffer, 10x	25 mM EDTA-Na_2 (Titriplex III), 900 mM Tris base and 889 mM boric acid were dissolved in H_2O.

TE buffer, 1x	10 mM Tris-HCl pH7.5, 1 mM EDTA, in H_2O, autoclaved.
Tris-HCl, 50 mM, pH 7.5, 500 ml	3,03 g Tris was dissolved in ~400 ml H_2O, adjusted to pH 7.5 using 6 N HCl, filled up to 500 ml with H_2O and autoclaved.
Urea stock solution, 1 mg/ml	1.0 g urea was dissolved in 10 ml 50 mM Tris-HCl pH7.5, diluted 1:100 in 50 mM Tris-HCl pH7.5 and used immediately for arginase enzyme activity assays.

2.9 Cell culture reagents and media

Cell culture reagents

2-Mercapthoethanol, 50 mM (1000x)	Invitrogen, Karlsruhe, Germany
Amino acid solution, 50x, liquid	Invitrogen, Karlsruhe, Germany
DMEM+Glutamax I, 4.5 g/l Glc	Invitrogen, Karlsruhe, Germany
D-PBS, Dulbecco's PBS without Ca/Mg (1x)	PAN Biotech GmbH, Aidenbach, Germany
Fetal calf serum (FCS): FBS superior	Biochrom, Berlin, Germany
FCS (heat-inactivated)	FCS was heat-inactivated before use by incubation at 56°C in a water bath for 30 minutes.
Harvest medium	RPMI1640 with Glutamax I, 10% heat-inactivated FCS, 1% PenStrep
Hank's balanced salt solution (HBSS)	Sigma-Aldrich, Schnelldorf, Germany
Non-essential amino acids, 100x, liquid	Invitrogen, Karlsruhe, Germany
Penicillin/Streptomycin 10 ku/ml Penicillin, 10 mg/ml Streptomycin	PAA Laboratories, Vienna, Austria
RPMI1640+Glutamax I	Invitrogen, Karlsruhe, Germany
Trypan blue, 0.4%	Sigma-Aldrich, Schnelldorf, Germany
Trypsin-EDTA, 1x 0.5 g/l Trypsin, 0.22 g/l EDTA in D-PBS.	PAA Laboratories, Pasching, Austria

Cell culture media

PM medium

Composition: RPMI1640+Glutamax I, 10% heat-inactivated FCS and 1% Penicillin/Streptomycin
Application: Cultivation of peritoneal macrophages

HS medium

Composition: DMEM+Glutamax I (4.5 g/l Glc), 10% heat-inactivated FCS and 1% Penicillin/Streptomycin
Application: Harvesting of splenocytes

SSB medium

Composition: DMEM+Glutamax I (4.5 g/l Glc), 10% heat-inactivated FCS, 1% Penicillin/Streptomycin, 1x non-essential amino acid solution, 1x amino acid solution and 50 µM 2-ME
Application: Stimulation of splenocytes and BMDM

BBM medium

Composition: DMEM+Glutamax I (4.5 g/l Glc), 10% heat-inactivated FCS, 1% Penicillin/Streptomycin and 50 µM 2-ME
Application: Harvesting of bone marrow cells
Removal of stromal cells and mature macrophages from bone marrow cells
Differentiation of bone marrow cells in Petri dishes (= BBM medium + 20 ng/ml murine recombinant M-CSF)
Stimulation of BMDM

DBM medium

Composition: DMEM+Glutamax I (4.5 g/l Glc), 15% L929s-conditioned medium, 10% heat-inactivated FCS, 5% heat-inactivated horse serum, 1% Penicillin/Streptomycin and 50 µM 2-ME
Application: Differentiation of bone marrow cells in Teflon bags

L929 medium

Composition: DMEM+Glutamax I (4.5 g/l Glc), 10% heat-inactivated FCS, 1% Penicillin/Streptomycin
Application: Cultivation of L929 cells

CC medium

Composition: DMEM+Glutamax I (4.5 g/l Glc)/50% heat-inactivated FCS/10% DMSO
Application: Cryoconservation medium for BMDM

2.10 Consumables

6 well plates for bacteriology	Greiner Bio-One, Frickenhausen, Germany
6 well plates for tissue culture	Corning Costar, Lowell, Massachusetts, USA
10 cm plastic dish for bacteriology	Greiner Bio-One, Frickenhausen, Germany
10 cm plastic dish for tissue culture	TPP, Trasadingen, Switzerland
12 well plates for tissue culture	Corning Costar, Lowell, Massachusetts, USA
15 cm plastic dish for tissue culture	TPP, Trasadingen, Switzerland
Cell strainer, 100 µm	BD Biosciences, Heidelberg, Germany
Centrifugation tubes, 15 ml, polyprop.	BD Falcon, Heidelberg, Germany
Centrifugation tubes, 50 ml, polyprop.	BD Falcon, Heidelberg, Germany
Cryovials, 2.0 ml, free standing	Alpha Laboratories, Eastleigh, UK
FACS tubes, round bottom, 5 ml	BD Biosciences, Heidelberg, Germany
Forceps, sterile and disposable	Seidel Medizin, Gauting-Buchendorf, Germany
Gauge needle, 20Gx1½″, BD Microlance 3	BD Biosciences, Heidelberg, Germany
Gauge needle, 26Gx½″, BD Microlance 3	BD Biosciences, Heidelberg, Germany
lumox™ film fluorocarbon foil, 25 µm	In Vitro Systems & Services, Göttingen, Germany
MagNA Lyser Green Bead tubes	Roche Diagnostics, Mannheim, Germany
Microscope slides, SuperFrost, grinded	Menzel, Braunschweig, Germany
Microtiter plates, 96 well, flat bottom	Greiner Bio-One, Frickenhausen, Germany
Paper pad cards for cytospin	Shandon Inc., Pittsburgh, Pennsylvania, USA
PCR tubes, 0.5 ml, DNase/RNase-free	Trefflab, Degersheim, Switzerland
Reaction tubes, PCR clean, 1.5 ml	Eppendorf AG, Hamburg, Germany
Scalpels, sterile and disposable, No. 11	Feather, Osaka, Japan
Scalpels, sterile and disposable, No. 20	Feather, Osaka, Japan
Sterile filter, PES, 0.22 µm, 250 ml	TPP, Trasadingen, Switzerland

Syringes, BD Plastipak, 1 ml	BD Biosciences, Heidelberg, Germany
Syringes, BD Discardit II, 10 ml	BD Biosciences, Heidelberg, Germany
Syringe filter, 0.45 µm pore size	Nalgene, Roskilde, Denmark
TissueTek	Leica Microsystems, Nussloch, Germany

