Genetic and functional characterisation of killer cell immunoglobulin-like receptors (KIR) of rhesus macaques (*Macaca mulatta*)

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no materials previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the university or other institute of higher education, except where due acknowledgment has been made in the text.

Signature	**Name**	**Date and place**
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Ever tried? Ever failed? No matter.

Try again. Fail again. Fail better.

- Samuel Beckett

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Kruse PH, Rosner C, Walter L: Characterization of rhesus macaque *KIR* genotypes and haplotypes; Immunogenetics (2010), Volume 62, Number 5, 281-293

Rosner C, Kruse PH, Lübke T, Walter L: Rhesus macaque MHC class I molecules show differential subcellular localizations; Immunogenetics (2010), Volume 62, Number 3, 149-158

List of abbreviations

APS	ammonium persulfate
bp	base pairs
BSA	Bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
Cy	cyanine
DAP12	DNAX-activation protein-12
DDT	dichlorodiphenyltrichloroethane
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
FACS	Fluorescence-activated cell scanning
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
GFP	green fluorescent protein
HLA	human leukocyte antigen
Ig	immunoglobulin
INF	interferon
IPTG	isopropyl β-D-1-thiogalactopyranoside
ITAM	immunoreceptor tyrosine-based activating motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
KIR	killer cell immunoglobulin-like receptor
LB	lysogeny broth
LCR	leukocyte-receptor complex
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MOPS	3-(N-morpholino)propanesulfonic acid

mRNA	messenger RNA
NK cell	natural killer cell
OD	optical density
PAA	polyacrylamide
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
p-CA	p-coumaric acid
PCR	polymerase chain reaction
PE	phycoerythrin
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SIV	Simian immunodeficiency virus
SOC	super optimal broth with catabolite repression
SSC	side scatter
TBE	Tris/Borate/EDTA
TBS	Tris Buffered Saline
TC	tricolour
TEMED	tetramethylethylenediamine
TNF	tumour necrosis factors
UTR	untranslated region
UV	ultraviolet

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1 Introduction

1.1 *The immune system*

Almost all living species possess some sort of a defence system that serves to protect them from potentially harmful influences of their environment. In addition to systems that serve an immune function secondary to their main role (e.g., skin), humans possess an immune system consisting of two interconnected parts: innate and adaptive immune system.

The innate immune system is responsible for the first response of the body to a pathogen. While innate immune responses are unspecific with regard to the pathogen, the adaptive immune system represents the pathogen-specific defence system of the body that is responsible for a slow acting, but highly specific defence.

1.1.1 The adaptive immune system

Cells of the adaptive immune system (B and T lymphocytes) possess highly variable receptors, which are produced by rearrangement of gene segments during cell development (Kasahara et al., 2004; Kelley et al., 2005b). This leads to expression of a specific receptor for each B or T cell and their clonal daughter cells. B and T cells with self-reactive or useless receptors are already eliminated during development in the bone marrow and thymus, respectively.

The adaptive immune system is also characterised by immunological memory, which plays an important role in repeated exposure to the same pathogen.

1.1.2 The innate immune system

Unlike the adaptive immune system, the innate immune system responds to pathogens within minutes. Therefore, it is essential for the primary response of the organism against pathogens. In most cases, the innate immune system itself prevents or limits the spreading of pathogens all over the body. Hence, most infections can be abolished without causing any clinical symptoms. The innate immune system represents the evolutionary older part of the immune system because it is present in both vertebrates

and invertebrates, although in different forms. In vertebrates, both innate and adaptive immunity are functionally linked (Janeway and Medzhitov, 2002).

Besides different cell populations (e.g. natural killer (NK) cells, neutrophils and macrophages) as well as serum proteins (e.g. cytokines and complement factors) belong to the innate immune system. Macrophages can eliminate pathogens by phagocytosis; they also attract other immune cells to the site of inflammation by secreting cytokines and chemokines.

The innate immune system has no genetic rearrangement of the receptors. Therefore, it was initially perceived to be less important compared to the adaptive immune system and was less characterized than the adaptive immune system. However, this view has changed since it has become obvious that the innate immune system is a very important and fast reacting tool for prevention of pathogen invasion and is able to boost the adaptive immune system.

1.2 *NK cells*

The NK cells are lymphocytes that represent about 10 % of the lymphocyte fraction in the blood. They are functionally characterised by their ability to rapidly and efficiently kill target cells and to release various cytokines, in particular interferon-γ (INFγ). NK cells show some functional similarities with T cells. Cytotoxic T cells and NK cells share the same mechanism for eliminating cells (the secretion of TNF, granzyme and perforin) and can secrete INFγ like cytotoxic T cells and T_{H1} helper cells (Lanier, 2005). The detection of these cells takes place via different germline-encoded cell surface receptors. In humans, the repertoire of these receptors includes the killer cell immunoglobulin-like receptors (KIR) and the C-type lectin-like receptors (e.g. CD94:NKG2 heterodimers and NKG2D homodimers). Both families possess inhibitory and activating receptors (Kelley et al., 2005b).

Major histocompatibility complex (MHC) class I molecules were identified as interaction partners of T cells quite early (Dunlop and Blanden, 1977). However, the interaction between MHC class I molecules and receptors on the cell surface of NK cells were first described by Klas Kärre and colleagues (1986). When the receptors of a NK cell are interacting with MHC class I molecules on the cell surface of a healthy target cell, the inhibitory signal suppresses the activating signals and thereby prevents the target cell from being killed (Karlhofer et al., 1992; Moretta et al., 1993; Correa et

al., 1994; Kaufman et al., 1995; Vitale et al., 1995; Long, 1999). In virus-infected and tumour cells, the cell surface expression of MHC class I molecules is usually changed (by down-regulation). This down-regulation of specific MHC class I molecules is regulated by pathogens and prevents them from being detected by MHC class I restricted cytotoxic T cells. However, this down-regulation is also recognised by NK cells, i.e. by discontinuation of the inhibitory receptor signalling and involvement of activating receptors (Gasser and Raulet, 2006; Daeron et al., 2008; Long, 2008). Induction of activating NK cell receptors leads to release of granzyme B and perforin and finally to lysis of the target cell (Höglund et al., 1997; Valiante et al., 1997; Lanier, 1998; Delves and Roitt, 2000a; 2000b; Trowsdale, 2001). Therefore, NK cell activation is influenced by quantitative as well as qualitative expression of MHC class I molecules on the cell surface of the target cell. The regulation of NK cell activation was explained more than 20 years ago and is summarized by Klas Kärre in his ‚missing self' hypothesis (1986; 2002).

If the NK cell is interacting with a target cell, the NK cell receptors form an immune synapse with a peripheral KIR/MHC cluster and a central leukocyte function-associated antigen-1/intercellular adhesion molecule-1 cluster in the shape of a donut (Davis et al., 1999). The binding affinity of the NK cell receptors with their ligands is quite low in order to prevent hypersensitivity.

Although immunological memory was initially viewed as characteristic for the adaptive immune system, recent studies have shown that NK cells also have some sort of memory (Lanier and Sun, 2009). It was shown in mice that NK cells, expressing a receptor that recognises a glycoprotein encoded by the cytomegalovirus, expand during the viral infection. Some of these NK cells can survive in the host for months and can show a recall and more rapid response (Sun et al., 2009). This response is more efficient than the response of a “naive” NK cell.

In humans and many other primates KIR molecules are the most important NK cell receptors, whereas in mice the C-type lectin like Ly49 receptors represent the main NK cell receptor (Natarajan et al., 2002). In humans, only a single and non-functional Ly49 gene is described (Westgaard et al., 1998). On the other hand, only two *KIR* genes are described in mice, which most likely have a different function (Bryceson et al., 2005). Therefore, these two receptor families, which are neither structurally nor phylogenetically related, have developed independently since the last common ancestor of rodent and primates more than 87 million years ago (Springer et al., 2003).

1.3 *KIR genetics in humans*

KIR genes belong to the immunoglobulin superfamily and are located in the leukocyte-receptor complex (LCR) in a head-to-tail fashion on human chromosome 19 in 19q13.4 (Wende et al., 1999; Trowsdale, 2001). KIR receptors are expressed on the cell surface of NK cells and a subset of T lymphocytes (Moretta et al., 1990; Wagtmann et al., 1995; Gardiner, 2008; Lanier, 2008).

The characteristic features of *KIR* genes are their substantial polymorphism. This can be both allelic polymorphisms and gene copy number variation as well as their varied expression (Uhrberg et al., 1997; Shilling et al., 2002a; Marsh et al., 2003). In humans, 16 *KIR* genes have been identified. However, the number of *KIR* genes present in the genome of different individuals varies extremely (Wilson et al., 2000; Hsu et al., 2002b; Uhrberg et al., 2002) due to the variable number of *KIR* genes per haplotype, which varies between nine and fifteen (Middleton and Gonzelez, 2010). Although the set of *KIR* genes determines the overall *KIR* repertoire of an individual, the NK cells also differ in their expression patterns of these genes (Valiante et al., 1997). Each cell expresses an individual repertoire of *KIR* genes, which is passed to the daughter cell by DNA methylation of the transcriptional start site of the unexpressed *KIR* genes (Lanier, 1998; Santourlidis et al., 2002; Chan et al., 2003).

The heredity of the *KIR* genes is independent of the heredity of the *MHC class I* genes which are located on chromosome 6 in 6p21.3 (Kelley et al., 2005a). This independent heredity makes co-evolution of *KIR* and *MHC class I* genes complicated. The *MHC class I* genes have to adapt to the evolution of pathogens. On the other hand *KIR* genes have to react to the evolution in the *MHC class I* genes to maintain the possibility to interact with their ligands. This adaptation has happened on the basis of gene duplication. Therefore, the number of *KIR* genes is higher than the number of *MHC class I* genes to warrant at least some interaction partners. Nevertheless, it may occur that NK cells express KIR molecules that possess no ligand in a specific individual (Gumperz et al., 1996). Furthermore, it may happen that NK cell lack inhibitory receptors specific for MHC class I molecules. Yet, due to an education each NK cell possesses some sort of tolerance towards normal cells (Gasser and Raulet, 2006; Yokoyama and Kim, 2006). Due to this education, NK cells which lack inhibitory receptors that are specific for MHC class I molecules are hyporesponsive to stimulations of target cells (Fernandez et al., 2005; Cooley et al., 2007).

