Renal allograft rejection in chemokine receptor Ccr1$^{-/-}$, Ccr5$^{-/-}$ and Ccr1$^{-/-}$/Ccr5$^{-/-}$ mice

and

Impact of Ccr5 deficiency on macrophage polarization

Von der Fakultät für Energie-, Verfahrens- und Biotechnik
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Stefan Dehmel

geboren in Altötting

Hauptberichter: Prof. Dr. Klaus Pfizenmaier
Mitberichter: Privatdozent Dr. Bruno Luckow

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To a hammer every problem looks like a nail;

to an immune system every problem looks like an infection.

Rot and von Andrian,
Annu. Rev. Immunol.
2004. 22:891–928.

for Melanie

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Declaration

I herewith declare that this thesis comprises only my own original research towards the degree of Doctor rerum naturalium (Dr. rer. net.) except as indicated under 'External Contribution' (see appendix 7.1) and that due acknowledgement has been made in the text to all other materials used. This thesis has not been submitted previously for a degree or any other qualification at this University or any other institution.

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Stefan Dehmel

München, _____

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Abbreviations

2-ME	2-Mercaptoethanol
7-AAD	7-Actino-aminomycin D
Ab	antibody
AAM	alternatively activated macrophages
AIDS	acquired immunodeficiency syndrome
AMA	alternative macrophage activation
APC	allophycocyanine or antigen-presenting cell
α-SMA	alpha-smooth muscle actin
bp	base pair/s
BMDM	bone-marrow derived macrophages
BSA	bovine serum albumin
BUN	blood urea nitrogen
c-Ets	cellular erythroblastosis virus E26 oncogene homolog (avian)
Ca^{2+}	calcium ions, double positive-charged
CAM	classically activated macrophages
cAMP	cyclic adenosine monophosphate
CCL	chemokine (CC-motif) ligand
CCR	chemokine (CC)-motif receptor
cM	centi Morgan
CREB	cAMP response element binding protein
CXCL	chemokine (CXC-motif) ligand
CXCR	chemokine (CXC-motif) receptor
CNS	central nervous system
Da	Dalton, unified atomic mass unit; 1 Da = 1 u = $1.660538782\times10^{-24}$ g. 1 Da is the equivalent of 1/12 of the mass of an unbound atom of carbon-12 (^{12}C) at rest in its ground state [10].
DAG	diacylglycerol
DARC	duffy blood group, chemokine receptor
DEPC-H_2O	diethylpyrocarbonate-treated H_2O
DMEM	Dublecco's modified Eagle medium
DMSO	dimethylsulfoxide
DNP	dinitrophenol
dNTP	desoxyribonucleotide triphosphate
DTH	delayed-type hypersensitivity
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
ER	endoplasmatic reticulum
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FcR	Fc (fragment, crystallizable) receptor

FDA	U.S. Food and Drug Administration
FITC	fluorescein isothiocyanat
FL	fluorescence
GAGs	glycosaminoglycans
GAP	GTPase activating protein
GDP	guanosine diphosphate
GM-CSF	granulocyte/macrophage-colony stimulating factor (= CSF2)
GTP	guanosine triphosphate
GTPase	GTP hydrolyzing enzyme
Glc	glucose
GPCRs	G protein-coupled transmembrane receptors
GRKs	G protein-coupled receptor kinases
HBSS	Hank's buffered salt solution
HMCV	human cytomegalovirus
HSP	heat shock protein
HIV	human immunodeficiency virus
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
IP_3	inositol triphosphate
IRF	interferon regulatory factor
ITC	isotype- and concentration-matched antibody control
JAK	Janus kinase
K^+	potassium ion, single positive-charged
ko	knock-out
LPS	lipopolysaccharide
LTA	lymphotoxin α
MΦ	macrophage
M-CSF	macrophage colony stimulating factor (= CSF1)
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein
MDSCs	myeloid-derived suppressor cells
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MNP	MIP-1α nuclear protein
MMPs	matrix metalloproteinases
MOPS	3-N-(morpholino)-propanesulfonic acid
MPS	mononuclear phagocyte system
NK cells	natural killer cells
N-terminal	amino-terminal, NH_2-terminal
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National center for biotechnology information

NF-IL6	nuclear protein Il6
NFκB	nuclear factor kappaB
NO	nitric oxide
NOD	nucleotide oligomerization domain
NOX system	NADPH oxidase system
ORF	open reading frame
PAS	periodic acid-Schiff stain
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
pH	power of hydrogen; pH is defined as the negative decimal logarithm of the hydrogen ion activity in an aqueous solution at RT, $pH = -\log_{10}[H_3O^+_{aq}]$, [11]
$PI(4,5)P_2$	phosphatidylinositol 4,5-bisphosphate
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	peritoneal lavage cells or phospholipase C
qPCR	quantitative PCR
RANTES	regulated upon activation, normal T-cell expressed, and secreted; new designation CCL5 (actually named after the alien protagonist in the movie *Man Facing Southwest*; [12])
REL	relative expression level
ROS	reactive oxygen species
RT	room temperature
SD	standard deviation
SLOs	secondary lymphoid organs
STAT	signal transducers and activators of transcription
TAM	tumor-associated macrophages
TBE	tris-borate-EDTA
TC	tissue culture
TGF-β	transforming growth factor beta
TNF	tumor necrosis factor
TNP-KHL	Trinitrophenol keyhole limpet hemocyanin
Tris	Tris-(hydroxymethyl-)aminomethane
TLR	Toll-like receptor
wt	wildtype
Xcr	chemokine (XC-motif) receptor

Nomenclature

According to the recommendations of the Mouse Genomic Nomenclature Committee (MGNC), mouse gene symbols used in this study begin with an uppercase letter followed by all lowercase letters (*e.g.* Ccr5), whereas protein symbols use all uppercase letters (*e.g.* CCR5) (see http://www.informatics.jax.org/nomen and [13]).

For symbols of human genes and proteins the recommendations of the HUGO Gene Nomenclature Committee (HGNC) are followed (http://www.genenames.org). Thus, symbols for human genes are all uppercase and italicized (*e.g. CCR5*), whereas human protein names are non-italicized and all uppercase (*e.g.* CCR5).