2.11 Kits

ABC detection system	Vector Laboratories, Burlingame, USA
BD Fix & Perm kit	BD Biosciences, Heidelberg, Germany
DC protein assay, detergent compatible	Bio-Rad, München, Germany
DiffQuick staining kit	Dade Behring, Marburg, Germany
PureLink RNA Mini Kit	Invitrogen, Karlsruhe, Germany
RNase-free DNase set	Qiagen, Hilden, Germany
RNeasy Mini Kit	Qiagen, Hilden, Germany
RiboGreen (20x)	Invitrogen, Karlsruhe, Germany
Superscript I kit	Invitrogen, Karlsruhe, Germany
Superscript II kit	Invitrogen, Karlsruhe, Germany

2.12 Devices

ABI Prism 7000 Sequence Detection System	Applied Biosystems, Weiterstadt, Germany
ABI Prism 7700 Sequence Detection System	Applied Biosystems, Weiterstadt, Germany
Bag sealer Polystar 100 GE and tongs 30D	Rische & Herfurth, Hamburg, Germany
Camera for Leica DMIL, Jenoptic ProgRes CF	Jenoptic, Jena, Germany
Camera for Leica DMRBE, DC300F	Leica Microsystems, Nussloch, Germany
Centrifuge, bench top, Rotanta 460R	Hettich, Tuttlingen, Germany
Centrifuge, bench top, Universal 16	Hettich, Tuttlingen, Germany

Centrifuge, refrigerated, model 5417R	Eppendorf, Hamburg, Germany
Centrifuge, Cytospin	Shandon Southern, Runcorn, UK
CO_2 incubator	Heraeus, Hanau, Germany
Cryo freezing container	Nalgene, Roskilde, Denmark
FACSCalibur flow cytometer	BD Biosciences, Heidelberg, Germany
Electrophoresis chamber	MBT Brand, Gießen, Germany
Hemocytometer, Neubauer improved, 0.1mm	Marienfeld, Lauda-Königshofen, Germany
Hitachi 9-17E autoanalyzer	Hitachi, Frankfurt, Germany
Hot air oven	Heraeus, Hanau, Germany
Image documentation system CS1	Cybertech, Berlin, Germany
Image capture computer ICC/4	Cybertech, Berlin, Germany
MagNA Lyser	Roche Diagnostics, Mannheim, Germany
Microplate reader, GENios plus	TECAN, Crailsheim, Germany
Microscope, Leica DMIL	Leica Microsystems, Nussloch, Germany
Microscope, Leica DMRBE	Leica Microsystems, Nussloch, Germany
Power supply, PowerPack 300	Bio-Rad, München, Germany
Pipettes, Pipetman 2/20/200/1000 µl	Gilson, Middleton, Wisconsin, USA
Pipettor, accu-jet pro	Brand, Wertheim, Germany
Qubit fluorometer	Invitrogen, Karlsruhe, Germany
Spectrophotometer UV/Vis, DU 530	Beckman Coulter, Fullerton, California, USA
Thermocycler, RoboCycler Gradient 96	Stratagene, La Jolla, California, USA
Thermo mixer, Thermomixer comfort	Eppendorf, Hamburg, Germany
Thermo mixer, Thermomixer 5436	Eppendorf, Hamburg, Germany
UV transilluminator, (254 nm)	Bachofer, Reutlingen, Germany
Vortex mixer, Vortex-Genie 2	Scientific Industries, New York, USA

2.13 Software

CellQuest 3.3 for Mac	BD Biosciences, Heidelberg, Germany
Endnote X for Mac	Thomson Reuters, Carlsbad, California, USA
FlowJo 6.4.7 for Mac	Tree Star, Ashland, Oregon, USA
HUSAR software package (version 5)	Genetics computer group, University of Wisconsin, Madison, USA [180, 181]
Illustrator 10.0.3 for Mac	Adobe, Dublin, Ireland
Office 2004 for Mac	Microsoft, Redmond, Washington, USA
Photoshop 7.0 for Mac	Adobe, Dublin, Ireland
Primer3	Steve Rozen, Helen Skaletsky, Whitehead Institute for Biomedical Research, Cambridge, USA [181]
Prism 4.0c for Mac	GraphPad, La Jolla, California, USA

2.14 Mouse genotyping

1-2 mm of sample material were cut off the end of a mouse tail using sterile surgical scissors and placed in 1.5 ml reaction tubes on ice. To each sample 200 µl of PBND buffer containing 1 µl Proteinase K solution (20 mg/ml) were added and incubated in a thermo mixer for 4 h at 56°C and 1000 rpm to degrade tissue material. Afterwards, indigestible material was pelleted by centrifugation at 20,000xg for 1 minute and 150 µl of the supernatant were transferred to new 1.5 ml reaction tubes and stored at -20°C until analysis. 1 µl of the obtained lysates was used in PCR reactions. The PCR reaction mix was prepared on ice and contained:

2.50 µl	10x PCR buffer (ThermoPol)
4.00 µl	1.25 mM dNTPs
1.00 µl	10 pmol/µl forward primer
1.00 µl	10 pmol/µl reverse primer
1.00 µl	tail DNA lysate
0.20 µl	5 u/µl AmpliTaq polymerase
15.30 µl	H_2O (Aqua ad injectabilia)
25.00 µl	

For multiplex reactions (up to 4 primer pairs) the amount of H_2O added was adjusted accordingly. PCR reactions were carried out in a RoboCycler using the following program for the detection of Ccr1 and Ccr5 wildtype and knock-out alleles:

1 cycle	3 minutes at 94°C
30 cycles	30 seconds at 94°C, 30 seconds at 60°C and 30 seconds at 72°C
1 cycle	5 minutes at 72°C

10 µl of the PCR reaction were separated on 2 % agarose gels prepared with 0.5x TBE. The running buffer and the gel contained ethidium bromide at a concentration of 0.1 µg/ml. Expected amplicon sizes are listed in **Table 2** (see Materials section 2.7).

2.15 Orthotopic kidney transplantation (DKFZ)

Wildtype, $Ccr1^{-/-}$, $Ccr5^{-/-}$ and $Ccr1^{-/-}/Ccr5^{-/-}$ C57BL/6 (H-2^b) mice were used as recipients of fully MHC-mismatched BALB/c (H-2^d) renal allografts (n≥10 per group). For isograft controls C57BL/6 donor kidneys were transplanted into C57BL/6 recipient mice (n=5). Orthotopic kidney transplantation was performed as described elsewhere [183]. Briefly, the left kidney attached to a segment of the aorta and the renal vein along with the ureter was removed from the donor animal *en bloc*. The donor aorta and inferior vena cava were then anastomosed end to side to the recipient abdominal aorta and inferior vena cava below the level of the native renal vessels, respectively. The native left kidney was removed before revascularization. Donor and recipient ureter were anastomosed end to end at the border of the renal pelvis. The native right kidney was immediately removed after grafting. The animals did not receive any immunosuppressive therapy throughout the experiment. Grafts were explanted and further processed either on post-transplantation day 7 or on day 42. For histology, graft samples were fixed in buffered 4% formaldehyde or zinc fixative and embedded in paraffin. For immunohistochemistry the samples were embedded in TissueTek and for RNA analysis the samples were snap-frozen in liquid nitrogen.