1.3.1 Structure of KIR molecules

KIR genes encode type I transmembrane glycoproteins with either two or three extracellular Ig-like domains as well as stem, transmembrane and cytoplasmic regions (Kelley et al., 2005b). The nomenclature of the *KIR* genes is based on the structure of the receptors they encode. The "KIR" acronym is followed by the number of the Ig domains and a "D" for domains. The next capital letter indicates whether the KIR receptor possesses a long ("L") or a short cytoplasmic tail ("S"). The first number represents the number of the gene and is followed by a star and the allele number (Marsh et al., 2003).

KIR molecules with three extracellular Ig domains possess a D0 (membrane distal), a D1 (middle) and a D2 domain (membrane proximal) (Moretta et al., 2002). On the other hand, molecules with only two Ig domains can possess either the D0 and D2 domain (e.g. KIR2DL4 and KIR2DL5) or the D1 and D2 domain (e.g. KIR2DL1 and KIR2DL2/3) (Martin et al., 2002).

In KIR molecules, the signal peptide is coded by the first two exons. The three Ig domains are coded by exons 3, 4 and 5, respectively. The stem is coded by exon 6, the transmembrane region by exon 7, while the cytoplasmic tail is coded by exons 8 and 9 (Figure 1)

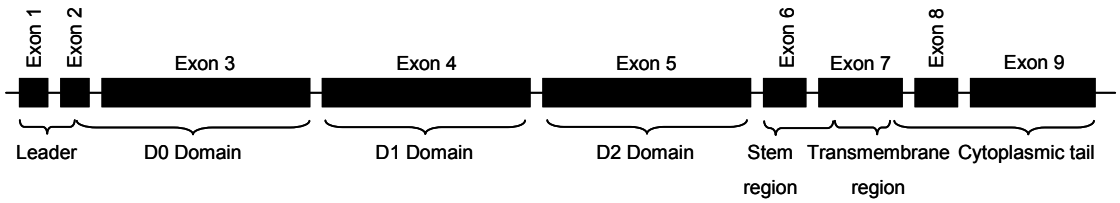

Figure 1: Schematic diagram of the exons of a *KIR* gene coding for a three Ig domain molecule with long cytoplasmic tail (Figure modified from the IPD database).

KIR molecules with long cytoplasmic tails possess two immunoreceptor tyrosine-based inhibitory motifs (ITIM) (Figure 2). Upon interaction with a ligand, these ITIMs are phosphorylated, which recruits intracellular phosphatases and leads to dephosphorylation of signal molecules that are important for the activating pathways. By this mechanism, the KIR molecules with a long cytoplasmic tail prevent activation of the NK cell (Blery et al., 2000; Vyas et al., 2002; Lanier, 2003; Stebbins et al., 2003).

Due to a premature stop codon, KIR molecules with short cytoplasmic tails do not possess any ITIMs. Instead, these KIR molecules contain a positively charged amino

acid (lysine or arginine) in the transmembrane region (Figure 2). This amino acid can interact with DNAX-activation protein-12 (DAP12), an adaptor molecule that possesses immunoreceptor tyrosine-based activating motifs (ITAM) (Lanier et al., 1998; Long, 1999; Feng et al., 2005). The ITAMs of the adapor molecule recruit phosphorylated ZAP-70 or Syk tyrosine kinase which finally leads to an activation of the NK cell (Lanier et al., 1998; Blery et al., 2000; Colucci et al., 2002).

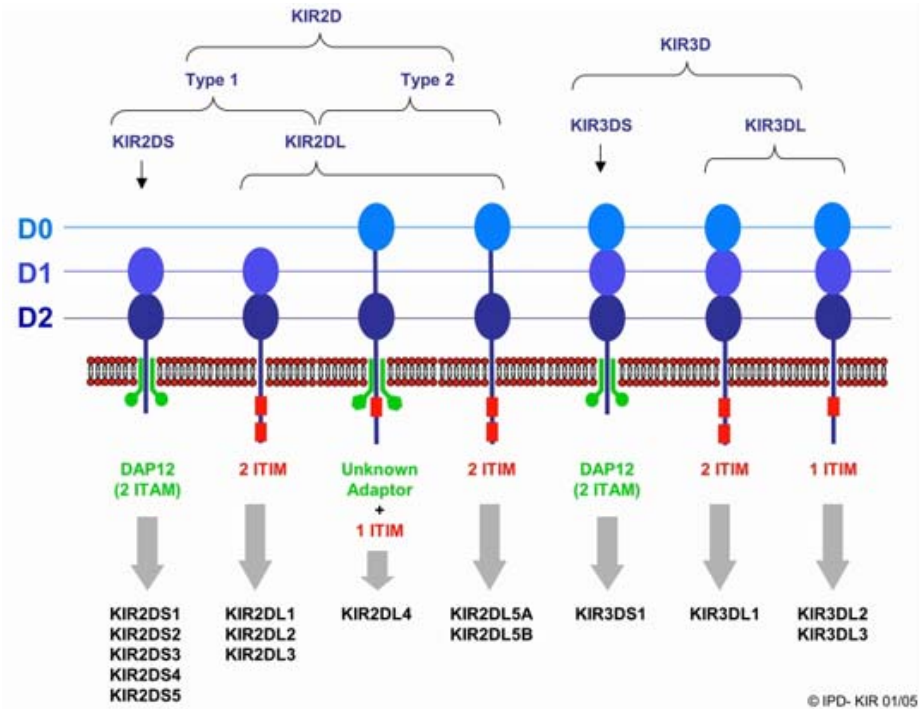

Figure 2: Schematic diagram of KIR molecules (Figure taken from the IPD database).

KIR2DL4 is an exception. KIR2DL4 contains a long cytoplasmic tail with only one ITIM as well as a charged amino acid in the transmembrane domain (Figure 2). Therefore, KIR2DL4 can act as both an inhibitory as well as an activating receptor, depending on whether the interacting FcRG adaptor protein is present.

1.3.2 Variability of human *KIR* genes

The diversity and the polymorphism of human *KIR* genes is quite high as it can be seen in differences in gene content and in large numbers of alleles, respectively (Uhrberg et al., 1997; Valiante et al., 1997; Shilling et al., 2002b; Yawata et al., 2002; Martin et al., 2004). The diversity of the *KIR* genes is also expanded by alternative splicing, whereby parts of the Ig domains (Döhring et al., 1996; Chwae et al., 1999) or even complete

domains (e.g. one Ig domain, the stem, transmembrane or cytoplasmic region) can be deleted (D'Andrea et al., 1995; Döhring et al., 1996; Selvakumar et al., 1997; Vilches et al., 2000). In humans, 16 *KIR* genes have been described and numerous *KIR* genotypes and haplotypes are known (Vilches and Parham, 2002; Gardiner, 2008; Robinson et al., 2010). Whereas the gene content and number varies between these haplotypes, *KIR3DL3* and *KIR3DL2* are present on all haplotypes at the centromeric and telomeric end of the *KIR* locus, respectively. Most haplotypes also possess the *KIR3DP1*, *KIR2DL4* and *KIR2DL2/3* in a central position. These five genes are referred to as the framework genes.

In humans the *KIR* haplotypes are divided into two groups, namely the A and B haplotypes, depending on the genes they possess (Figure 3). B haplotypes are defined by the presence of at least one of following genes: *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5* and *KIR3DS1*. On the other hand, group A haplotypes are defined by the absence of all of these genes and, except for *KIR2DS4*, possess no activating *KIR* genes. (Uhrberg et al., 1997; Hsu et al., 2002a).

Figure 3: Comparison of human *KIR* A haplotype and a representative B haplotype. Group A and B haplotypes are indicated in brackets. Gene designations are shown within the corresponding boxes (Figure modified from Middleton and Gonzelez, 2010).

The diversity of haplotypes is due to unequal crossing over during meiosis (Martin et al., 2003; Uhrberg, 2005; Norman et al., 2009). The gene content of *KIR* haplotypes can be determined by sequence-specific primers (Houtchens et al., 2007; Vilches et al., 2007; Martin et al., 2008).

Besides the high diversity of *KIR* genes, these genes also show high allelic variability. At present, 335 different alleles are known (Middleton and Gonzelez, 2010). The *KIR3DL* genes show the highest grade of variability and most of the *KIR3DL* genes (*3DL1*, *3DL2* and *3DL3*) possess over 40 different alleles.

1.4 *KIR molecules in primates*

KIR molecules have been identified not only in humans, but also in non-human primate species. This includes gorillas (Rajalingam et al., 2004), chimpanzees (Khakoo et al., 2000), bonobos (Rajalingam et al., 2001), orangutans (Guethlein et al., 2002), rhesus macaques (Grendell et al., 2001; Hershberger et al., 2001; Sambrook et al., 2005; Blokhuis et al., 2009a), cynomolgus macaques (Bimber et al., 2008) and African green monkeys (Hershberger et al., 2005). In these species, only few *KIR* genes are conserved (Sambrook et al., 2005). Only *KIR2DL4* and *KIR3DX1* represent orthologous genes which are conserved in almost all higher primates analysed so far (Sambrook et al., 2005; Sambrook et al., 2006). *KIR3DX1*, however, represents an ancestral *KIR* gene of unknown function in primates (Sambrook et al., 2006).

It was shown that the *KIR* genes are very diverse among primates (Khakoo et al., 2000; Hershberger et al., 2001; Guethlein et al., 2002). One reason for this diversity in primates is repeated duplication of *KIR* genes (Martin et al., 2004), leading to haplotypic diversity and recombinations between different genes (Toneva et al., 2001; Rajalingam et al., 2004). Interestingly, the duplication rate of *Ly49* genes in rodents, which have a function analogue to *KIR* genes, is comparable to the duplication rate of *KIR* genes in primates (Hao and Nei, 2005). Therefore, it is likely that the rapid evolution of the *KIR* and *Ly49* genes is connected with the rapid evolution of their ligands. Since the KIR molecules need to interact with their ligands, the MHC class I molecules, they have to be able to adapt to rapid evolutionary changes in these ligands (Parham, 1997). Hence, *KIR* genes have to keep pace with the known rapid evolution of *MHC class I* genes (Parham, 2005b).