Abstract

Previous studies showed that loss of the chemokine receptor Ccr1 or Ccr5 has a beneficial effect on survival of cardiac, carotid, corneal and islet allografts in mice. Additionally, human renal allograft recipients homozygous for a null allele of *CCR5* (*CCR5Δ32*) experience significantly prolonged allograft survival. To analyze the mechanisms underlying reduced allograft rejection in $Ccr1^{-/-}$ and $Ccr5^{-/-}$ recipient mice, a renal transplantation model was utilized, that allowed the study of the acute (7d) and the clinically more important phase of chronic (42d) allograft rejection. $Ccr1^{-/-}/Ccr5^{-/-}$ mice were included to analyze whether loss of both receptors is accompanied by additional improvements. Reduced graft fibrosis, leukocyte infiltration and improved histopathology were observed in all knock-out groups, but additional improvements in $Ccr1^{-/-}/Ccr5^{-/-}$ recipients were limited to certain aspects at 42d. $Ccr1^{-/-}$ and $Ccr5^{-/-}$ recipients showed significantly diminished mRNA levels of Th1-associated cytokines and chemokines at 7d. Expression of these genes was restored to wildtype levels in $Ccr1^{-/-}/Ccr5^{-/-}$ recipients at 7d explaining the few additional improvements in $Ccr1^{-/-}/Ccr5^{-/-}$ recipients. $Ccr1^{-/-}$ recipients showed Th17-shifted immune responses, while $Ccr5^{-/-}$ recipients exhibited dramatically increased alternative macrophage (MΦ) activation (AMA) at 42d which might explain the beneficial effects observed in $CCR5^{\Delta 32/\Delta 32}$ human transplant recipients. AMA was also observed in unchallenged spleens and elicited peritoneal MΦ of $Ccr5^{-/-}$ mice indicating a general role for CCR5 in MΦ polarization. Results of bone marrow-derived MΦ suggest that strain differences influence MΦ polarization and that BALB/c allografts favor the induction of AMA in $Ccr5^{-/-}$ MΦ. Ccr1 or Ccr5 deficiency in recipients has beneficial effects on renal allograft rejection though the underlying mechanisms may be different and might lead to the observed ambiguous effects in $Ccr1^{-/-}/Ccr5^{-/-}$ recipients. Furthermore, no indication for redundant functions of CCR1 and CCR5 was found during renal allograft rejection.

1 INTRODUCTION

1.1 Chemokines and chemokine receptors

The members of the chemokine family are small, mostly basic, heparin-binding secreted proteins with a molecular weight between 8 and 14 kDa. Chemokines are *chemo*tactically active cyto*kines* that induce directed migration (chemotaxis) of leukocytes and other cells to sites of inflammation or injury. During chemotaxis cells follow a concentration gradient of a chemotactically active substance. The direction of migration depends on the nature of the substance: toxic substances will defer cells whereas nutrients and chemokines attract cells.

Chemokine structure is largely determined by the formation of intramolecular disulfide bonds between the thiol (-SH) groups of cysteine residues in the N-terminal and central regions of the amino acid backbone. Therefore, the presence and arrangement of the N-terminal cysteine residues has been used as a criterion to classify chemokines into four major subfamilies: XC- (only one N-terminal cysteine, γ-chemokines), CC- (two adjacent N-terminal cysteines, β-chemokines), CXC- (two N-terminal cysteines separated by one amino acid, α-chemokines), CX_3C- (two N-terminal cysteines with three amino acids in between, δ- chemokines – whose sole member is called Fractalkine/CX_3CL1). CC-, CXC- and CX_3C-chemokines all have 4 conserved cysteine residues forming two disulfide bonds, whereas C-chemokines lack the first and third cysteine of the other chemokine subfamily members resulting in only one intramolecular disulfide bond [9]. The rapid progress in the field of chemokine biology at the end of the 1990s resulted in considerable confusion when different groups reported the same molecule under different names. Therefore, a systematic nomenclature of chemokines and chemokine receptors was introduced in 2000. Since chemokine receptors usually bind chemokines of a specific subfamily, this nomenclature is based on the N-terminal cysteine motif determining subfamily membership [9, 14]. For instance, CCR5 binds members of the CC-chemokine

subfamily, whereas CXCR4 binds several members of the CXC-subfamily. Thus, CCR5 stands for chemokine (CC-motif) receptor 5 [9]. Accordingly, chemokines are designated by their respective N-terminal cysteine motif, *e.g.* the chemokine RANTES (regulated upon activation, normal T-cell expressed, and secreted) is now called CCL5, due to the presence of a CC-motif in the N-terminal region. However, to refer to the historical denomination, the previous designation is sometimes set in brackets behind the systematic name.

Chemokines are involved in diverse biological processes such as wound healing, lymphoid trafficking, organ development, Th1/Th2 development, angiogenesis/angiostasis, inflammation, cell recruitment and metastasis [15]. Functionally, chemokines come in two different flavors: homeostatic and inflammatory. Homeostatic chemokines are constitutively expressed and regulate homing of leukocytes within and between lymphoid organs as well as the development of lymphoid organs [16]. Additionally, homeostatic chemokines guide T and B lymphocytes to localize antigen-presenting cells (APCs) within lymphatic organs [15]. On the other hand, inflammatory chemokines are produced at a site of infection or tissue injury and induce the migration of leukocytes towards such sites where they contribute to leukocyte activation and wound healing [17]. However, the division of chemokines into homeostatic and inflammatory is not an absolute one. Some homeostatic chemokines are up-regulated during inflammation and there are inflammatory chemokines that are constitutively produced and secreted into *e.g.* milk, saliva, tears or sweat. Notably, homeostatic chemokines (*e.g.* CCL20 and CCL22) bind to only one receptor – but, one receptor may bind more than one homeostatic chemokine (compare **Figure 1**), whereas inflammatory chemokines (*e.g.* CCL5 and CCL8) appear to bind to more than one chemokine receptor [6].