2.16 Determination of blood urea nitrogen and creatinine (DKFZ)

Blood was obtained either 7 or 42 days after transplantation from the heart of anesthetized mice using heparinized glass capillaries. After centrifugation plasma was collected and stored at -20°C. Blood urea nitrogen (BUN) and plasma creatinine levels were measured using a Hitachi 9-17 E autoanalyzer.

2.17 Histopathology and lesion scores (DKFZ)

Light microscopy was performed on 3 µm sections stained by PAS. A person trained in nephropathology performed the histopathologic analysis in a blinded manner. Histopathologic inspection of sections from allo- and isografts included counting of mononuclear infiltrates and assessment of graft injury in three compartments of renal grafts: endothelia covering the inner surface of blood vessels as well as the glomerular and the tubulointerstitial compartment. While scores for vascular rejection, tubulointerstitial inflammation and acute glomerular damage are a composition of scored infiltration by mononuclear cells and tissue damage, the scores for tubulointerstitial damage and transplant glomerulopathy do not include infiltration by mononuclear cells (see below for details). Different lesion scores were calculated as described in the following ([184, 185] and personal communication with Dr. Eva Kiss, nephropathologist, DKFZ Heidelberg).

Acute and chronic vascular rejection score

The vascular rejection was evaluated in whole kidney sections including cortex and outer stripe of outer medulla. Acute vascular rejection was assessed as no injury (0), sticking of the mononuclear cells to the endothelium (0.5), subendothelial location of the mononuclear cells (1), inflammation of the media, including transmural infiltration (2), fibrinoid necrosis of the vessel wall and/or thrombosis of the vessel in addition to the inflammatory reaction (3).

Chronic vascular rejection was evaluated as negative (-) or positive (+), determined as narrowing of the luminal area by fibrointimal thickening with or without presence of foam cells and expressed as the percentage of (+) vessels. The vascular rejection index was defined as the percentage of vessels with respective degree of the rejection encountered in a whole kidney section. The vascular rejection score was calculated as the sum of all specific vascular rejection indices, whereby the index of vessels with degree 0.5 was multiplied by 0.5, that of degree 1 by 1, that of degree 2, by 2, that of degree 3 by 3.

Acute tubulointerstitial damage score

Acute tubulointerstitial damage was evaluated as nonexistent (0), as thinning of the brush border with or without interstitial edema (0.5), thinning of the tubular epithelia with or without interstitial edema (1), denudation of the tubular basement membrane with or without interstitial edema (2), and tubular necrosis with or without interstitial edema (3). Tubulointerstitial damage was judged in 20 high-power fields of cortex (objective 40x), and the tubulointerstitial damage score was calculated in the same way as described for the vascular rejection score.

Chronic tubulointerstitial damage score

Chronic tubulointerstitial damage was defined as broadening of the basement membrane of the tubuli with flattened epithelium, tubular atrophy, and interstitial matrix increase. It was evaluated as 0.5, focal chronic damage and 1, diffuse chronic damage. Tubulointerstitial damage was judged in 20 high-power fields of cortex (objective x40), and the tubulointerstitial damage score was calculated as described for the transplant glomerulopathy score.

Tubulointerstitial inflammation score

The interstitial inflammation score was described by degree: no mononuclear cells in the interstitium or tubuli (0), focal mononuclear cell infiltration in the interstitium (0.5), focal mononuclear infiltration in the interstitium with tubulitis (1), diffuse mononuclear infiltration of the interstitium (2), and diffuse mononuclear infiltration in the interstitium with tubulitis (3). Tubulitis was defined as one or more mononuclear cells per tubular cross-section. The tubulointerstitial inflammation was judged in 10 fields (objective 20x) of cortex; the total tubulointerstitial inflammation score was calculated as described for the total vascular rejection score.

Acute glomerular damage score

Acute glomerular damage was described as no injury (0), sticking of mononuclear cells to the capillary endothelium in less than 50% of the convolute (0.5), sticking of the mononuclear cells to the capillary endothelium in more than 50% of the convolute (1), mesangiolysis with or without sticking of the mononuclear cells (2), aneurysm, thrombosis, or necrosis of the capillary loops (3). The acute glomerular damage score was calculated as described for the total vascular rejection score.

Transplant glomerulopathy score (=glomerulosclerosis score)

Transplant glomerulopathy was defined as 0, no sclerosis; 0.5, sclerosis of less than 25% of capillary loops; 1, sclerosis of 26 to 50% of the capillary loops; 2, sclerosis of 51 to 75% of the capillary loops; 3, sclerosis of more than 75% of the capillary loops. The transplant glomerulopathy score was calculated as the sum of all specific injury indices, whereby the index of glomeruli with degree 0.5 was multiplied by 0.5, that of degree 1 x 1, that of degree 2 x 2, that of degree 3 x 3.

2.18 Immunohistochemistry (DKFZ)

Immunohistochemical staining was performed on 5 µm sections of frozen- or zinc-fixed tissue. Antibodies and antibody concentrations used are listed in the Materials chapter (see 2.5, Table 1). The rabbit polyclonal antiserum against murine Chi3l3 peptides [186] was kindly provided by Dr. Kimura (NCI, Bethesda, MD). An ABC detection system was applied for visualization. Controls, omitting the first antibody or replacing the first antibody by a nonimmune IgG, were negative for each section tested. The number of cells staining positive for the above markers were counted for all glomeruli and in 10 high power fields (HPF, 40x) of the tubulointerstitium.

2.19 Isolation and purification of total RNA

RNA extraction from different sources

For the preparation of total RNA from tissues (spleen or renal allografts) snap frozen organs or parts thereof were transferred into prechilled 2 ml screw tubes filled with 1.4 mm ceramic beads (MagNA Lyser, Green Bead tubes, Roche) and 1 ml of lysis buffer containing 2-ME. Tubes were loaded into the rotor of a MagNA Lyser instrument (Roche) and tissue was disrupted by two runs at 6500 rpm for 1 minute separated by 2 minutes on ice. Lysates were transferred into new tubes and the volume was increased by the addition of lysis buffer (*e.g.* for RNA extraction from a complete spleen or renal allograft, volume was adjusted to 4 ml lysate). Lysates were stored at -20°C and cleared by centrifugation at 4300rpm and 4°C for 10 minutes before RNA purification.