1.4.1 KIR molecules in rhesus macaques

Rhesus macaques are important non-human primate models of human diseases (Bontrop and Watkins, 2005). Several groups have already analysed the diversity of rhesus macaque *KIR* genes (Grendell et al., 2001; Hershberger et al., 2005; Sambrook et al., 2005; Blokhuis et al., 2009a; Blokhuis et al., 2009b), but most of these studies have analysed cDNA of *KIR* genes, whereas only one haplotype has been sequenced completely (Sambrook et al., 2005). With only five *KIR* genes, this haplotype is smaller than the smallest *KIR* haplotype in humans (LaBonte et al., 2001; Shilling et al., 2002a; Sambrook et al., 2005). Furthermore, it possesses no activating *KIR* genes. Nevertheless,

other studies have shown that every analysed animal possesses at least one activating *KIR* gene (Blokhuis et al., 2009a).

Following phylogenetic analyses, the functional *KIR* genes have been grouped in different lineages. *KIR2D* genes with a D0 D2 configuration (*KIR2DL4* and *KIR2DL5*) belong to lineage I, whereas *KIR2D* genes with a D1 and D2 configuration belong to lineage III. *KIR3D* genes belong to the KIR lineage II, with the exception of *KIR3DL3* which represents a separate lineage (lineage V) (Rajalingam et al., 2004; Guethlein et al., 2007). In humans and great apes, an expansive duplication of lineage III *KIR* genes has occurred (Guethlein et al., 2007). Yet, in rhesus macaques the KIR lineage II has undergone several duplications while lineage III genes are completely missing (Hershberger et al., 2001; Sambrook et al., 2005; Blokhuis et al., 2009a). Only one *KIR2D* gene is known (*KIR2DL4*). This is in contrast to humans where most of the genes code for KIR2D receptors and only four *KIR3D* genes are known.

The activating KIR molecules in rhesus macaque also display differences when compared to humans. There are two different types of activating KIR molecules known in the rhesus macaque, formerly known as KIR3DH (Hershberger et al., 2001) and KIR3DM (Blokhuis et al., 2009a) In the meantime, both of them are referred to as KIR3DS according to the official KIR gene nomenclature committee. It is likely that both genes evolved independently from each other (Blokhuis et al., 2009a). Nevertheless, both molecules act as activating receptors due to a charged amino acid in the transmembrane domain (Hershberger et al., 2001; Blokhuis et al., 2009a). Activating receptors in humans possess a lysine whereas activating KIRs in rhesus macaques possess an arginine in the transmembrane region.

1.5 *MHC molecules*

Molecules encoded in the *major histocompatibility complex* (*MHC*) (in humans also known as *HLA*) are an essential part of the immune system. The *MHC* region is located on chromosome 6 in 6p21.3 (Morton et al., 1984) and is divided into three regions. Molecules of the class I region are expressed on all nucleated cells and present intracellular antigens. These antigens can be derived either from degraded cellular proteins or from endogenous pathogens (Dunlop and Blanden, 1977; Rammensee et al., 1995). In humans, the MHC class I molecules are designated as HLA-A, -B, -C, -E, -F, and -G. MHC class II molecules are only expressed on the cell surface of professional

antigen-presenting cells and present antigens of extracellular origin (self-proteins or proteins derived from pathogens). The class III region encodes molecules with different functions such as inflammatory cytokines, heat shock proteins and tumour necrosis factor α and β (Kelley et al., 2005a).

1.5.1 MHC class I molecules

MHC class I molecules consist of a heavy chain composed of three extracellular domains (α1, α2, α3), a transmembrane and a cytoplasmic region (Natarajan et al., 1999) and the β2 microglobulin, which is non-covalently bound to the heavy chain. The α1 and α2 domains form the peptide-binding groove, which harbours most of the polymorphism seen in the various alleles.
The MHC class I molecules can be grouped into classical and non-classical MHC class I molecules. The classical molecules are the highly polymorphic HLA-A, -B, and -C. The non-classical MHC class I molecules (HLA-E, -F, and -G) are less polymorphic and perform specialised functions.

1.6 *MHC molecules in the rhesus macaque*

The *MHC* of the rhesus macaque is designated *Mamu* and is located on chromosome 6 in 6p24 (Hüber et al., 2003). Human and rhesus macaque *MHC*s show differences in their size. While the human *MHC* spans 3.7 Mb (The MHC Sequencing Consortium, 1999), the rhesus macaque *MHC* encompasses 5.3 Mb (Daza-Vamenta et al., 2004). This difference in size is mainly due to considerable expansion of *MHC class I* genes of the *Mamu-A* and *-B* type (Figure 4) (Daza-Vamenta et al., 2004; Otting et al., 2007). Rhesus macaques can possess between two and four *Mamu-A* genes which display different levels of polymorphism (Otting et al., 2007). Nineteen *Mamu-B* genes have been identified on a completely sequenced haplotype (Daza-Vamenta et al., 2004). The *Mamu-B* region is characterised by substantial copy number variation (Otting et al., 2005; Otting et al., 2008). The so-called *Mamu-I* genes were previously named *Mamu-B*09* and are characterised by low variability and low cell surface expression (Urvater et al., 2000).

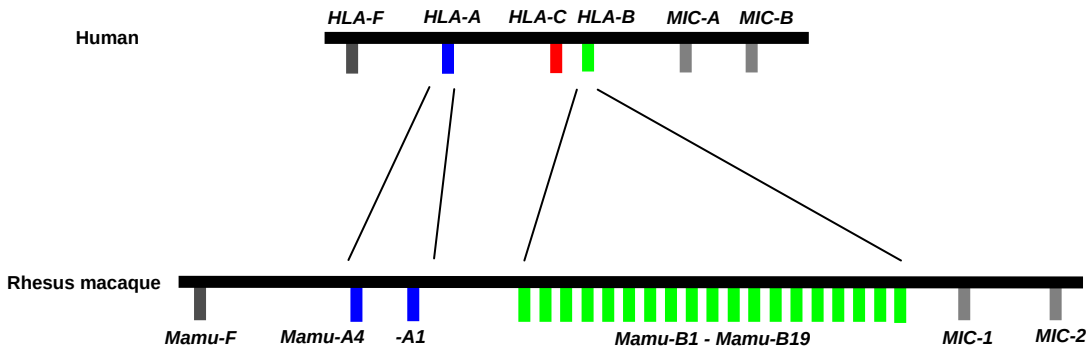

Figure 4: Schematic comparison of the genomic structure of the classical *MHC* genes of humans and rhesus macaques. The *MHC-A*, *-B* and *-C* genes are highlighted in blue, green and red, respectively. The *MHC-F* and *MIC* genes are highlighted in grey. The telomeric end is located on the left and the centromeric on the right side (Figure modified from Daza-Vamenta et al., 2004).

In contrast to humans, the rhesus macaque possesses no *MHC-C* gene (Boyson et al., 1996; Parham, 2005b). The *MHC-C* gene is fixed in gorilla, chimpanzee and human, but not in orangutans (Adams et al., 1999). The *MHC-C* gene likely developed from a duplication of the *MHC-B* gene (Mizuki et al., 1997). Analyses could show that the *MHC class I* genes vary considerably between the different primate species (Adams and Parham, 2001), as in the case of the *KIR* genes (Khakoo et al., 2000; Hershberger et al., 2001; Guethlein et al., 2002; Rajalingam et al., 2004).

1.7 *Interaction between KIR and MHC class I molecules*

MHC class I molecules are the only known ligands for KIR molecules in humans. Most of the interaction partners of human inhibitory KIR molecules are known. KIR2DL4 interacts with HLA-G (Rajagopalan and Long, 1999; Rajagopalan et al., 2006). KIR3DL1 binds ligands with the Bw4 epitope (Litwin et al., 1994; Dohring et al., 1996; Hansasuta et al., 2004). KIR2DL1 and KIR2DL2/3 interact with HLA-C2 and HLA-C1 types, respectively (Colonna et al., 1993; Boyington et al., 2001; Yawata et al., 2006; Moesta et al., 2008). It has also been shown that some interactions between KIR and MHC class I molecules are influenced by the peptide bound by the MHC molecules (Hansasuta et al., 2004; Stewart et al., 2005; Thananchai et al., 2007). For example, binding of KIR3DL2 is not only influenced by its ligands (HLA-A3 and -A11), but also by the bound peptide of the HLA molecules (Hansasuta et al., 2004).

The interaction partners of activating KIR molecules are not well characterised compared to the interaction partners of their inhibitory counterparts. This might be due to the fact that activating KIR molecules show a lower binding affinity to MHC

molecules and also display a peptide dependency (Stewart et al., 2005). For instance, KIR2DS1 shows weak binding with HLA-C2 molecules despite its structural similarities to KIR2DL1 (Biassoni et al., 1997; Vales-Gomez et al., 1998). On the other hand, no binding between KIR2DS2 has been detected, even though this molecule possesses structural similarities with KIR2DL2/3 which are interacting with HLA-C1 molecules. An interaction was also found between KIR2DS4 with HLA-A molecules as well as HLA-C (Graef et al., 2009). Interestingly, binding specificity is neither limited to HLA-C1 nor to -C2 molecules. Therefore, it is likely that the binding specificity of KIR2DS4 is peptide-dependent (Graef et al., 2009).

The D1 and D2 domains of KIR molecules are responsible for the interaction with MHC class I molecules (Boyington et al., 2001). Interestingly, the KIR molecules interact with parts of the MHC class I protein that are also involved in binding of T cell receptors. This includes the α1 and α2 domains and the Bw4/Bw6 epitopes located in the α1 domain at amino acid position 77-83 (Müller et al., 1989).

Rhesus macaques show substantial expansions of *KIR3D* genes (Hershberger et al., 2001; Blokhuis et al., 2009a). This is believed to be due to co-evolution with *Mamu-A* and *-B* genes, which are also expanded in rhesus macaques (Otting et al., 2007; Otting et al., 2008). On the other hand, rhesus macaques lack *KIR2D* genes, with the exception of *KIR2DL4*. This can be attributed to the fact that KIR2D molecules interact with MHC-C molecules, which are absent in rhesus macaques. Thus, the prevalent types of MHC class I ligands obviously affect which type of KIR molecules are dominant within a particular species.