Chemokines of both groups mediate their function via binding to a subfamily of seven-transmembrane, G protein-coupled receptors (GPCRs) expressed at the plasma membrane surface of leukocytes. These so-called chemokine receptors are peptide-binding members of

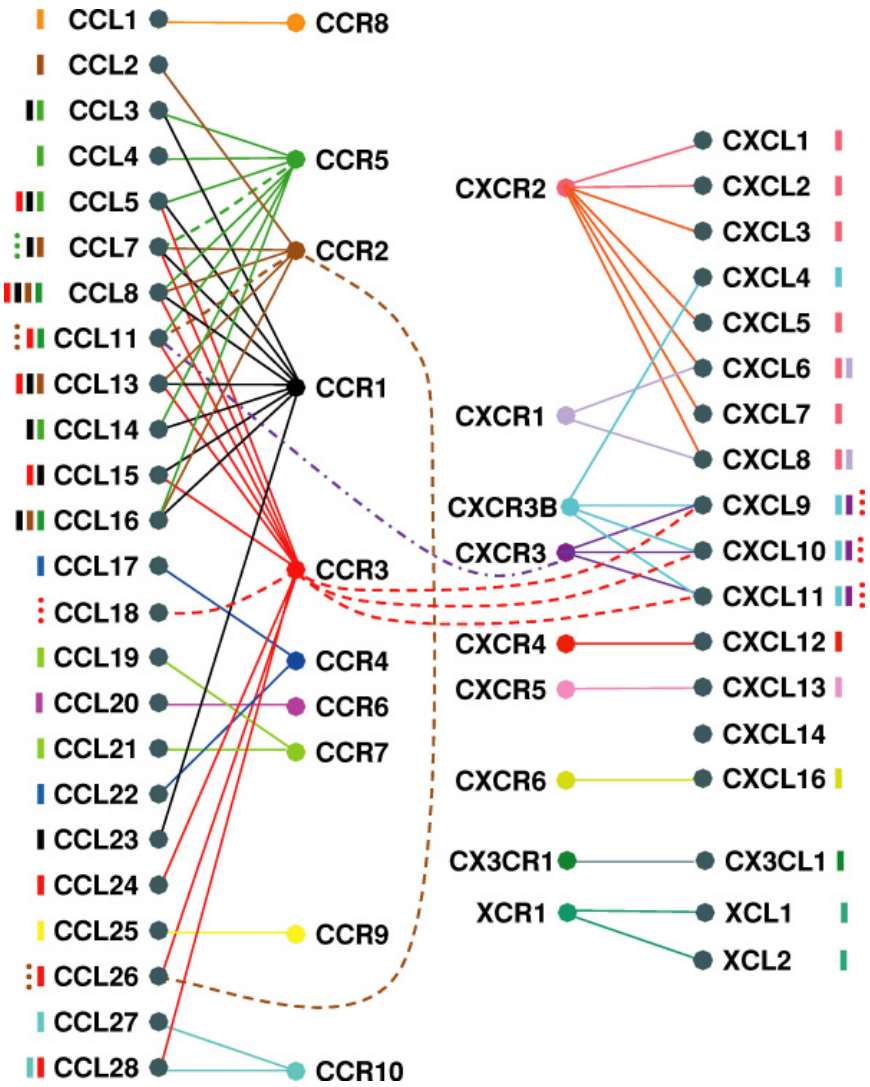

Figure 1. Overview of human chemokines and their receptors (adapted from Rot and von Andrian 2004, [6]). Solid and dashed lines connect receptors with their agonists and antagonists, respectively, and are color coded to correspond with the colors of individual receptor hubs. The bars next to individual chemokine numbers reflect the colors assigned to their apposite receptors. CXCR7, which binds to CXCL11 and CXCL12 [8], is not shown because it was unknown at the time of publication of this review. Furthermore, the so called "interceptors" DARC, D6 and CCX-CKR – chemokine receptors that bind chemokines without further signaling [6] – are excluded from the official nomenclature [9] and were therefore omitted here.

the class A Rhodopsin-like superfamily of GPCRs. They range in size from 340 to 370 amino acids and exhibit common structural features [3]. Some of these structural features are exampled in **Figure 2** by an illustration of the human CCR5 receptor protein. Chemokine receptors display the same classification already mentioned for chemokines. Some chemokine