Lysates from in vitro cultured cells (*e.g.* bone marrow-derived and peritoneal macrophages) were prepared by completely removing supernatant and adding an appropriate amount of lysis buffer containing 2-ME to the cell culture vessel (*e.g.* $1.5x10^6$ BMDM were lysed in 1.0 ml of lysis buffer per well of a 6 well plate). After incubation for 2-3 minutes at RT lysates were mixed by pipetting, transferred into prechilled tubes and stored at -20°C until RNA extraction.

For the generation of lysates from splenocytes growing adherently and in suspension the suspension cells were centrifuged (500xg, 4°C, 5 minutes) and during this time, adherent cells were lysed by adding 1.0 ml lysis buffer containing 2-ME to each well of a 6 well plate, incubation for 2-3 minutes at RT and pipetting. This lysate was transferred into the tube containing the corresponding pellet of suspension cells and pelleted cells were disrupted by pipetting. Lysates prepared in this manner were stored at -20°C until RNA extraction.

Total RNA was extracted from lysates using commercial kits either from Qiagen (RNeasy Mini Kit) or Invitrogen (PureLink Mini RNA kit) according to manufacturer´s instructions. Briefly, lysates were loaded onto columns containing ion exchange material and washed once with buffer included in the respective kit. Contaminating genomic DNA was removed by on column-digestion with RNase-free DNase (Qiagen) for 20 minutes and RNA was eluted in 60-100 µl RNase-free H2O after several washing steps.

RNA quantification

Determination of RNA concentrations was done spectrophotometrically. RNA samples were appropriately diluted in RNase-free H_2O and absorbance was measured at 260 nm using a spectrophotometer (Beckman Coulter, model DU530). The RNA concentration was calculated by using the Beer-Lambert law and consideration of the dilution factor:

$A_{260} = \varepsilon \times C \times L$

A: absorbance at 260 nm

ε: extinction coefficient (for RNA: 0.025 $(mg/ml)^{-1}cm^{-1}$)

C: concentration of nucleic acid

L: path length of the spectrophotometer cuvette

Since an A_{260} of 0.1 corresponds to ~4 µg/ml RNA this method is often not practical for RNA samples with low concentrations. In such cases a fluorescent dye called RiboGreen (Invitrogen) was used in combination with the Qubit fluorometer (Invitrogen). Upon binding to nucleic acids

RiboGreen exhibits a large fluorescence enhancement and RiboGreen is relatively insensitive to contaminants of nucleic acid preparations (manufacturer's data). To determine the RNA concentration of a sample using the RiboGreen assay 10 µl of appropriately diluted RNA sample (1:200 – 1:2000 according to the expected yield) were added to 190 µl of working solution in RNase-free 0.5 ml PCR tubes. The working solution consisted of 5 µl RiboGreen (1x) and 185 µl 1x TE buffer. The sample was vortexed shortly and incubated for 5 minutes in the dark. The Qubit fluorometer was calibrated by using RNA standards with 0 and 10 ng/µl in high sensitivity (HS) mode. Samples were measured and the indicated RNA concentration (ng/ml) was multiplied with the total dilution factor and converted to µg/µl.

RNA quality control

Integrity of isolated RNA was verified by analytical agarose gel electrophoresis. A 1% agarose 1x MOPS gel was prepared and 0.5-1 µg of RNA sample (in a maximum volume of 10 µl) was added to 17 µl RNA loading buffer. After incubation at 65°C for 15 minutes to remove secondary structures the denatured RNA was chilled on ice and centrifuged. RNA samples were electrophoretically separated at 90 V for 30-45 minutes and RNA quality was assessed by visualization using an UV transilluminator and a digital camera system.

Optimal RNA quality is indicated if (1st) the upper 28S ribosomal RNA band is about double the intensitiy of the lower 18S ribosomal RNA band and only a weak or no smear is visible in each lane (signs of RNA degradation) and (2nd) if no further bands above the 28S ribosomal are visible that would indicate contamination with genomic DNA.

2.20 Reverse transcription of total RNA

Renal allograft RNA samples

To obtain cDNA from renal allograft RNAs the Superscript I kit from Invitrogen was used according to the manufacturer's instructions with the following modifications. The reaction mix for reverse transcription was prepared on ice and contained:

8.00 µl	5x first strand buffer
0.80 µl	25 mM dNTPs
2.00 µl	100 mM DTT
0.50 µl	15 µg/ml linear acrylamid
0.43 µl	1.56 µg/µl hexanucleotide mix
1.00 µl	40 u/µl RNasin
0.87 µl	200 u/µl Superscript I
13.60 µl	

2 µg of total RNA were added and the final volume was adjusted to 40 µl using DEPC-H_2O. RNA was reverse transcribed at 42°C for 60 minutes. The samples were centrifuged and stored at -20°C until analysis.

RNA Samples from spleen and in vitro cultured cells

During the course of this thesis, Superscript I became unavailable by the manufacturer. Therefore, Superscript II (Invitrogen) was used instead and the procedure of reverse transcription was altered in a way to obtain comparable results as with Superscript I. 1-2 µg of RNA were adjusted to a volume of 26.4 µl with DEPC-H_2O and RNA was incubated for 5 minutes at 65°C to remove secondary structures. The RNA samples were transferred to ice, spun down and 13.6 µl of reaction mix (as used for renal allograft RNA samples) containing Superscript II instead of Superscript I were added. The samples were vortexed for 2-3 seconds, centrifuged and put into a heating block set to 42°C for 90 minutes. Afterwards the samples were incubated

at 85°C for 5 minutes to inactivate the Superscript II enzyme. The samples were centrifuged and stored at -20°C until analysis.

2.21 Real-time RT-PCR

The mRNA expression levels of selected genes were quantified by real-time RT-PCR using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Pre-designed cDNA-specific TaqMan Gene Expression Assays (Applied Biosystems) were applied in combination with the TaqMan Universal PCR master mix (Applied Biosystems) according to manufacturer's instructions in a final volume of 20 µl. Thermal conditions were set according to the specifications of the manufacturer. Self-designed cDNA-specific primers were used in combination with a SYBRgreen buffer (see buffers and solutions). For this approach one reaction mix contained:

10.00 µl	2x SYBRgreen buffer for TaqMan
0.60 µl	10 pmol/µl forward primer
0.60 µl	10 pmol/µl reverse primer
0.12 µl	5 u/µl Taq polymerase
2.00 µl	cDNA sample
6.68 µl	H_2O (Aqua ad injectabilia)
20.00 µl	

Depending on the expression level of the target gene cDNA samples were used either undiluted or diluted 1:10 in TE buffer. The following thermal conditions were used for self-designed primers at the ABI Prism 7700 Sequence Detection System:

1 cycle	5 seconds at 50°C
1 cycle	5 minutes at 95°C
40x cycles	15 seconds at 95°C, 1 minute at 60°C

Usually, mRNA expression in each sample was determined in duplicates and expression of target genes was normalized to 18S ribosomal RNA using the comparative CT method in all

cases [187]. The resulting relative expression levels (REL) were multiplied by a factor of 10^5 to avoid very small numbers.