1.7.1 Associations of diseases with KIR/MHC combination

It has been shown in humans that certain combinations of highly variable KIR and MHC molecules are significantly associated with susceptility/resistance to various infectious and autoimmune diseases as well as with graft rejection and even affect reproductive success. (Khakoo et al., 2004; Nelson et al., 2004; Martin et al., 2007; Hiby et al., 2008)

For example, patients who possess a combination of *HLA-C1* and *KIR2DL3* are more likely to survive hepatitis virus C infection (Khakoo et al., 2004). Combinations of *KIR* and *MHC* genes also have an effect on the progression of acquired immunodeficiency syndrome (AIDS). Humans possessing *KIR2DL2*, *2DS2* or *2DS3* exhibit the first

symptoms of AIDS significantly earlier than other patients, whereas the combined expression of *KIR3DL1* and its specific ligand leads to a significantly later outbreak of AIDS symptoms (Gaudieri et al., 2005; Martin et al., 2007). In addition to the abovementioned effects, specific *HLA-B* allotypes are known to influence susceptility to AIDS. *HLA-B*27* and *-B*57* are associated with longer survival (Migueles et al., 2000; Gao et al., 2001), whereas *HLA-B*35* is linked to faster progression to AIDS (Scorza Smeraldi et al., 1988; Carrington et al., 1999; Jin et al., 2002).

In addition to virus infections, autoimmune diseases are also influenced by possession of specific KIR and MHC molecules. 83% of rheumatoid vasculitis patients possess *KIR2DS1*, whereas only 47 % of the control population possess this gene (Yen et al., 2001). Also, the occurrence of psoriasis vulgaris is associated with *KIR2DS1* and *HLA-Cw*06* (Luszczek et al., 2004).

There are reports that link leukemia with the presence of *KIR* genes. In one study, *KIR2DS2* and *KIR2DL2* appeared more frequently in leukemia patients compared to the control group (Verheyden et al., 2004). Still, both genes are in linkage disequilibrium (Hsu et al., 2002b; Uhrberg et al., 2002). Therefore, it is not clear which gene (if any) has an influence on the etiology of leukemia.

Combinations of *KIR* and *HLA* also influence the outcome of hematopoietic stem cell transplantation in patients suffering from different forms of leukemia. Certain donor *KIR* and recipient *HLA* genotypes are associated with less relapse, but lead to increased frequency of graft-versus-host-diseases (Miller et al., 2007).

Specific combinations of *KIR* and *HLA* genes can also cause complication during pregnancy termed pre-eclampsia. During pregnancy, NK cells normally activate trophoblast cells, which enable proper blood supply to the foetus. In mothers homozygous for group A *KIR* haplotypes that carry foetuses homozygous for HLA-C2 ligands, which show strong interaction with KIR2DL1 molecules, occurrence of pre-eclampsia is significantly increased (Hiby et al., 2004).

1.8 *Aim of the study*

Rhesus macaques are a commonly used non-human primate model for several diseases like AIDS and are often used for the development of vaccines (Del Giudice et al., 2001; Lukashevich et al., 2003; Bontrop and Watkins, 2005). Therefore, it is important to extrapolate results obtained with this animal model to humans. In humans, it has been

shown that distinct *KIR* genes or haplotypes have an influence on the outcome of different diseases (Parham, 2005b). Up to now, however, the knowledge of the diversity of *KIR* genes of the rhesus macaque is still limited compared to humans. Therefore, no association between *KIR* genes and the progress of different diseases is known in he rhesus macaque. Also, specific interaction between KIR and MHC class I molecules in the rhesus macaque are unknown.

Therefore, this study is divided into two parts: 1) determination of the *KIR* diversity and establishment of a *KIR* genotyping method and 2) characterisation of specific interactions between KIR and MHC class I molecules.

2 Materials and methods

2.1 Materials

2.1.1 Animals and blood samples

Rhesus macaques (*Macaca mulatta*) are housed in the facilities of the German Primate Centre. The EDTA treated whole blood of the rhesus macaques was obtained during regular health checks and was made available by the Cost Centre of the German Primate Centre.

The following animals are used for the establishment of the cDNA library: 1748, 1818, 1577, 1874, 1989, 2142, 2148, 2154, 2156, 2185, 2196, 2206, 2229, 2237, 2239, 2240, 2251, 2255, 2257, 2274, 2282, 2285, 2288, 2289, 2306, 2317, 2324, 2326, 2328 and 2340.

The following animals are used for the *KIR* genotyping: 1577, 1594, 1635, 1683, 1748, 1818, 1840, 1858, 1908, 1926, 1927, 1930, 1999, 2019, 2020, 2022, 2051, 2056, 2078, 2084, 2089, 2115, 2122, 2123, 2127, 2129, 2131, 2137, 2138, 2142, 2148, 2183, 2185, 2200, 2204, 2217, 2225, 2227, 2229, 2232, 2242, 2243, 2258, 2275, 2277, 2278, 2289, 2291, 2299, 2304, 2305, 2309, 2320, 2321, 2322, 2323, 2332, 2342, 2350, 2353, 2354, 2360, 2371, 2380, 2387, 2396, 2401, 4989, 4992 and 9309.

2.1.2 Equipment

Equipment	Version	Manufacturer
Autoclave	Varioklav® 400E	H+P Labortechnik
Autoradiography cassette	Cronex-Kassette	DuPont de Nemours
Blotter, semi-dry	V20-SDB	Roth
Centrifuges	Centrifuge 5414 R	Eppendorf
	Centrifuge 5415 D	Eppendorf
	Centrifuge 5810 R	Eppendorf
	Multifuge 1 S-W	Heraeus
Centrifuge rotors	A-4-62	Eppendorf
	F 45-24-11	Eppendorf

Equipment	Version	Manufacturer
	75002000	Heraeus
Cryo-container	Mister Frosty	Nalgene
Developer	M35 X-Omat Processor	Kodak
Electrophoresis chamber	Horizon 58	Gibco BRL
Electroporation device	Gene Pulser II	Bio Rad
FACS	BD LSR II	BD Bioscience
	FACS-Scen	Becton Dickinson
Geiger counter	LB122	Berthold Technologies
Gel imager	Gel Jet Imager 2000	Intas
Hamilton syringes	Mikroliter Syringes	Hamilton
Hood, sterile	KR-125 Safety	Bio-Flow
Hybridisation flask	GL 45	Ochs
Hybridisation oven	430	Bachofer
Incubators	B12	Heraeus
	B6060	Heraeus
Liquid-Scintillation Counter	1414	Wallac
Magnet stirrer	M32	GLW
Microscopes	Axiovert 405M	Zeiss
	CK40	Olympus
pH meter	pH 535 Multi Cal®	WTW
Photometer	Gene Quant II	Pharmacia Biotech
Pipettes	Finnipipette® 300 µl	Heinemann
	Reference 10 µl	Eppendorf
	Research 100 µl	Eppendorf
	Research 1000 µl	Eppendorf
Power supply	Etron-S	Etron
	Power Pack P25	Biometra
Rotary shakers	3033	GFL
	Incubator 1000	Heidolph
	Unimax 1010	Heidolph
Scale	BP 310 s	Sartorius
SDS-PAGE chamber	EPH-1010-V	Bridge
Sequencer	ABI3130XL	Applied Biosystem

Equipment	Version	Manufacturer
Shaker	Thermomixer comfort	Eppendorf
Speed vac	Speedvac	H. Saur
Thermocycler	GeneAmp PCR System 2700	Applied Biosystem
	Labcycler	Sensoquest
	PTC-200	Bio-Rad
Thermomixer	Thermomixer comfort	Eppendorf
UV-Crosslinker	UV-Stratalinker 2400	Stratagene
Vortexer	L46	GLW
Waterbath	WB7	Memmert

2.1.3 Consumables

Consumables	Manufacturer
1,4-Dithiothreitol	Sigma
2-Propanol	Roth
6-well plates for adherent cells	Sarstedt
Acrylamid 40 % / Bisacrylamid	Biorad
Agar-Agar	Roth
Agarose	Cambrex (Biozym)
Amicon Ultra 30 columns	Millipore
Ammoniumchlorid	Merck
Ammoniumperoxodisulfat	Sigma
Ammoniumpersulfat	Sigma
Ampicillin	Sigma
Autoradiography emulsion NTB2	Kodak
BD Advantage™ 2 PCR Enzyme System	BD Biosciences
BigDye® Terminator v1.1 Cycle Sequencing Kit	Applied Biosystem
Biotherm buffer (10 x)	GeneCraft
Biotherm Tag DNA Polymerase	GeneCraft
Biotinylated protein A	Pierce
Boric acid	Roth
Bovine serum albumin	Sigma
Bromphenolblue	Sigma

Consumables	Manufacturer
CD 16 Microbeads non-human Primate	Miltenyi Biotec
Chloramphenicol	Sigma
CHO-Ultra medium	Lonza
Creator™ SMART™ cDNA Library Construction Kit	BD Biosciences
Cryo tube	Sarsted
Culture flask for adherent cells (10 cm)	Sarstedt
Diethylpyrocarbonat	Roth
Dimethylsulfoxid (10 %)	Gibco
Dinatriumhydrogenphosphat	Roth
DNA Ladder 100bp Plus	Fermentas
DNA polymerase	Promega
DNase	Promega
dNTP	Invitrogen
Dulbecco's modified Eagle medium (High Glucose)	PAA
EcoRI	New England Biolabs
Electroporation cuvette	Biozym
Eppendorf tube, 0,5 ml	Sarstedt
Eppendorf tube, 1,5 ml	Sarstedt
Eppendorf tube, 2 ml	Eppendorf
Ethanol, impure	Roth
Ethanol, pure	Roth
Ethidiumbromid	Roth
Ethylendiamintetraacetat	Sigma
FACS flasks	BD Falcon
Falcontube, 15 ml	Greiner
Falcontube, 50 ml	Greiner
Fetal calf serum	PAA
Ficoll 400	Sigma
Filter Storage Bottle 0.45 membrane, 500 ml	Corning
FITC-coupled mouse IgG1 Isotype Ab	Becton Dickinson
Fixer Polymax	Kodak
Formaldehyde	Roth
Formamide	Roth