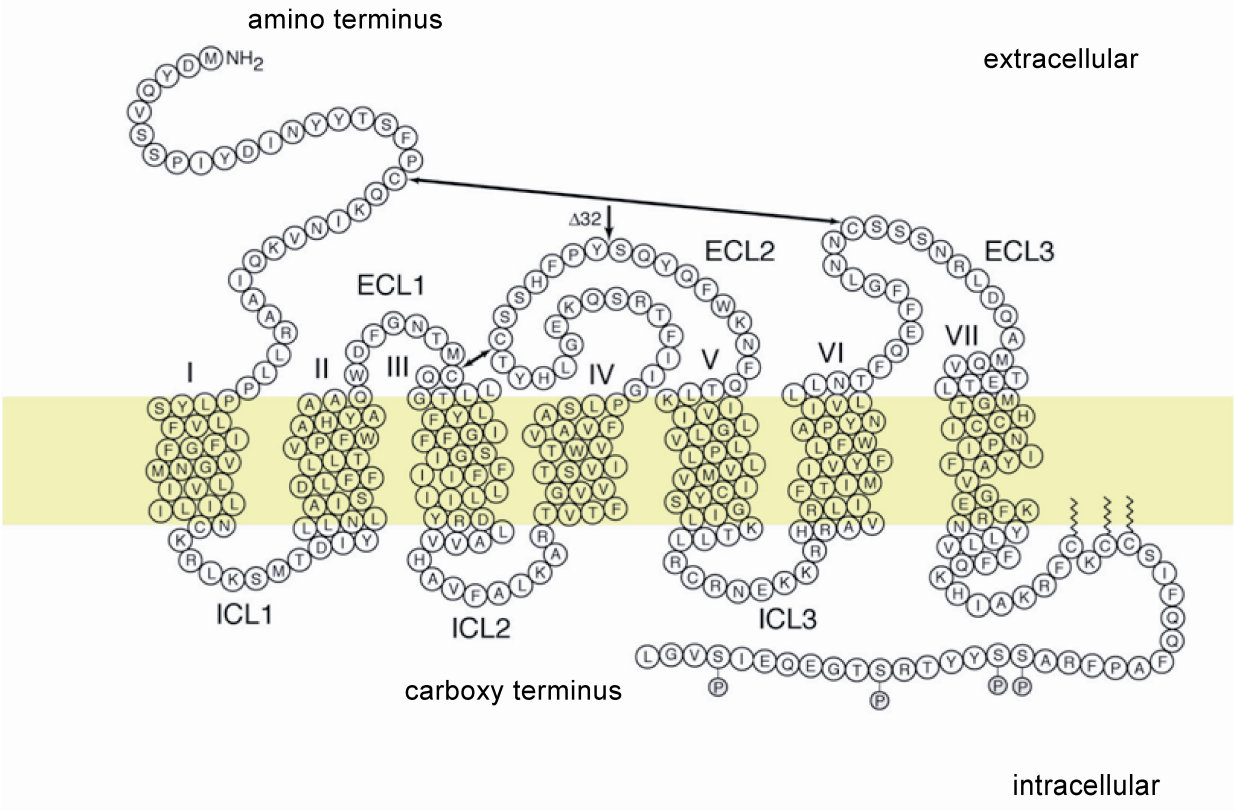

Figure 2. Proposed membrane topology of the human chemokine receptor CCR5 (adapted from Blanpain *et al.* 2002, [4]). Amino acids are indicated in one-letter code and the shaded area specifies the plasma membrane. The seven transmembrane regions of CCR5 are designated by roman numerals (I to VII). The intra- and extracellular loops between the seven transmembrane regions are indicated as ICL1-3 and ECL1-3, respectively. Two disulfide bonds link the extracellular domains of the receptor together (indicated by arrows). Δ32 indicates the location of a 32 bp deletion found at a frequency of about 1% in populations of European origin. This mutation results in a truncated, non-functional receptor that is not expressed at the surface of natural leukocytes or transfected cells but is retained in the endoplasmatic reticulum. The carboxy-terminal domain features a cluster of three palmitoylated cysteine residues anchoring this region to the plasma membrane and four phosphorylation sites for G protein–coupled receptor kinases (GRKs).

receptors – such as CCR1 and CCR5 – are up-regulated upon inflammatory stimuli or during infection, while others (*e.g.* CCR6 and CXCR4) are constitutively expressed (*i.e.* homoeostatic chemokine receptors) [6].

Chemokine receptors function as allosteric transmitters by communicating chemokine binding through modification of the tertiary structure into the inside of the cell. Ligand binding to chemokine receptors results in conformational changes and allows receptor dimerization upon

which complex signaling cascades are initiated (**Figure 3**). Receptor dimerization induces rapid phosphorylation of intracellular tyrosines residues by associated of Janus kinases (JAK) as well as subsequent recruitment and activation of STAT molecules (signal transducers and activators of transcription) [3]. Another major effect of chemokine triggering is the dissociation of receptor-associated heterotrimeric $G_{\alpha\beta\gamma}$ proteins into G_{α} and $G_{\beta\gamma}$ subunits upon exchange of G_{α}-bound GDP *vs.* GTP. Both subunits thereby acquire an activated state and are able to regulate the activity of plasma membrane-bound enzymes like adenylyl cyclase or phospholipase C (PLC). Chemokine receptors are usually coupled to G proteins with an $G_{\alpha i}$ subunit that exerts an inhibitory effect on adenylyl cyclase resulting in reduced intracellular cAMP (cyclic adenosine monophosphate) amounts, decreased cAMP-dependent protein kinase A activity and inhibition of CREB (cAMP response element binding protein)-mediated transcription. Besides these $G_{\alpha i}$-triggered effects, $G_{\beta\gamma}$ mediates the activation of phospholipase C. PLC catalyzes the hydrolytic cleavage of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] to inositol 1,4,5-trisphosphate [IP_3] and diacylglycerol (DAG) – two important secondary lipid messenger molecules. In turn, IP_3 opens Ca^{2+} channels in the membrane of the endoplasmatic reticulum thus activating calmodulin and other Ca^{2+} dependent cytoplasmic proteins. Increasing Ca^{2+} levels also induce the translocation of protein kinase C (PKC) isoforms from the cytoplasm to the plasma membrane where it is activated by Ca^{2+}, DAG and phosphatidylserine. PKC then phosphorylates downstream target proteins and initiates nuclear signal transduction events [3]. Furthermore, the finding that chemokine-induced MAPK (mitogen-induced protein kinases) and PI3K (phosphoinositide 3-kinase) cascades are inhibited by Pertussis toxin or PI3K inhibitors, respectively, indicated that both pathways are involved in chemokine signaling. Chemokine induced PI3K activity results in production of 3-phosphorylated lipids and subsequent initiation of PKC, Akt and Ras signaling. These pathways promote NADPH oxidase activity, polarization of adhesion molecules and re-organization of actin cytoskeleton. Arrestin proteins have been