2.22 Determination of alloreactive antibody levels

Flow cytometry was used to determine alloreactive antibody levels in plasma samples from C57BL/6 wildtype (n=3) and $Ccr5^{-/-}$ mice (n= 3), which had been immunized on day -21 and -14 by i.p. injection of $4x10^7$ BALB/c splenocytes. In parallel, plasma samples from wildtype (n=5) and $Ccr5^{-/-}$ (n=3) renal allograft recipients 42d post transplantation were analyzed using a modification of published methods [113, 188]. Aliquots containing $4.5x10^5$ J558L BALB/c plasmacytoma cells ($H\text{-}2^d$) [189] in 150 µl HBSS were mixed with 150 µl HBSS diluted plasma samples from wildtype (1:4) or $Ccr5^{-/-}$ (1:4, 1:16, 1:64, 1:256) C57BL/6 recipients ($H\text{-}2^b$) and this mixture was incubated for 1 h on ice. After 3 washing steps with D-PBS/2% FCS, the cells were stained for 30 min on ice with a 1:20 dilution of a FITC-conjugated goat anti-mouse IgG and IgM specific antiserum (BD Biosciences) in 200 µl D-PBS/2% FCS. The cells were washed twice, fixed using Cytofix (BD Biosciences), and analyzed by flow cytometry using a FACSCalibur instrument (BD Biosciences). Plasma from naive C57BL/6 mice served as a control. For each sample the mean channel fluorescence was determined. Flow cytometry data were collected using CellQuest and analyzed using FlowJo software. Antibodies used in these experiments are listed in **Table 1** in the Materials section (see 2.4).

2.23 Preparation of protein lysates from cultured cells

Culture medium was removed completely and cells were washed once with D-PBS. Protein lysates for the determination of arginase enzyme activity were generated by adding 300 µl protein lysis buffer to each well of a 6 well plate containing $2x10^6$ cells and incubation for 30

minutes at 4°C on a horizontal shaker. Lysates were either directly quantified for protein content or snap frozen in liquid nitrogen and stored at -80°C.

2.24 Protein quantitation

Protein concentrations of cell lysates were determined using the detergent compatible (DC) protein assay (Bio-Rad) in a microplate scale according to the manufacturer's instructions.
25 µl of solution A' (consisting of 1 ml solution A plus 20 µl solution S) were prefilled in the wells of a 96 well microtiter plate. A standard curve was prepared in protein lysis buffer using triplicates of ten BSA standards in concentrations ranging from 0 to 2.0 µg/µl. 5 µl BSA standard or sample and 200 µl solution B were added per well and mixed by pipetting up and down. The plate was incubated at RT in the dark for 15 minutes and absorbance was determined at 690 nm using a microplate reader.

2.25 Determination of arginase enzyme activity

Arginase enzyme activity was determined as previously described with slight modifications [190-192]. Determination of arginase activity is based on the measurement of urea production rates. For each sample protein content was determined and duplicates of each sample were used to determine arginase enzyme activity. Arginase enzyme was activated by adding 20 µl 10 mM $MnCl_2$ solution to 100 µl protein sample (containing 3.0 µg protein in protein lysis buffer) and incubation for 8 minutes at 56°C. Afterwards 100 µl 0.5 M L-arginine (pH 9.7) were added and the sample was incubated at 37°C for 1h to allow conversion of L-arginine to urea and ornithine by arginase. During this time ten urea standards were prepared in duplicates at a concentration ranging from 0 to 10 mM urea. Urea production was stopped by adding 780 µl acid mix to protein samples and 900 µl acid mix were added to 100 µl of each urea standard. By addition of 40 µl 6% α-ISPF and two incubation steps (45 minutes at 95°C followed by 30

minutes at 4°C in the dark) a colorimetric reaction was induced that changed sample color from clear/transparent to pink-violet depending on the urea concentration. 200 µl of standard or sample were transferred into the wells of a 96 well microtiter plate and absorption was measured at 540 nm in a microplate reader. Arginase enzyme activity was calculated as nM urea per µg protein per hour (nM µg protein^{-1} h^{-1}).

2.26 Determination of NO production

Griess' reagent was used to determine nitrite concentrations in cell culture supernatants. Nine nitrite standards ranging from 0 to 6.4 ng/µl were prepared in duplicates from a fresh 25 µg/ml $NaNO_2$ stock solution in DMEM medium. The wells of a 96 well microtiter plate were prefilled with 100 µl of Griess' reagent and mixed with 100 µl nitrite standard or cell culture supernatant by pipetting up and down. After incubation for 10 minutes in the dark the absorbance was measured at 540 nm using a microplate reader. Nitrite production was calculated as nmol nitrite per mg protein per 48 h (nmol NO_2^- mg protein^{-1} 48h^{-1}).

2.27 Cytospin and differential leukocyte staining

For cytospins cell suspensions were prepared containing 2.0x10^5 cells/ml in a buffer consisting of 90% FCS and 10% D-PBS. 200 µl cell suspension was spun down onto clean, degreased microscope slides mounted with paper pad cards for 2 minutes at 500 rpm in a cytospin centrifuge and air dried. For differential staining the DiffQuick kit from DADE Behring was used. The samples were stained by dipping the slide 5x for 1 second into fixative solution, holding the slide 8 seconds into solution 1 and 3 seconds in solution 2. The slides were rinsed in distilled water and air dried before microscopic inspection.

2.28 Experimental peritonitis and cultivation of elicited peritoneal macrophages

Mice were injected i.p. with 2.5 ml of 4% Brewer's thioglycollate medium and euthanized by CO_2 asphyxiation at 1.5, 5.0 and/or 96h after injection. The abdomen was sterilized by wetting with 70% ethanol and a small midline incision was made with sterile scissors. The abdominal skin was carefully retracted leaving the abdominal wall intact. Avoiding perforation of intestines 10 ml ice cold harvest medium was injected using a syringe with 26G needle. The needle was retracted from the peritoneum and the slat with the pinned down mouse was put on a vortex mixer for 30 seconds to increase the amount of cells in suspension. To collect the harvest medium containing the detached cells a syringe with a 20G needle was inserted into the peritoneal cavity and the peritoneal fluid was carefully withdrawn. The cell suspension was transferred into 50 ml polypropylene centrifugation tubes on ice. Numbers of viable cells were determined using the dye exclusion method with trypan blue and a hemocytometer. The cells were pelleted by centrifugation at 250xg for 4 minutes and resuspended in PM medium at an appropriate concentration for the respective experiment. In some experiments harvested peritoneal lavage cells were lysed directly using 1 ml of RNA isolation lysis buffer for $4x10^6$ cells. Peritoneal lavage cells harvested after thioglycollate-induced peritonitis were cultivated in tissue culture 6 well plates at a density of $10x10^6$ per well in 3 ml PM medium. Cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere for the times indicated in each experiment.