Consumables	Manufacturer
GelBond® Film	B W Molecular Applications
GeneRuler 100bp DNA Ladder Plus	Fermentas
Genescan HD ROX size standard	Applied Biosystem
Glucose	Sigma
Glycerin	J. T. Baker
Glycine	Sigma
Goat anti mouse IgG antibody	SantaCruz
Hemocytometer (0,1 mm)	Optik-Labor
Hepes	Roth
Hi-Di™ Formamid	Applied Biosystems
Human IgG	Sigma
Hydrogenperoxide (30 %)	Roth
Hyperfilm™ ECL	Amersham
Isopropyl-β-D-thiogalaktopyranoside	Roth
Liquid nitrogen	Vesper
Luminol	Fluka
Magnesiumchloride	Merck
Magnesiumsulphate	Merck
Map Trap Kit	GE Healthcare
Metafectene™	Biontex
Methanol	Merck
Milk powder	Frema
MiniMACS Starting Kit	Miltenyi Biotec
Morpholinopropionic acid	Roth
N, N-Dimethylformamide	Sigma
Natriumacetate	Roth
Natriumchloride	Roth
Natriumcitrate	Roth
Natriumdodecylsulphate	Roth
Natriumhydroxide	Roth
NotI	New England Biolabs
PageRuler Prestained Protein Ladder	Fermentas
PBS – Dulbecco (10 x)	Biochrom AG

Consumables	Manufacturer
p-Cumaric acid	Sigma
PeCy5-labeled streptavidin	BD
Penicillin/Streptomycin (100 x)	PAA
Petri dishes	Greiner
pFUSE-hIgG1-Fc2	Invitrogen
pGEM-T Easy Cloning Kit	Promega
Photoemulsion NTB2	Kodak
Polyvinylpyrrolidone	Sigma
Potassium acetate	Roth
Potassium chloride	Merck
Potassium dihydrogen phosphate	Merck
Prime-a-Gene Labeling System	Promega
Pronase	Sigma
pUC19 DNA	Fermentas
QIAquick Gel extraction Kit	Qiagen
QIAquick Plasmid Midi Kit	Qiagen
QuickChange II Site-Directed Mutagenesis Kit	Stratagen
Rabit anti human IgG HRP antibody	Dako
RNase A	Sigma
RNeasy Plus Mini Kit	Qiagen
RNeasy Plus Mini Kit	Qiagen
Roswell Park Memorial Institute Medium 25 mM Hepes	PAA
Saccharose	Merck
Salmon sperm	Roche
Shrimp alcaline phosphatase	USB
Shrimp alcaline phosphatase buffer	USB
Streptavidin-microbeads	Miltenyi Biotec
Streptomycine	Sigma
Suprasil® Precision quartz cuvettes	Hellma
TC-coupled mouse IgG1 anti-human CD16 antibody	Becton Dickinson
Triple Flasks	Nunc
Tris	Roth
Triton-X	Sigma

Consumables	Manufacturer
Trypanblue	PAA
Trypton / Pepton	Roth
Tween 20	Sigma
W6/32 antibody	ATCC
Water (HPLC-Quality)	Merck
Wax	Adams Istruments
Whatman paper	Schleicher & Schuell
X-Gal	Roth
Xylencyanole	Sigma
Yeast extract	Roth
Zeocin	Invivogen
α-^{32}P-dCTP	Amersham

2.1.4 Vectors and size standards

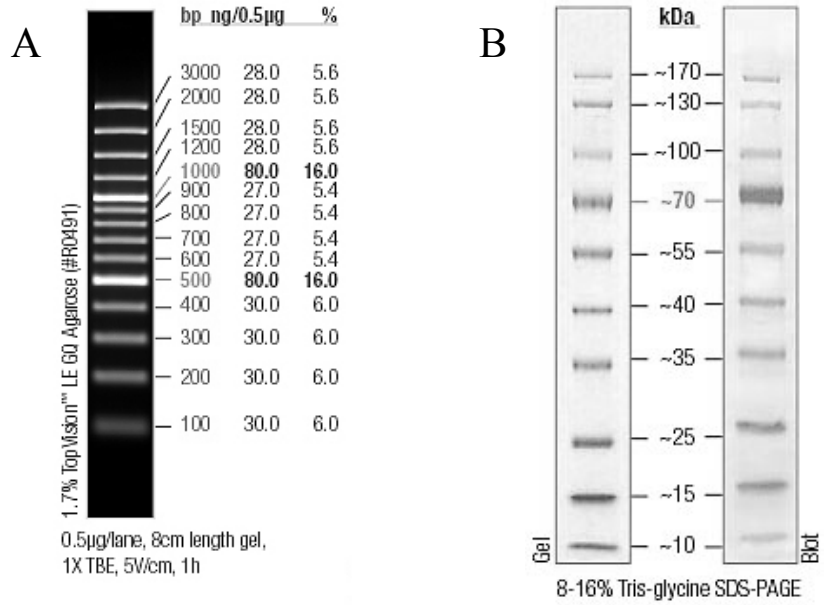

Figure 5: A) GeneRuler 100bp DNA Ladder Plus, B) PageRuler Prestained Protein Ladder (Figure taken from Promega catalogue).

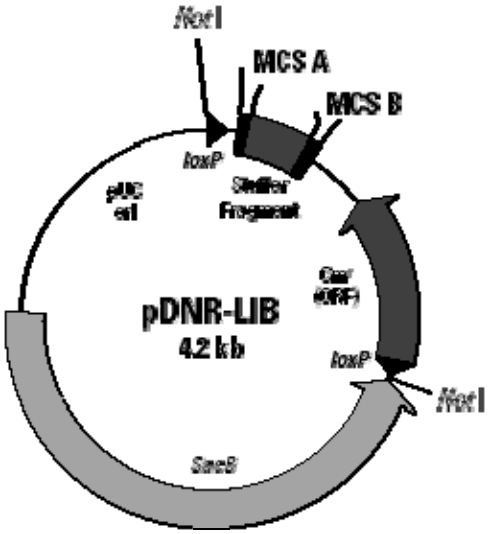

Figure 6: pDNR-LIB vector (Figure taken from Clontech catalogue).

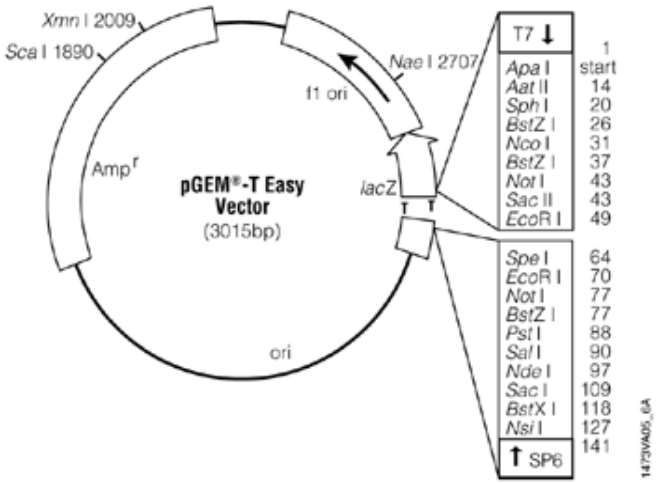

Figure 7: pGEM-T Easy vector (Figure taken from Promega catalogue).

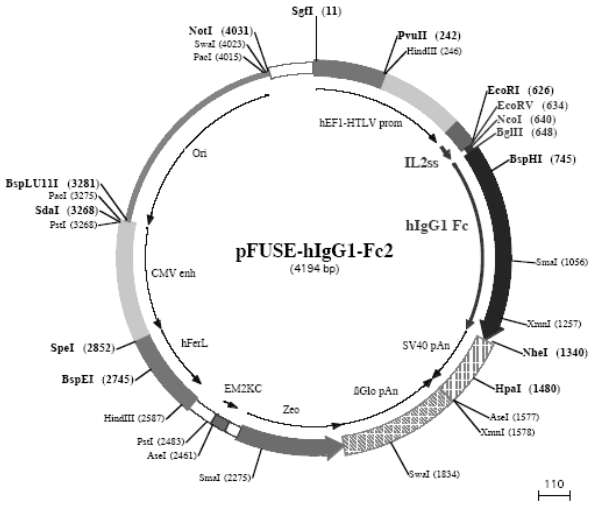

Figure 8: pFUSE-hIgG1-Fc2 vector (Figure taken from Invivogen catalogue).

2.1.5 Bacterial strains

Escherichia coli TOP10 (Invitrogen)
F⁻ mcrAΔ (mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara, leu)7697 galK rpsL (Str^R) end A1 nupG

Escherichia coli ElectroMAX™ DH10B (Invitrogen)
F⁻ mcrAΔ (mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galKλ⁻ rpsL nupG

2.1.6 Cell lines

HEK-293 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). They were generated by the transformation of embryonic kidney cells of a human foetus with the sheared adnovirus 5 DNA.
K562 cells were obtained from American Type Culture Collection (ATCC). They are derived from a female CML patient and are an immortalised myelogenous leukemia cell line. A cell surface suppression of classical MHC class I molecules in K562 cells is not detectable.