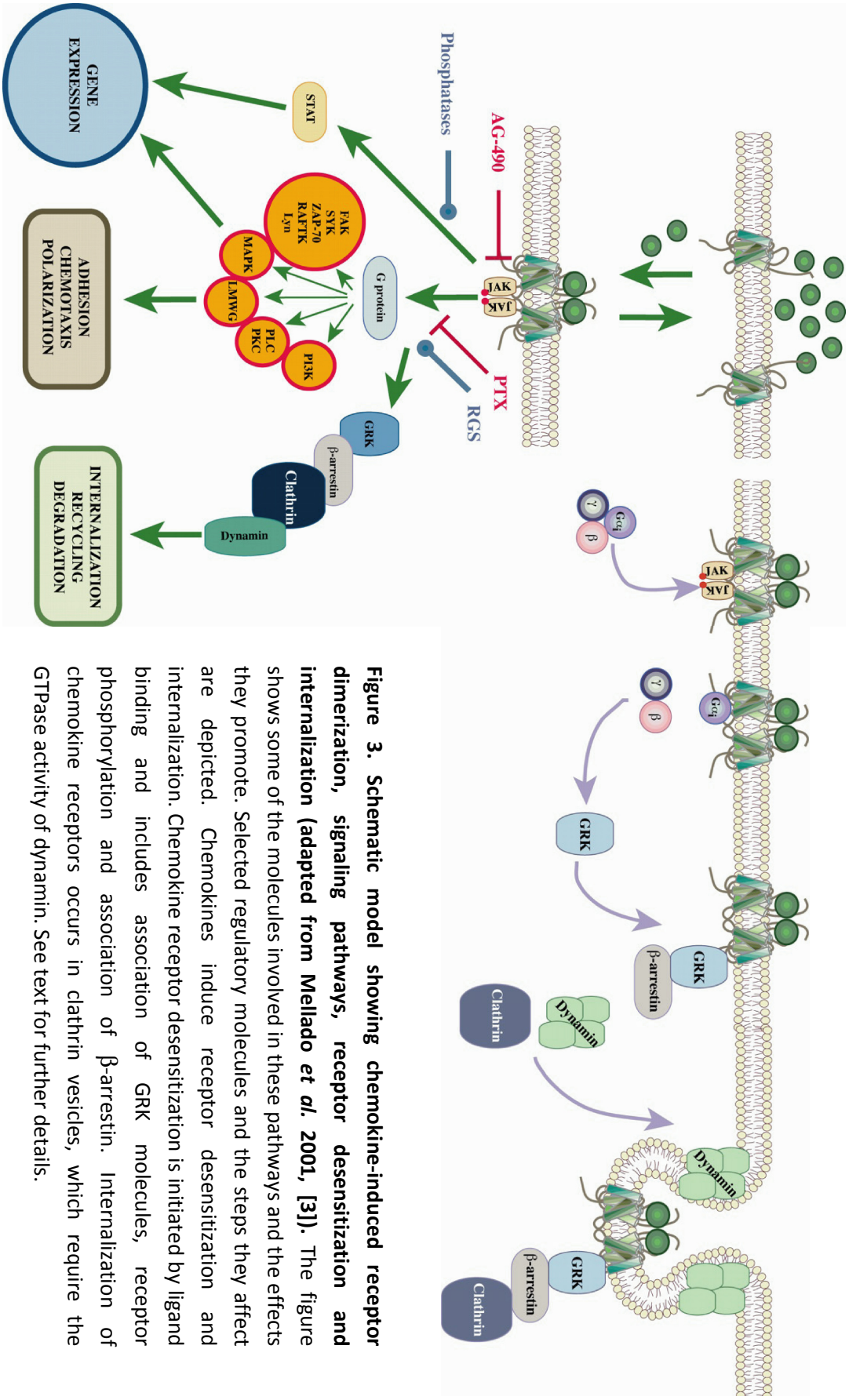

Figure 3. Schematic model showing chemokine-induced receptor dimerization, signaling pathways, receptor desensitization and internalization (adapted from Mellado *et al.* 2001, [3]). The figure shows some of the molecules involved in these pathways and the effects they promote. Selected regulatory molecules and the steps they affect are depicted. Chemokines induce receptor desensitization and internalization. Chemokine receptor desensitization is initiated by ligand binding and includes association of GRK molecules, receptor phosphorylation and association of β-arrestin. Internalization of chemokine receptors occurs in clathrin vesicles, which require the GTPase activity of dynamin. See text for further details.

shown to work as scaffold proteins mediating MAPK cascade initiation at activated chemokine receptors. Activation of phospholipase A_2 (PLA_2) through MAPK-mediated pathways results in the release of arachidonic acid which is important for the production of leukotrienes that promote actin polymerization. In addition, MAPK regulate several different protein kinases and transcription factors [3].

Chemokine receptor desensitization is thought to be an essential process that preserves the ability of cells to detect minute differences in chemokine gradients (**Figure 3**). Activated GPCRs induce their own shutdown by two processes. First, the slow intrinsic GTPase activity of activated G_α subunits leads to the re-association of $G_{\beta\gamma}$ with GDP-G_α subunits yielding an inactive heterotrimeric $G_{\alpha\beta\gamma}$ protein. GTPase-activating proteins (GAPs) accelerate this process and are therefore known as RGS proteins (regulators of G protein signaling). Second, $G_{\beta\gamma}$ subunits provide an interface for the docking of GRK proteins (G protein-coupled receptor kinases) at the receptor. The GRK protein sterically hinders G protein activation and induces phosphorylation of Ser/Thr residues at the intracellular regions of the receptor. These phosphorylation events recruit β-arrestin and adaptin molecules which uncouple receptors from G protein signaling and lead to receptor sequestering and down-regulation by internalization into clathrin-coated pits or caveolae. Receptor containing vesicles are then transported to perinuclear endosomes where the receptor is either degraded or recycled and brought back to the plasma membrane in a dephosphorylated from [3, 6, 18]. These complex regulatory pathways and signaling cascades amplify chemokine signals and result in modified gene expression patterns, cytoskeletal re-organization, increased integrin adhesiveness (a prerequisite for leukocyte transmigration), cell polarization and activation as well as directed migration towards a chemotactic source.

Chemokines and their receptors represent an extremely complex network. The human genome contains at least 53 genes encoding chemokines and 21 chemokine receptor genes (**Figure 1**).

To date, 39 chemokine genes and 20 chemokine receptor genes (Ccr1 – Ccr10, Ccr1l1, Cxcr1 – Cxcr7, Cx3cr1 and Xcr1) were identified in the genome of the mouse (Kumamoto Cytokine Database, http://cytokine.medic.kumamoto-u.ac.jp/CFC/CK/Chemokine.html).

Many chemokines bind to more than one receptor and many chemokine receptors are able to bind multiple chemokines (**Figure 1**). An agonist for one receptor might exhibit an antagonistic behavior on another receptor [19, 20]. Depending on the extracellular environment specific proteases truncate amino acids from the N-terminal domain thereby dramatically influencing receptor affinity or even specificity of chemokines [21, 22]. Receptor heterodimerization adds another layer of complexity, although the exact consequences of these interactions are unclear at present [23-26]. Furthermore, non-chemokines have been found to bind to chemokine receptors. For instance, human β-defensin 2 interacts with CCR6 [27, 28]. But it also works the other way round: chemokines bind to non-chemokine receptors: CCL16 binds to the histamine H4 receptor [29]. Cross-desensitization further complicates deciphering chemokine interactions, *e.g.* activation of CCR5 desensitizes μ-opioid receptors and vice versa [30]. Some chemokine receptors were shown to have overlapping ligand specificity and mouse models employing chemokine receptor deficient mice often show only mild effects between wildtype and knock out animals. Redundancy in the chemokine receptor network is therefore thought to be another factor contributing to this complexity. Additionally, heterodimerization of GPCR receptors – as proposed for CCR5 and CXCR4 as well as CCR5 and CCR2 - was also reported to influence signaling properties of chemokines [31-33].