2.29 Preparation of splenocyte suspensions and cultivation of splenocytes

For the generation of single cell suspensions from spleens mice were euthanized by CO_2 asphyxiation and the spleen was explanted under aseptic conditions. The spleen was transferred into a 10 cm bacteriological plastic dish filled with 5 ml ice cold HS medium and was minced on ice using two disposable sterile forceps. The suspension was sieved through a 100

µm cell strainer into a 50 ml polypropylene tube and remaining material was forced through the strainer using the plunger of a syringe. The strainer was then rinsed with medium and the cells were centrifuged (10 minutes, 75xg, RT). Afterwards the splenocytes were resuspended in SSB medium, seeded in duplicates into the wells of a tissue culture 6 well plate at a concentration of $5x10^6$ cells per well and incubated at 37°C and 5% CO_2 in a humidified atmosphere (3 ml medium per well) for 24 h. In case of low RNA yields, RNA lysates were prepared by combining lysates from duplicate wells. Therefore, the supernatants of duplicate wells were combined and the suspension cells were pelleted (5 minutes, 250xg, RT). Adherent cells were lysed by adding 1 ml of RNA isolation lysis buffer to one of the duplicate wells and then transferring to the second well. This lysate was then combined with the pelleted suspension cells of both wells. In this way 1 ml RNA lysate was generated from the total splenocytes (adherent and non-adherent cells) of duplicate wells from one mouse. RNA isolation and reverse transcription was carried out as described above.

2.30 Preparation of splenocyte suspensions for FACS analysis

Single cell suspensions of splenocytes were prepared as described in 2.29 with the distinction that medium was replaced by staining buffer. After centrifugation (10 minutes, 75xg, RT) erythrocytes were lysed by resuspending the cell pellet in 5 ml TAC erythrocyte lysis buffer for 4 minutes at RT. After this time 25 ml staining buffer were added and the suspension was filtered through a 100 µm cell strainer. The cells were pelleted again (5 minutes, 400xg, 4°C) and resuspended in staining buffer. After determining the number of viable cells using a hemocytometer and the dye exclusion method the cell concentration was adjusted to $20.0x10^6$ cells/ml. This cell suspension was used for extra- and intracellular staining procedures as outlined in flow cytometry.

2.31 Generation of bone marrow-derived macrophages (BMDM)

Harvesting of bone marrow cells

Mice were euthanized by cervical dislocation and skin was removed from hind legs using disposable sterile scalpels and forceps. Femur and tibia were explanted, muscle tissue removed from the bones and the articular capsules were cut off at both ends. A syringe with a 26G needle was inserted into the bone cavity and bone marrow was flushed out with BBM medium until the bone cavity appeared white. Wash medium from one mouse was collected in a 50 ml polypropylene centrifugation tube on ice and cell aggregates were separated by pipetting up and down. Bone marrow cells were pelleted by centrifugation (10 minutes, 200xg, 4°C) and used to generate bone marrow-derived macrophages as described below.

Differentiation of bone marrow cells in Petri dishes

To remove stromal cells and mature macrophages the bone marrow cell suspension of one mouse was first resuspended in 10 ml BBM medium and then seeded in 10 cm tissue culture dishes and incubated at 37°C and 5% CO_2 in a humidified atmosphere for 4h. After this time non-adherent cells were centrifuged (10 minutes, 200xg, RT) and resuspended in 30 ml BBM medium containing 20 ng/ml murine recombinant M-CSF. The cell suspension was seeded on a 15 cm tissue culture dish and incubated at 37°C and 5% CO_2 in a humidified atmosphere for 7 days. During this time non-adherent cells were removed every 24h and half of the medium was replaced by 15 ml fresh medium containing 40 ng/ml murine recombinant M-CSF. Cells grown in a confluent monolayer (**Figure 11**) were harvested by washing once with 10 ml D-PBS and incubation with 5 ml 5 mM EDTA for 10 minutes at RT. After detachment EDTA was inactivated with medium and cells were pelleted (5 minutes, 200xg, RT) to remove EDTA before seeding the cells for stimulation experiments or FACS analysis. This method yielded 10-15x10^6 bone

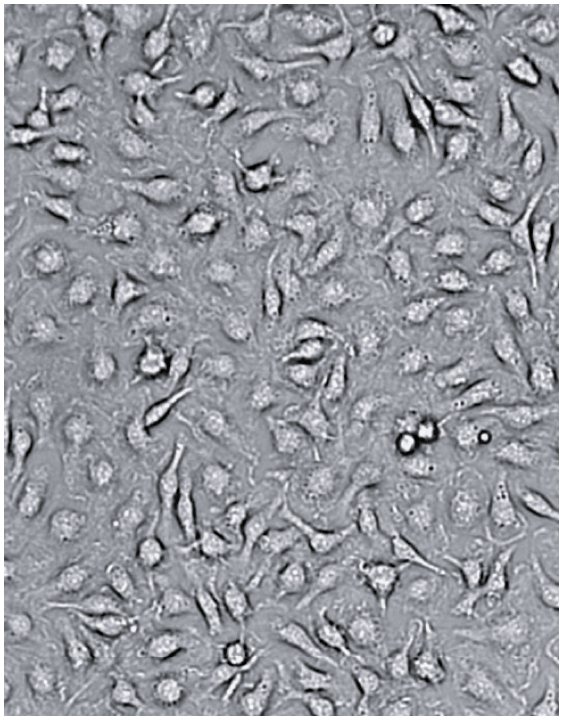

Figure 11. Micrograph showing morphology of BMDM 7 days after seeding of bone marrow cells onto tissue culture plastic dishes (200x). Bone marrow cells were harvested and cultivated as outlined in the text (see 2.31).