2.1.7 Buffers and solutions

Buffer	Substance of content
Blocking solution	1 x TBS 5 % milk powder 0,05 % Tween
BSA/Tritron-X buffer (10x)	500 mg BSA 250 mg Triton-X dissolve in 50 ml H_2O

Buffer	Substance of content
Denhardt´s solution (50 x)	5 g Ficoll 400 5 g Polyvinylpyrrolidon 5 g BSA dissolve in 500 ml H_2O
DEPC-H_2O	50 µl DEPC solution (97 %) dissolve in 50 ml H_2O autoclave
Developing solution 1	100 µl 250 mM Luminol 44 µl 90 mM p-CA 1 ml 1 M Tris 8,8 ml H_2O
Developing solution 2	6 µl 30 % H_2O_2 1 ml 1 M Tris 9 ml H_2O
Electrophoresis buffer (5 x)	15,1 g Tris 94 g Glycine 50 ml 10 % SDS dissolve in 1000 ml H_2O
Erythrocyte lysis buffer	155 mm NH_4Cl 10 mm $KHCO_3$ 0,1 mm EDTA (PH 8,0) dissolve in 1000 ml H_2O adjust pH 8,0
Hybridisation buffer	150 ml 20 x SSC 50 ml 50 x Denhardt´s solution 30 ml 10 % SDS dissolve in 500 ml H_2O

Buffer	Substance of content
Laemmli buffer (2 x)	1,51 g Tris 4 g SDS 5 g Saccharose dissolve in 80 ml H_2O adjust pH 6,8 30 mg Bromphenolblue add H_2O to 100 ml filtering
Loading buffer (DNA)	6,6 g Sucrose 0,04 g Bromphenolblue dissolve in 10 ml H_2O
Loading buffer (RNA)	0,4 % Bromphenolblue 0,4 % Xylencyanol 50 % Glycerin 1 mm EDTA (PH 8,0)
Lysis puffer	155 mM NH_4CL 10 mM $KHCO_3$ 0.1 mM EDTA pH 8.0
MACS buffer	1 x PBS 0,5 % BSA 2 mM EDTA (PH 8,0) degas before use
Mini prep solution I	50 mm Glucose 25 mm Tris 10 mm EDTA (PH 8,0)

Buffer	Substance of content
Mini prep solution II	400 ml 10 M NaOH 100 ml 20 % SDS dissolve in 1000 ml H_2O
Mini prep solution III	3 M KAc (pH 5,2)
MOPS buffer (5 x)	20,6 g MOPS 6,804 g NaAc dissolve in 800 ml H_2O adjust pH 7,0 10 ml EDTA (pH 8,0) add H_2O to 1000 ml
PBS (10 x)	80 g NaCl 2 g KCl 15,4 g Na_2HPO_4 x 12 H_2O 2 g KH_2PO_4 dissolve in 1000 ml H_2O
Pre washing solution	5 x SSC 0,5 % SDS 1 mm EDTA (pH 8,0)
RNA gel	1,5 g Agarose 22,5 ml 5 x MOPS 78,5 ml DEPC-H_2O heat to 60 °C and cool down 10 ml Formaldehyde 4 µl EtBr

Buffer	Substance of content
Sample buffer (RNA)	20 µl Formamid 7 µl Formaldehyde 4 µl 5 x MOPS buffer 3 µl loading buffer (RNA)
SDS separation gel (10 %)	3,33 ml 30% PAA 2,5 ml 4x Tris / SDS (pH 8,8) 4,16 ml H_2O 50 µl 10% APS 15 µl TEMED
SDS stacking gel (4 %)	0,54 ml 30 % PAA 1 ml 4x Tris / SDS (pH 6,8) 2,46 ml H_2O 50 µl 10% APS 20 µl TEMED
SE buffer	75 mm NH_4Cl 5 mm EDTA (PH 8,0)
Semi-Dry transfer buffer	3 g Tris 11,3 g Glycine 100 ml Methanol dissolve in 1000 ml H_2O
SSC (20 x)	175,3 g NaCl 88,2 g Na-Citrate dissolve in 100 ml H_2O
TBE Agarose gel (1 %)	1 g Agarose add 1x TBE-Puffer to 100 ml heat to 60 °C and cool down 5 µl EtBr

Buffer	Substance of content
TBE buffer (10 x)	1 M Tris 0,8 M boric acid 10 mm EDTA (PH 8,0)
TBS (1 x)	8,5 g NaCl dissolve in 900 ml H_2O 20 ml 1 M Tris HCl (pH 7,2) adjust pH 7,2 add H_2O to 1000 ml
TE buffer	10 mm Tris 1 mm EDTA (PH 8,0) adjust pH 7,4
TEN buffer	50 mm Tris (pH 8) 100 mm EDTA (pH 8) 50 mm NaCl
Tris / SDS (pH6,8) (4 x)	6,05 g Tris 4 g SDS adjust pH 6,8 dissolve in 1000 ml H_2O adjust pH 6,8 after two days again
Tris / SDS (pH8,8) (4 x)	45,5 g Tris 1 g SDS adjust pH 8,8 dissolve in 250 ml H_2O adjust pH 8,8 after two days again
Tris hybridisation buffer	6 X SSPE 0,1 % Tween-20 50 mM Tris HCl

Buffer	Substance of content
Trypanblue solution	1 x PBS 0,5 % Trypanblau
Washing buffer (Hybridisation)	2 x SSC 0,1 % SDS
Washing buffer (Western-Blot)	1 x TBS 0,05 % Tween

2.1.8 Media

All media except DMEM were autoclaved before use. All sterile filtered antibiotics were added after the media were cooled down to 50 °C.

Medium	Substance of content
DMEM medium	450 ml 1 x DMEM (High Glucose) 50 ml FCS Gold (45 min deactivated at 65°C) 5 ml Penicillin/Streptomycin (100 x)
LB medium	1 % Tryptone 0,5 % yeast extract 1 % NaCl
LB-Amp Agar plats with IPTG and X-Gal	1000 ml LB medium 15 g Agar-Agar 1 ml Amp (50 mg/ml) 2 ml X-Gal (2 % in Dimethylformamid) 1 ml IPTG (24 mg/ml)
LB-Amp medium	1000 ml LB medium 1 ml Amp (50 mg/ml)

Medium	Substance of content
LB-Cm agar plates	1000 ml LB medium 15 g Agar-Agar 30 g Chloramphenicol
LB-Cm medium	1000 ml LB medium 30 g Chloramphenicol
LB-Zeo medium	1000 ml LB medium 25 mg Zeocin
LB-Zeo agar plates	1000 ml LB medium 15 g Agar-Agar 25 mg Zeocin
SOC medium	2 % Trypton 0,5 % yeast extract 10 mm NaCl 2,5 mm KCl 10 mm $MgCl_2$ 10 mm $MgSO_4$ 20 mm Glucose

2.1.9 Oligonucleotides

All oligonucleotides were ordered at Metabion.

Table 1: Used oligonucleotides

Name	Sequence (5'-3')	Temp.
M13F	GTAAAACGACGGCCAGT	52 °C
M13R	AACAGCTATGACCATG	52 °C
G23	GTTTTCCCAGTCACGAC	52 °C
G24	GGATAACAATTTCACACAGG	52 °C
MYCF1	ACACCATGGGAGCTGGTAAT	55 °C
MYCR1	CTTCWTCGACTTYCAGACCCAAGGCAT	55 °C

Name	Sequence (5'-3')	Temp.
MYCF2	GTTCTTTGAAAACTGAAT	55 °C
MYCR2	GCATCCACCAWAWACTCT	55 °C
Microsatellite I fw	CTTAATTCCTGAAGTCTCACTTGTAAA	59 °C
Microsatellite I rev	CATTTCTAGGTGAACCCATCC	59 °C
Microsatellite II fw	CTCCTGCTGGAATTCACTCG	59 °C
Microsatellite II rev	TTTCTGTGTGAGGGCTTGAG	59 °C
Microsatellite III fw	TTCTTGGTCCGAAAAAGGTG	59 °C
Microsatellite III rev	TGTACTTCTCCATGGGTGTGG	59 °C
Microsatellite IV fw	GTTGTGACTGGCGTCTTGGT	59 °C
Microsatellite IV rev	AGGGTCTCCCCTTCCCTACT	59 °C
Microsatellite V fw	AAAAGTGTGGCTGGGTGAAG	59 °C
Microsatellite V rev	GTTGACTTGGGGCTCTCTTG	59 °C
Microsatellite VI fw	GGCACGAAAACAGGGAAAG	59 °C
Microsatellite VI rev	TGATCTTCCTCTTTCTTTAATTCTGA	59 °C
Microsatellite VII fw	AGAGGCCCAAGGACAGAGA	59 °C
Microsatellite VII rev	TATCAATTGTCTATCTATCCGTCAA	59 °C
Microsatellite VIII fw	CACATTCAGGGGAACCTCAG	59 °C
Microsatellite VIII rev	TCTGCAAGCTTCATGTAGGG	59 °C
Microsatellite IX fw	GAGGCAGAGAAAGACAAGGAGA	59 °C
Microsatellite IX rev	TCTGTTTTGTTCATAACCTTCTGC	59 °C
β-Actin fw	ACGGGGTCACCCACACTGTGC	---
β-Actin rev	CTAGAAGCATTGCGGTGGACGATG	---
KIR pFUSE2 fw	GGTGAATTCGCACACGGGTGGTCAGGAC	58 °C
KIR pFUSE2 rev	GGTGAATTCGAATGCAGGTGTCTGGGGATAC	60 °C
KIR3DL05 K144E fw	GTGAGGTGACCTTTGAGAAGTCCGTGCACC	---
KIR3DL05 K144E rev	GGTGCACGGACTTCTCAAAGGTCACCTCAC	---
KIR3DL05 K204E fw	GATCACAGGTATATATGAGAAACCTTCTCTCTCAGC	---
KIR3DL05 K204E rev	GCTGAGAGAGAAGGTTTCTCATATATACCTGTGATC	---
KIR3DL05 R230C fw	GTCCTGCAGCTCCCGGTGCTCCTTTGACATGTAC	---
KIR3DL05 R230C rev	GTACATGTCAAAGGAGCACCGGGAGCTGCAGGAC	---
KIR3DL05 Y203A fw	CATCGTGATCACAGGTATAGCTAAGAAACCTTCTCTCTC	---
KIR3DL05 Y203A rev	GAGAGAGAAGGTTTCTTAGCTATACCTGTGATCACGATG	---

Name	Sequence (5'-3')	Temp.
KIR3DL05 D233H fw	CTCCCGGCGCTCCTTTCACATGTACCATCTATC	---
KIR3DL05 D233H rev	GATAGATGGTACATGTGAAAGGAGCGCCGGGAG	---
KIRconsfw1	ACAAAGACGACAGAAGCCAC	---
KIRconsfw2	ACGGTCACCCTACAATGTTC	---
KIRconsfw3	TGTTGTCAGCTCCCAGTGAC	---
KIRconsfw4	CAGATGCTTCGGTTCTTTCC	---
KIRconsfw5	GCTGGTGCTCCAACAAAAAG	---
KIRconsfw6	CCAAATGCTGAGCCCAGATC	---
KIRconsfw6 b	CTGAAGGTGTGAGTCTGCAT	---
KIRconsfw7	ACACACTCCTTTGCTTAGCC	---