1.2 Chemokine receptors Ccr1 and Ccr5

Chemokine receptor CCR1

CCR1 was identified in 1993 as the first CC-motif chemokine receptor [34]. The murine gene encoding Ccr1 has a two-exon structure with the complete ORF on exon 2 and is located on the

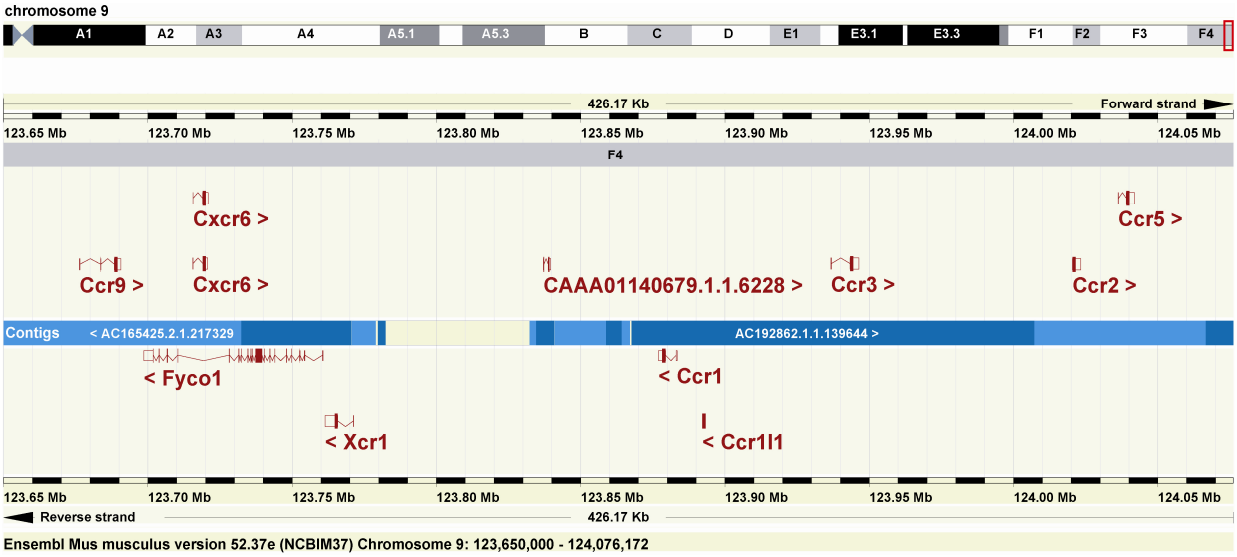

Figure 4. Map of the murine chemokine receptor cluster at the distal end of chromosome 9. The top panel shows the banding structure of chromosome 9. The lower panel represents an enlargement of the highlighted region (red box) at the end of band F4. Gene exon-intron structure and annotation is based on the Ensembl/Havana projects. Filled boxes are exons and lines connecting boxes are introns. Open boxes are untranslated regions. Annotations above the contig bar are in forward strand direction, while annotations below the contig bar are in reverse strand direction (Source: http://www.ensembl.org/; Mus musculus version 52.37e).

distal part of chromosome 9 in a cluster with seven other chemokine receptor genes: Ccr9, Cxcr6, Xcr1, Ccr1l1, Ccr3, Ccr2 and Ccr5 (**Figure 4**). Interestingly, the human cytomegalovirus encodes a functional homolog of CCR1 called US28 thereby manipulating cellular chemokine responses [35, 36]. The overall structure of the G protein-coupled chemokine receptor CCR1 resembles the structure of CCR5 (**Figure 2**) having seven transmembrane domains and two disulfide bonds that link the extracellular domains. However, some features distinguish the molecular structures of both receptors. CCR1 has a comparatively short intracellular C-terminal domain and palmitoylated cysteines which would connect the C-terminus to the plasma membrane are absent [37]. The murine as well as the human CCR1 protein has a size of 355 amino acids and CCL3 (MIP-1α), CCL5 (RANTES) and CCL7 (MCP-3) are high affinity ligands for CCR1 [9, 38]. Ccr1 expression has been reported on neutrophils, eosinophils, monocytes, T and B lymphocytes [39], basophils [40], mast cells [41], NK cells [42, 43], tissue macrophages [44,

45], immature dendritic cells [46-49], as well as erythroid progenitor cells [50] and platelets [51].

Due to its association with inflammatory and autoimmune diseases CCR1 is an interesting pharmaceutical target and several candidate antagonists are currently tested in clinical trials [52, 53]. Several studies using Ccr1-deficient mice suggest a role for this receptor in mobilization of neutrophils and hematopoietic progenitor cells [39, 54, 55]. Other studies showed a tendency towards decreased Th1 functions and/or increased Th2 immune responses in mice lacking functional CCR1 [39, 43, 56]. But these effects appear to depend on the particular disease model since other models showed increased Th1 responses in Ccr1-deficient mice [57, 58]. In a pathogen-free environment Ccr1-deficient mice develop normally but display opposite immune responses depending on the disease context.