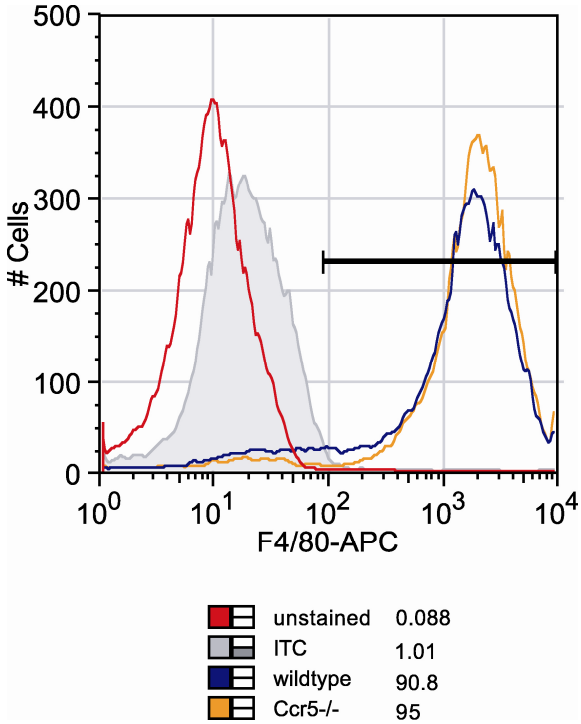

Figure 12. Flow cytometric analysis of F4/80 surface expression on BMDM generated in Petri dishes. Numbers below the histogram indicate percentages of cells within the plotted region.

marrow-derived macrophages per mouse and ≥90% of these cells stained positive for the macrophage marker F4/80 (**Figure 12**).

Differentiation of bone marrow cells in Teflon bags

Numbers of viable bone marrow cells were determined using the dye exclusion method with trypan blue and a hemocytometer. Cell concentrations were adjusted to $5x10^6$ cells/ml in DBM medium. Teflon bags were filled with 50 ml differentiation medium, 2 ml of bone marrow cell suspension were added and bags were sealed by heat using a bag sealer. The Teflon bags were placed on plastic racks to allow gas exchange from both sides (**Figure 13**) and incubated at 37°C and 10% CO_2 in a humidified atmosphere for 10 days. After this time bone marrow-derived macrophages were detached by incubating the Teflon bags for 30 minutes on ice and gentle

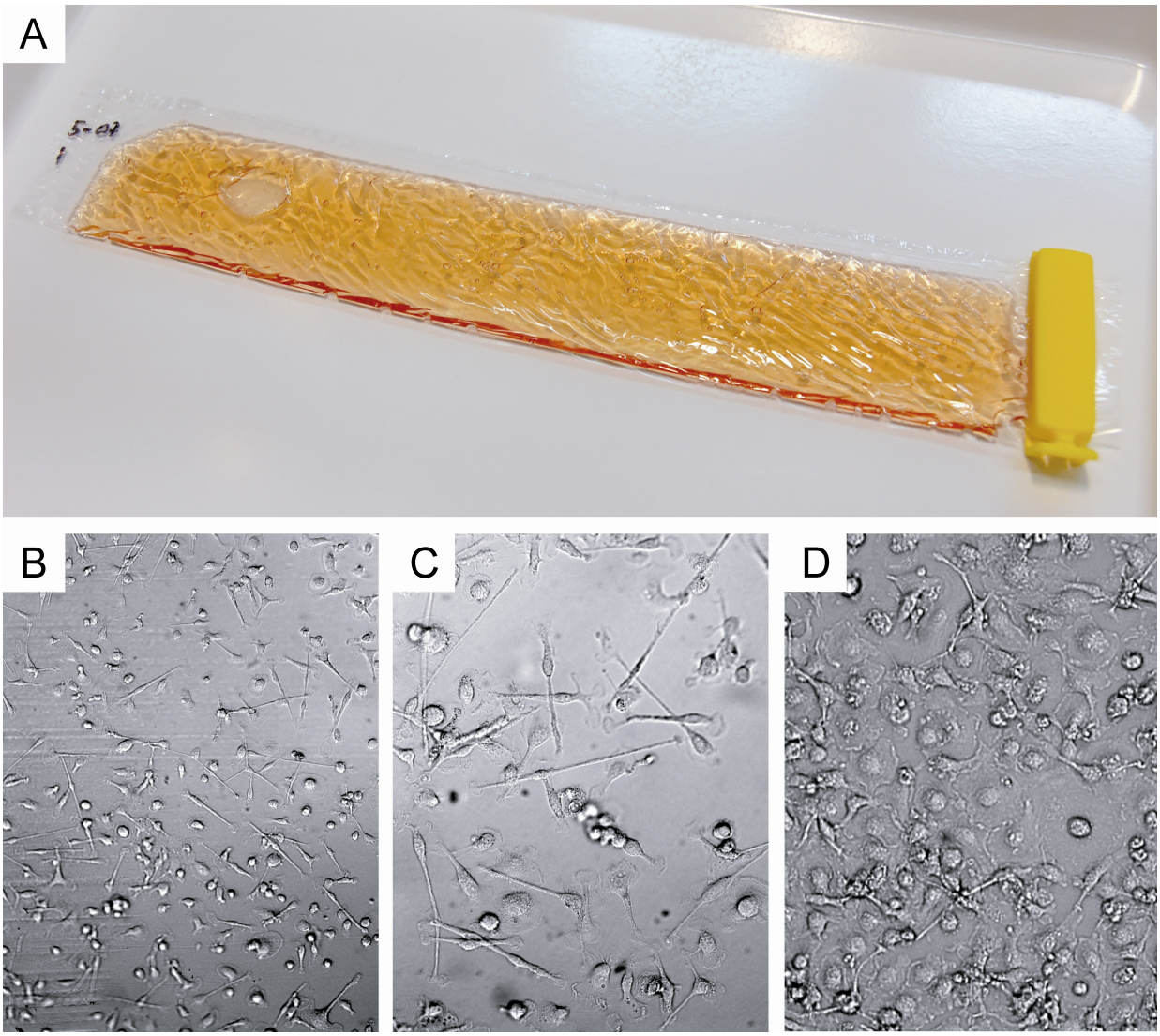

Figure 13. Cultivation and morphology of BMDM generated in Teflon bags. Teflon bag **(A)** and microscopic images of BMDM 1 day after re-seeding into Teflon bags (**(B)** 200x, **(C)** 400x). **(D)** BMDM cultivated 24h on tissue culture plastic dish (400x). Bone marrow cells were harvested and cultivated as outlined in the text (see 2.31).

massage and stretching of the bags. The bags were opened using sterile scissors and the suspension of BMDM was transferred into 50 ml polypropylene centrifugation tubes for further use in stimulation experiments or FACS analysis. In this manner, up to $30x10^6$ BMDM were harvested from one Teflon bag. Excess BMDM not used immediately for experiments were either seeded into fresh Teflon bags and further cultivated or cryopreserved (see 2.32) and stored frozen in liquid nitrogen.