Table 2: KIR-typing primer

No.	*KIR* gene(s)	Primers (5'→3')	Annealing temp.	Length (bp)	Domain
1	*KIR2DL04*001*	GGTCAGGACAAGCCCTTCTG ACCAGGGGGTTGCTGGGTG	62°C	269	D0
2 [a]	*KIR3DL01*002*, FJ562109[b]	TATCGTCGTGGGCTTTACAAC TGACCTGTGACCATGATCGT	58°C	218	D0
3	*KIR3DL01* (**003*, **004*, **006*, **008N*, **009N*, **010*)	TGTCACTATCGTCGTGGGCTTT CCAGGGGTTCACTGAGTGCT	62°C	205	D0
4	*KIR3DL01*011*, FJ562110[b]	TGCCTCAGGGAGGACACGTA CCTGATCGCCAGGGGGTCG	62°C	242	D0
5	*KIR3DL02*001*	TCGTGGTGGGTTTAACAACTTC GGGAGTCGACCACTCAGTGA	62°C	175	D0
6	*KIR3DLW03* (**004*, **005*)	TACAAAGACGACAGAAGCCACA CCAGGGGGTTGCTGGGAGT	62°C	160	D0
7	*KIR3DL05*007*, FJ562120[b], FJ562121[b]	GGAGTCCACAGAAAACCTTC TCTCCAACAAGGTGCACGGA	58°C	155	D1
8	*KIR3DL06*001*	GGTGTCACTATCGTCGTGGC GTCCCTGCGTGTGCCTGG	60°C	142	D0
9	*KIR3DL07*001*	CCTACAATGTTCTTCAGATATG GGAGCTGACAACACATAGTC	58°C	204	D1
10	*KIR3DL07*002*	TATGAGAAACCTTCGCTCTCAT AAGCATCTGTAGGTTCCTCCA	60°C	221	D2
11 [a]	*KIR3DL08**001	AAGACCTCCCTGTCTGCCCA GACCTGTGACCCTGATCACG	62°C	286	D0
12	*KIR3DL08* (**002*, **003*)	TCTCTCAGCCCAGCCGGGA GTAGTGGGTCACTCGGGTG	60°C	260	D2
13	*KIR3DL10*001*, FJ562113[b]	CCTGTCTGCCCGGCCTAGT CGTGTGCCGGGGTCACAGT	62°C	188	D0
14 [c]	*KIR3DL10*002*, FJ562112[b]	CTCAGGGAGGACACGTGACC GTCCCTGCGTCTGCCGGG	62°C	166	D0
15	*KIR3DL11*001*, FJ562116[b]	TCGTCAGATACCGTGTTTGG GTCACTGGGAGCTGACAAG	58°C	201	D1
16	*KIR3DL11*002*	TCTCAGCCCAGCCGGGCCT TTTGACAGAAACGGGCAGTGG	62°C	272	D2
17	*KIR3DL20* (**002*, **003*)	CTTAGGCTCCCTGCAATGCCA GTCACTCGGGTGTGACCACA	62°C	138	D2
18	AF334646, AF334647[b]	TTAGGCTCCCTGCAGTGCCG GTCACTCGGGTGTGACCACA	62°C	137	D2
19	*KIR3DS01* (**00101*, **002*, **003*)	ACGGTGCAGGCAGGAGAGG GACCACTGGTAGGGTGCGGA	62°C	218	D2
20 [c]	*KIR3DS02* (**001*, **008*)	GTCAGGACAAGACCTTCTTGTT CTGCGTGTGCCGGGGTCAT	62°C	207	D0

No.	*KIR* gene(s)	Primers (5'→3')	Annealing temp.	Length (bp)	Domain
21	*KIR3DS03* (**00101*, **002*, **003*)	GGTGCCTCAGGGAGGACACA GGTCCCTGCGTCTGCCGGA	64°C	172	D0
22	*KIR3DS04*001*	GAAATCAGGAGAGACGGTCAT GATGTCCAGGGTGTCACTC	58°C	244	D1
23 [c]	*KIR3DS05* (**00201*, **00202*, **003*)	GTCAACGGAACATTCCAGGA TGTGACAGAAACGGGCAGTG	60°C	132	D2
24	*KIR3DS06*004*	CCCAGGTCCCTTGGTGAAATT ACCTGTGATCACGATGTCCC	60°C	271	D1
25	*KIR3DSW07*001*	CCCTGGTGAAATCAGGAGAT ACCGTAGCATCTGTAGGTCT	58°C	197	D1
26	*KIR3DSW07*002*	AATCAGGAGAGACGGTCACA CTCTGCAAGGTCAGACGTCT	58°C	170	D1
27	*KIR3DSW08*005*	AAAACCTTCCCTCCTGGCCT CTGGGAGCTGACAACACATC	60°C	264	D1
28	*KIR3DSW08*006*	CTGCCCGGCCCAGCGCTG CCGACATCTGTAGGTCCCTGT	64°C	203	D0
29	*KIR3DSW08*007*	TGTCACTATCGTCGTGGGCTTT CCGACATCTGTAGGTCCCTGT	64°C	153	D0
30	*KIR3DSW09*004*	CGGTCACCCTACAATGTTCC GAGTGAGTGACAGAACCGTAA	60°C	190	D1
31	*KIR3DSW09*005*	TGCAGCTCCCGGTGCTCGG GGTCACTCGGGTCTGACCAT	62°C	199	D2

[a] Primer pairs 2 and 11 reach into the intron region.
[b] In those cases where an official designation is not yet available, the database accession numbers were included.
[c] Primer pairs 14, 20 and 23 recognize besides alleles of one locus also recombinants of different *KIR* genes.

2.1.10 Database and programs

Database

NCBI:	http://www.ncbi.nlm.nih.gov/
IP Database	http://www.ebi.ac.uk/ipd/index.html

Online programs

Expasy Translate Tool	http://au.expasy.org/tools/dna.html
Reverse Complement	http://bioinformatics.org/sms/rev_comp.html
Webcutter 2.0	http://rna.lundberg.gu.se/cutter2/

Software

BioEdit	(Hall, 1999)
ClustalX	(Thompson et al., 1997)
CodonCode Aligner	(CodonCode Corporation)
FlowJo	(Tree Star Inc.)
Free Workbench2	(CLC bio A/S)

MEGA 3.1	(Kumar et al., 2004)
Sequencing Analysis	(Applied Biosystem)
GeneMapper v4.0	(Applied Biosystem)

2.2 Molecular biology methods

2.2.1 Polymerase chain reaction

The polymerase chain reaction (PCR) was performed as a hot-start PCR, whereby the template and the polymerase was separated from the oligonucleotides and the dNTPs by a wax layer. The oligonucleotides can bind to the DNA and the amplification starts only after reaching the denaturation temperature after the wax is melted. Thereby, specificity of primer binding increases and the polymerase can start the amplification only upon reaching the optimal temperature, which reduces the possibility of amplification artefacts.

The PCR reaction was set up by using 1 µl Biotherm 10 x reaction buffer, 0.2 µl dNTP mix (each 25 mM), 1 µl forward primer, 1 µl reverse primer and 6.8 µl H_2O. Afterwards the mix was covered with wax and the second mix was added: 2 µl 10 x reaction buffer, 4 µl 10 x BSA/Triton-X buffer, 0.2 µl Biotherm Taq DNA polymerase (5 U/µl), 1 µl DNA and 12.8 µl H_2O.

Table 3: PCR programs

PCR programmes	Sequencing PCR		Mycoplasm PCR		Colony-PCR	
Initial denaturation	-		3 min 94 °C		4 min 94 °C	
Denaturation	30 s 95 °C	25 cycles	30 s 94 °C	35 cycles	1 min 94 °C	30 cycles
Annealing	15 s 50 °C		2 min 55 °C		1 min 52 °C	
Elongation	4 min 60 °C		1 min 72 °C		1,5 min 72 °C	
Final Elongation	-		7 min 72 °C		5 min 72 °C	

2.2.2 *KIR* genotyping

KIR genotyping was performed by using an internal positive control (β-Actin). Therefore, *β-Actin* primers (Table 1) were added to the PCR reaction and only those PCR reactions were analysed where the positive control leads to positive results. The PCR reaction was set up by using 1 U Taq DNA polymerase, 3 µl 10 x buffer, 5 mM dNTPs and 50 ng DNA. Sequence-specific *KIR* primers (Table 2) and internal control primers were used in 0.16 pmol/µl and 0.06 pmol/µl concentrations, respectively. PCR conditions are the same for all primers, except for the annealing temperature (Table 2): initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, annealing for 30 s, and elongation for 45 s at 72°C, followed by a final elongation at 72°C for 5 min. As for normal PCRs, the *KIR* genotyping was performed as a hot-start PCR to increase the specificity (2.2.1). All PCR products were analysed by agarose gel electrophoresis (2.2.4).

2.2.3 Microsatellite markers

Short tandem repeats were identified in the sequenced *KIR* haplotype (Sambrook et al., 2005) by manual inspection. Flanking primers (Table 1) were designed and used in a PCR reaction consisting of an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 59 °C for 30 s, and 45 s at 72 °C, and a final extension step for 5 min at 72 °C. 6-FAM, HEX and TAMRA-labelled PCR products were analysed in an ABI3130xl sequencer along with the Genescan HD ROX size standard. Allele sizes were calculated with the Gene Mapper v4.0.

2.2.4 Gel electrophoresis of DNA in agarose gels

To determine the fragment size, the Gene Ruler 100 bp DNA Ladder Plus was used. The electrophoretic separation was performed in 1 % agarose gels supplemented with ethidiumbromide (0.4 µg / ml) and 120 V in 1 x TBE buffer for 15 minutes.

2.2.5 DNA extraction from agarose gels

To isolate DNA fragments from agarose gels, the DNA products were cut out of the gel and extracted using the QIAquick Gel Extraction Kit according to the supplier's recommendations.

2.2.6 DNA quantification

The concentration of DNA fragments was measured on an agarose gel. Therefore, a control plasmid (pUC19 vector) with known DNA concentration was separated along with the samples in Agarose gels. By comparing the bands under UV light the concentrations of the DNA fragments were determined.