Chemokine receptor CCR5

CCR5 shares many features with CCR1. The murine Ccr5 gene has a two-exon structure with the complete ORF on exon 2 and is located in the same gene cluster as CCR1 at the distal part of chromosome 9 (**Figure 4**). Murine CCR5 has a length of 354 amino acids, *O*-glycosylation sites, sulfated tyrosine residues and the intracellular C-terminal domain is plasma membrane-anchored via palmitoylated cysteines creating a fourth intracellular loop (compare topology of human CCR5 depicted in **Figure 2**) [18]. Human CCR5 shares about 82% amino acid identity with its murine ortholog [59]. Ccr5 expression was reported in progenitor as well as $CD4^+$ and $CD8^+$ thymocytes [9], NK cells [60], memory and effector T cells with a preferential Th1 polarization [4, 61, 62] and low levels of CCR5 were detected in circulating monocytes, whereas differentiated tissue macrophages express CCR5 at high levels [4, 45]. Furthermore, dendritic cells in the peripheral blood and immature dendritic cells as well as epidermal Langerhans cells [4], but CCR5 was absent from follicular dendritic cells in lymph nodes [9]. In addition, several

non-immune cells, such as neurons, astrocytes, endothelial and epithelial cells, smooth muscle cells and fibroblasts can express CCR5, but the functional role o this receptor in these cells remains to be determined [4, 9]. Interestingly, CCR5 expression was also found on sperm cells suggesting a role during fertilization [63, 64] and a potential way of transmitting HIV-1 by spermatozoa [65].

Pro-inflammatory stimuli (*e.g.* TNF, IL-12, LPS and ROS), Th1 cytokines like IFN-γ and IL-2 and growth factors (GM-CSF and M-CSF) were shown to up-regulate CCR5 expression on peripheral blood mononuclear cells (PBMCs), macrophages and lymphocytes [4]. Surprisingly, anti-inflammatory cytokines such as IL-10 and TGF-β can also induce CCR5 expression on monocytes, macrophages and dendritic cells [66-68].

High affinity agonists for CCR5 are CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES) and CCL8 (MCP-2), whereas CCL7 (MCP-3) acts a natural antagonist [9]. Ligand binding to CCR5 was reported to initiate heterotrimeric $G_{\alpha\beta\gamma}$ protein signaling and triggers tyrosine kinase initiated pathways and several different kinase cascades such as JAK/STAT, MAP and PI3 kinase pathways. These pathways result in inhibition of adenylyl cyclase, release of intracellular Ca^{2+}, opening of K^+ ion channels, chemotaxis and activation of leukocytes. However, none of these effects is specific for CCR5 owing to the redundancy and overlap of chemokine specificities in the chemokine receptor system. Agonist ligation finally leads to phosphorylation, internalization and receptor recycling [4, 18].

Mice with a Ccr5 deficiency appear healthy and display no developmental abnormalities. Moreover, humans homozygous for a null allele of CCR5 (*CCR5Δ32*, see below) do not show any overt phenotype; suggesting that pharmacological blockade of this receptor should result in little side-effects. CCR5 has been implicated in several important pathologic conditions like rheumatoid arthritis, multiple sclerosis, organ transplant rejection, asthma, AIDS and atherosclerosis [53]. About ~30% of all prescription drugs on the market such as beta blockers,

neuroleptics and antihistamines target GPCRs [69]. Due to these reasons CCR5 has become a promising target for pharmaceutical industry. A first CCR5 antagonist (Maraviroc, Pfizer) has already been approved for the therapy of antiviral treatment-experienced HIV patients by the FDA in 2007 [70, 71]. Several other antagonists are currently tested in clinical trials against autoimmune and inflammatory diseases [72].

Ccr5-deficient mice were used to study the effects of Ccr5 deficiency on the outcome of parasitic [60, 73-76] or viral [77, 78] infections. These studies reported significant reductions in the numbers of infiltrating macrophages, NK cells, $CD4^+$ and $CD8^+$ T cells as well as reduced numbers of regulatory T cells at the site of inflammation in animals that lacked functional CCR5. Interestingly, Glass *et al.* reported that CCR5 represents a critical antiviral and survival determinant in a model of cerebral West Nile virus infection [79]. In an Influenza infection model, Tyner *et al.* showed that CCL5 (RANTES) signaling through CCR5 generates an anti-apoptotic signal, supporting macrophage survival and virus elimination [80]. Other studies reported reduced IFN-γ production [81] and attenuated Th1 responses [58] in $Ccr5^{-/-}$ mice.

In 1996, five groups simultaneously reported that CCR5 is the major co-receptor for macrophage-tropic HIV-1 strains (**Figure 5**) [82-86]. Besides CCR5, other chemokine receptors were proposed to act as co-receptors for different HIV isolates: CXCR4 (T cell-tropic HIV-1 [87]), CXCR6 (HIV-1 and HIV-2 isolates [88, 89]), CCR2b and CCR3 (dual-tropic HIV-1 89.6 [83, 85]), the HMCV-encoded US28 receptor (HIV-1 and HIV-2 isolates [90]) and the orphan receptor GPR15 (HIV-1 and HIV-2 isolates [88]). Weissman and colleagues reported in 1997 that the envelope protein gp120 of CCR5 tropic (R5) HIV strains induces calcium flux and chemotaxis in $CD4^+$ T cells through CCR5 in a CD4-dependent manner [91]. Subsequently, it was shown that CCR5 - as well as CXCR4 - associates physically with CD4 [92] - which is the natural receptor of IL-16. Interestingly, allosteric cross-talk between CD4 and CCR5 seems to promote signaling of IL-16 as well as CCL4 and results in the preferential recruitment of Th1 polarized $CD4^+$ T cells to sites

of inflammation [93, 94]. Despite the high amino acid identity between human and murine CCR5, the murine ortholog does not confer HIV-1 entry capabilities [59]. However, some monoclonal anti-CCR5 antibodies, the chemokines CCL3, CCL4, CCL5 and several CCL5 derivatives were shown to act as HIV entry inhibitors [9, 95] (**Figure 5**). Furthermore, a 32 bp deletion allele of CCR5 (designated *CCR5Δ32*) confers resistance to infection by R5 HIV strains in homozygous carriers. However, the *CCR5Δ32* allele might have a dual effect on AIDS progression, since individuals heterozygous for *CCR5Δ32* exhibit increased AIDS-free survival time, but, upon onset of AIDS, those patients have an accelerated decrease of $CD4^+$ T cell counts and reduced survival time [96]. Interestingly, apart from their resistance to HIV-1 infection and reduced risk for asthma [97], carriers of a homozygous *CCR5Δ32* genotype - ~1% of individuals of the northern European population - do not display an obvious phenotype [9]. The 32 bp deletion leads to a truncated, non-functional receptor protein that lacks the last three transmembrane domains and is retained in the endoplasmatic reticulum [4] (compare **Figure 2**). The *CCR5Δ32* allele is frequently found in populations of Caucasian origin showing a north-south gradient in Europe.