Preparation of Teflon bags

Lumox™ film fluorocarbon foil (In Vitro Systems & Services) was cut into strips of 25x10 cm size and put onto a glass plate with the hydrophobic side facing upward. The stripe was folded at the long side and sealed by heat. In the next step the open long side was sealed. To facilitate opening of the bag after hot air sterilization a small stripe of aluminum foil was inserted into the upper part of the bag before wrapping it into aluminum foil as a container for sterilization. Sterilization was done in a hot air oven at 180°C for 1.5 h.

Long-term storage of BMDM in liquid nitrogen

Excess BMDM were pelleted (5 minutes, 200xg, RT) and resuspended in CC medium at a concentration of 10-20x10^6 cells per aliquot. Aliquots of 1.5 ml were filled into cryovials, stored in an isopropyl alcohol-filled cryo freezing container at -80°C over night and transferred to liquid nitrogen.

Preparation of L929s-conditioned medium

L929s fibroblast cells are known to produce high amounts of chemokines and growth factors like CCL2 (MCP-1) and CSF1 (M-CSF) that promote macrophage differentiation and survival [193, 194]. Therefore L929s-conditioned medium has been extensively used as a source for these factors and to generate bone marrow-derived macrophages [195]. For the generation of L929s-conditioned medium 0.25x10^6 L929s cells were seeded into 15 cm tissue culture dishes containing 25 ml of L929 medium. The cells were incubated at 37°C and 10% CO_2 in a humidified atmosphere until the cells formed a confluent monolayer (8-10 days). The supernatant (=L929s-conditioned medium) was sterilized using 0.22 µm sterile filters (TPP) and aliquots of 45 ml were stored at -80°C. The remaining cells were washed once with D-PBS and detached by adding 5 ml Trypsin-EDTA (1x) per dish for 10 minutes at RT. After detachment

Trypsin-EDTA was inactivated by addition of fresh medium and the cell concentration was determined using a hemocytometer. After pelleting (5 minutes, 200xg, RT) the cells were either seeded again as outlined above or resuspended in medium containing 7% DMSO at a concentration of $2.0x10^6$cells/ml for long term storage in liquid nitrogen.

2.32 Cultivation and stimulation of bone marrow-derived macrophages (BMDM)

BMDM were generated as described above and used for stimulation experiments at a concentration of $2.0x10^6$ cells per well of a tissue culture 6 well plate. Cells were rested over night in 3 ml medium per well (3) without M-CSF or L929s-conditioned medium before stimulation with reagents indicated in the respective experiment. Unstimulated cells served as controls. After the stimulation period medium was removed and BMDM cells were lysed using RNA isolation lysis buffer. For arginase enzyme activity assays $0.75x10^6$ BMDM cells were seeded in the wells of a tissue culture 12 well plate in a volume of 1.5 ml medium.

2.33 Flow cytometry

Dead cell discrimination

For the exclusion of non-viable cells the G-C base-specific DNA intercalating reagent 7-AAD (7-Amino-Actinomycin D, BD Biosciences) was used [196]. Like propidium iodide (PI) 7-AAD diffuses into dead and dying cells due to loss of membrane integrity, whereas living cells exclude these fluorescent DNA dyes. However, in contrast to PI 7-AAD has minimal spectral overlap with FITC and PE fluorescence emissions and is therefore ideally suited for use in conjunction with FITC- and PE-labeled antibodies in multicolor analysis. For the detection of 7-AAD fluorescence a 650 nm long-pass filter (FL3) in the far red spectrum was used. By using 7-AAD dead cells are excluded on the basis of their forward scatter (FSC) and FL3 properties. Dead cells have high 7-AAD intensities (FL3) and medium to low forward scatter intensities

[197]. Dead cell discrimination was done as the last step before analysis and after labeling cells with monoclonal antibodies. Therefore 1.0-3.0x10^6 cells were resuspended in 100 µl staining buffer containing 5 µl of 7-AAD. Cells were incubated for 10 minutes at RT in the dark and immediately analyzed at the flow cytometer without further washing steps. Cells fixed for intracellular staining were not stained with 7-AAD.

Fc receptor (FcR) blocking

Almost all immune cells express receptors that bind immunoglobulin molecules. During the adaptive immune response these receptors bind to sites located on the constant region of immunoglobulin (Ig) molecules [198]. Therefore it is important to block these receptors before staining with fluorescently labeled antibodies since binding to Fc receptors would lead to increased non-specific binding of test antibodies and hence high background staining. Blocking of Fc receptors was achieved by incubating the cells in staining buffer containing a non-labeled monoclonal antibody recognizing CD16 and CD32 (FcR gamma 3 and 2b, respectively). Cell suspensions were incubated with Fc blocking antibody (Abd Serotec, Mouse Seroblock FcR) at 4°C for 30 minutes before addition of the fluorescently labeled monoclonal antibodies. Depending on the origin of cells, Fc block was applied at a final dilution of 1:200 for BMDM or 1:400 for splenocytes. Fc block was not removed before addition of the fluorescently labeled monoclonal antibodies. Optimal dilutions of Fc block were determined by titration in halving steps (1:50 – 1:1600) against F4/80-APC antibody.

Determination of optimal staining concentrations of antibodies by titration

Using the optimal concentration of antibody is important in flow cytometric analysis. Factors that determine antibody binding rates (*i.e.* the affinity constant) are temperature, pH, buffer composition, antibody concentration and amount of available antigen. Very high antibody

concentrations can lead to diminished signal intensities due to self-quenching and cause increased non-specific binding ("high background") of test and isotype-matched control antibody [199]. Low antibody concentrations underestimate the presence of antigen thereby masking differences between samples of different origin (*e.g.* genotype, stimulation, etc.). To find optimal antibody concentrations test and isotype-matched control antibodies were titrated at equal concentrations and mean fluorescence intensities (MFI) were determined. The antibody concentration giving the best signal-to-noise ratio (i.e. MFI (test Ab) vs. MFI (control Ab)) was used as the optimal antibody concentration. Since antigen amounts vary depending on experimental conditions, titrations were performed for each experimental set individually.

Staining of extracellular antigens

Staining of cell surface antigens was done by incubating 1.0-3.0x10^6 cells in 100 µl staining buffer containing monoclonal antibodies for 30 minutes at RT or 1 h at 4°C in 5 ml round bottom tubes (BD Falcon) in the dark. Optimal antibody concentrations were determined by titration as outlined above. After incubation the cells were washed once in 3 ml ice cold staining buffer (5 minutes, 440xg, 4°C), resuspended in 200 µl staining buffer and stored on ice until flow cytometric analysis.

2.34 Statistical analysis

Values are presented as means ± SD. Pairwise statistical comparisons were performed either by two-tailed unpaired Student´s t test (parametric data) or Mann-Whitney U test (nonparametric data). $P < 0.05$ was considered significant.