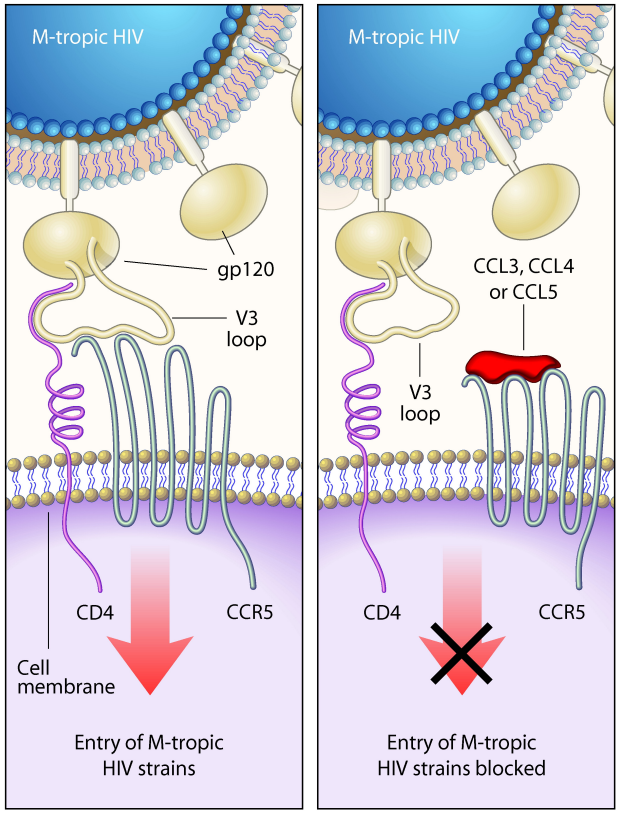

Figure 5. CCR5 mediates HIV entry and ligands of CCR5 block inhibition of HIV entry (adapted from Luster *et al.* 1998, [5]). HIV gp120 binds to CD4, inducing a conformational change that exposes the V3 loop and permits subsequent interaction with a chemokine receptor. To gain entry into cells, M-tropic HIV-1 uses CCR5 predominantly. Chemokine ligands for CCR5 (CCL3, CCL4 or CCL5) block M-tropic HIV-1 from entering cells.

Populations of northern Russia, Finland and Sweden have the highest allele frequencies (~15%) while residents in western and central European countries have allele frequencies of about 10% and around 1% homozygous carriers [98]. In southern countries such as Portugal, Greece and Turkey allele frequencies of 4-6% have been observed, and frequencies of *CCR5Δ32* drop to ~2% in northern Africa. Low allele frequencies are also found in the Middle East and India. *CCR5Δ32* does not occur in indigenous populations of other regions such as central and western Africa, China and Japan [4]. The relatively restricted distribution and the high incidence of the *CCR5Δ32* allele were attributed to a selection advantage associated with homo- or heterozygosity. Nevertheless, the driving force for such a positive selective pressure favoring the enrichment of *CCR5Δ32* remains unclear. Advantages in the resistance to pathogens like *Yersinia pestis* (plague) and *Variola* (smallpox) as well as to diseases such as rheumatoid arthritis, multiple sclerosis and asthma were postulated but not confirmed so far [4, 9]. Notably, recent studies suggested that the deletion first occurred about 7000 years ago. Thus, the observed allele frequencies might be the result of neutral evolution and genetic drift, though a possible influence of positive selection cannot be ruled out by these findings [99, 100].

1.3 Mechanisms of allograft rejection

An allograft is defined as transplanted cells, tissues or organs from a genetically different member of the same species as the recipient. Rejection of an allograft is mediated by cellular as well as humoral components of the immune system [1].

The main inductor of rapid rejection responses are allogeneic graft MHC (major histocompatibility complex) molecules. These proteins are recognized by recipient T cells by two possible pathways. In the direct pathway, intact MHC molecules of donor APCs present self- or allo-antigen either to naïve recipient T cells in the draining lymph node of the graft or to circulating memory T cells of the recipient generated during previous exposure to *e.g.* microbial

antigens. The recognition of self- or allo-antigen on foreign MHC molecules activates $CD4^+$ or $CD8^+$ T cells with reactivity towards allogeneic MHC. These T cells are self-restricted but recognize either the allogeneic MHC molecule itself or in combination with a bound peptide as a foreign structure. In a second, more indirect pathway, host dendritic cells process alloantigen within the graft or alloantigen that reached the lymph node. The alloantigen is usually presented on MHCII molecules by host APCs but presentation on MHCI molecules due to cross-priming has also been observed. Naïve T cells recognizing these alloantigens are activated and differentiate into $CD4^+$ or $CD8^+$ T cells. However, $CD8^+$ cytotoxic T cells generated by the indirect pathway are self-restricted and cannot directly kill the allograft cells [1].

Once activated by the direct or indirect pathway, alloreactive host T cells migrate into the graft and trigger graft rejection. Alloreactive $CD8^+$ cytotoxic T cells generated by the direct pathway kill graft cells which express allogeneic MHCI molecules. This reaction was suggested to be important during the acute phase of rejection. In contrast, alloreactive $CD4^+$ T helper cells might have a stronger relevance for the chronic phase of rejection by the production of cytokines which subsequently cause graft damage by inducing macrophage activation and inflammation in a manner resembling delayed type hypersensitivity (DTH) responses [1].

Polymorphic antigens other than MHC molecules induce weaker, more gradual rejection reactions, are usually presented via the indirect pathway and comprise the group of minor histocompatibility antigens. Besides these primary signals, activation of alloreactive T cells by co-stimulatory B7 molecules on APCs plays an important role during acute allograft rejection [1].

Humoral allograft rejection is initiated by preexisting host antibodies (generated during previous blood transfusion, transplantation or pregnancy) that bind to endothelial graft antigens and trigger complement activation. In turn, endothelial cell injury and exposure of the subendothelial basement membrane initiate platelet adhesion, thrombosis and vascular