2.2.7 Ligation of PCR products

The pGEM-T Easy cloning Kit was used for the ligation of PCR products according to the manufacturer's recommendations.

2.2.8 DNA restriction by endonucleases

For the restriction of 10 µg DNA 20 U of the restriction endonucleases and 2 µl of the respective 10 x reaction buffer were used. According to the manufacturer's recommendations BSA was added to the reaction and H_2O was added to a total volume of 20 µl. The reaction was incubated for 1 h at 37 °C and afterwards separated in a 1% agarose gel (2.2.4) and the fragment was isolated (2.2.5).

2.2.9 Dephosphorylation of DNA fragments

10 µg of DNA restricted by endonuclease enzymes (2.2.8) were mixed with 10 µl 10 x reaction buffer and 5 U shrimp alkaline phosphatase and added with H_2O to a total volume of 100 µl. The reaction was incubated for 2 h at 37 °C. Afterwards the enzyme was inactivated by 65 °C for 15 min. The DNA was precipitated by adding 250 µl 100 % EtOH and 10 µl 3 M sodium acetate (pH 4.8) and centrifuged for 15 min by 16,000 x

g at RT. The DNA was washed with 250 µl 70 % EtOH and centrifuged for 5 min by 16,000 x g at RT. After drying in the speed vac the DNA was resolved in H_2O.

2.2.10 Ligation of DNA fragments

For the ligation of DNA fragments 500 ng insert DNA and 100 ng of linearized expression vector DNA was mixed with 2 µl 10 x T4-ligase buffer as well as 0.2 µl T4-ligase and H_2O was added to a total volume of 20 µl. The reaction was incubated over night at 4 °C and used for a transformation of bacterial cells (2.2.12).

2.2.11 Establishment of competent cells for electroporation

For the establishment of competent cells *E. coli* TOP10 cells were added to 5 ml LB medium supplemented with streptomycin (100 ng/ml) and incubated in a shaker at 37 °C and 200 rpm over night. On the next day, the mixture was added to 450 ml pre-warmed LB medium supplemented with streptomycin (100 ng/ml) and incubated at the same conditions until the OD_{605} reached a value of 0.5-0.6. Afterwards the mixture was incubated for 30 min on ice and all following steps were performed on ice and pre-chilled solutions. Then the cells were centrifuged for 5 min at 4 °C and 1,500 x g and the supernatant was discarded. The cells were washed with 10 % glycerine solution and centrifuged for 15 min at 4 °C and 1,200 x g. The supernatant was discarded and the cells were resuspended in 1.5 ml 10 % glycerine solution and 50 µl aliquots were immediately frozen at -80 °C.

2.2.12 Transformation of electro-competent bacterial cells

For the transformation of bacterial cells, 50 µl *E. coli* TOP10 cells were thawed on ice. Afterwards, 1.5 µl of ligation reaction (2.2.7) was added. The transformation was performed in a pre-chilled electroporation cuvette with 1.6 kV, 25 µF und 200 Ω. 1 ml of SOC medium was added to the cells, which were incubated at 37 °C in the shaker (140 rpm). After one hour the cells were plated on a LB agar plate with the appropriate antibiotic and incubated over night at 37 °C. In order to define the insert size a colony PCR was performed. Therefore, the PCR reaction was set up by using 3 µl Biotherm 10 x reaction buffer, 4 µl 10 x BSA/Triton-X buffer, 0.2 µl Biotherm Taq DNA

polymerase (5 U/µl), 0.2 µl dNTP mix (each 25 mM), 1 µl forward primer, 1 µl reverse primer and 19.6 µl H_2O. Afterwards cells of a bacterial colony was directly transferred in the PCR reaction. The reaction was covered with wax and the PCR was performed (2.2.1).

2.2.13 Mini preparation of plasmid DNA

The bacteria were incubated over night at 37 °C in 3 ml LB medium with appropriate antibiotics. Bacteria were pelleted (16,000 x g, RT, 30 s) and resuspended in 150 µl solution I. Afterwards, 150 µl solution II was added and incubated for 5 minutes at RT. After that 150 µl solution III was added and mixed by inverting. The mixture was centrifuged twice and the supernatant was transferred in a new cup. The DNA plasmid was precipitated with 1 volume of Isopropanol (16,000 x g, RT, 10 min) and washed with 300 µl 70 % EtOH (16,000 x g, RT, 5 min). After drying the DNA in the speed vac the pellet was resuspended in 30 µl H_2O.

2.2.14 Midi preparation of plasmid DNA

The QIAquick Plasmid Midi Kit was used according to the manufacturer's instructions for a purification of plasmid DNA out of bacteria in higher amounts of DNA.

2.2.15 Total RNA isolation

Total RNA was isolated by using the RNeasy Plus Mini Kit according to the supplier's recommendations.

2.2.16 Quantification of RNA concentration

The quantification of RNA was carried out by photometric analysis. Therefore, the RNA was diluted with DEPC-H_2O with a ratio of 1:50 or 1:100 and measured in a photometer by 260 nm with DEPC-H_2O as reference. The calculation was carried out as followed:

RNA concentration [µg ss RNA /ml]= Extinction (OD_{260}) x dilution x 40

2.2.17 Mutagenesis of gene constructs

For the mutation of gene constructs the QuickChange II Site-Directed Mutagenesis Kit was used as described in the user manual.

2.2.18 Sequencing analysis

Sequence reactions contained 20-30 ng DNA of a PCR product or 200-300 ng plasmid-DNA. The BigDye® Terminator v1.1 Cycle Sequencing Kit was used according to supplier's recommendations. After the sequencing reaction (Table 3), the DNA was precipitated by adding 90 µl H_2O, 250 µl EtOH and 10 µl 3 M sodium acetate (pH 4.8) and the mixture was centrifuged (16,000 x g, RT, 15 min). The DNA pellet was washed by adding 250 µl 70 % EtOH and centrifuging again (16.000 x g, RT, 5 min). The pellet was dried in the speed vac, resolved in 4 µl Hi-Di™ formamide and analysed in an ABI3130XL sequencer. The analysis was performed with CodonCode Aligner.

2.2.19 Genomic DNA isolation from total blood

The cellular fraction of blood samples (5 to 15 ml) was incubated in erythrocyte lysis buffer for 20 min and centrifuged for 10 min at 7 °C and 200 x g. The pellet was washed with 10 ml lysis buffer and incubated over night at 37°C in 5 ml SE buffer, 250 µl 20% SDS and 20µl Pronase E. After adding 2 ml 5 M NaCl, the reaction was centrifuged for 10 min at 1,250 x g. The DNA was precipitated with 14 ml 100 % EtOH, washed with 5 ml 70 % EtOH and resolved in H_2O.

2.3 Cell culture

2.3.1 General work with cell cultures

Work with cell cultures was carried out under sterile conditions. Therefore, all working steps were performed using sterile hoods of the safety category 2. Media or solutions were autoclaved or sterile filtered before use.

2.3.2 HEK-293 cells

The HEK-293 cells were cultivated in DMEM high glucose medium with 15 % FCS and 1 % penicillin and streptomycin in the incubator at 37 °C and 5 % CO_2. Every 48-72 hour cells were split and reseeded.

2.3.3 K562 cells

The K562 cells were cultivated in DMEM high glucose medium with 10 % FCS and 1 % penicillin and streptomycin in the incubator at 37 °C and 5 % CO_2. Every 48-72 hour cells were split and reseeded.

2.3.4 Mycoplasma PCR

The cell cultures were checked regularly for contamination with mycoplasma. Therefore, 200 µl cell culture supernatant was harvested together with medium as negative control and a positive control (cell culture supernatant from mycoplasma-infected cells). The supernatants were incubated for 5 min at 95 °C and centrifuged for 1 min at 1,200 x g. Subsequently, 1 µl of the supernatant was used to perform a PCR (2.2.1) with the MYC1 primers (Table 1). Afterwards, a nested PCR was performed with the MYC2 primers (Table 1). If a band between 200 and 500 bp was present on the agarose gel (2.2.4), the cells were positive for mycoplasma and were discarded.

2.3.5 Determination of the viable cell number

To determine the viable cell number 10 µl of the cell suspension was mixed with the equal volume of trypan blue. The number of living, not coloured, cells was counted in a hemocytometer.

2.3.6 Transfection of eucaryotic cells

Cells were grown to the proliferation phase and 5 x 10^6 cells were incubated in a culture flask for 24 hours. Afterwards, 10 µg DNA was mixed with serum-free DMEM medium to a total volume of 50 µl. 50 µl Metafectene mixed with 450 µl serum-free DMEM

medium was carefully mixed with the DNA. After incubation for 20 minutes at RT, the mixture was slowly dropped on the cells. After 48 hours the transfected cells were either used as transiently transfected cells or selected by adding the appropriate antibiotic to gain stably transfected cells.

2.3.7 Cryoconservation of eukaryotic cells

4×10^6 cells were washed with 1 x PBS and the supernatant was carefully discarded. Cells were resuspended in 1 ml DMSO and transferred into a cryo-tube. The tubes were frozen for two days at -80 °C in a cryo container, which was filled with isopropanol. Due to the isopropanol, the temperature was decreased by 1 °C per hour and the cells were gently frozen. Afterwards the tubes were transferred in a liquid nitrogen tank for long-term storage.

2.3.8 MHC class I cell surface expression

Cell surface expression of MHC class I molecules was analysed by flow cytometry. 10^6 cells were transferred to FACS tubes and washed twice with 1 x PBS (10 min, 300 x g). Three different set-ups were performed. The negative control was used without adding any antibody; non-specific binding of the secondary antibody (*goat-anti-mouse* IgG) was checked in a second set; the third reaction contained first the primary antibody (W6/32) and after one hour the secondary antibody was added. Prior to the FACS analyses all reactions were washed twice with 1 x PBS. The FACS analyses were performed in a FACScan Flowcytometer and analysed with the FlowJo 5.7.1 software.

2.3.9 KIR/MHC class I binding assay

K562 cells were transiently transfected with AcGFP-tagged Mamu-A, -B and, -I molecules were used, kindly provided by Dr. Cornelia Rosner (Primate Genetics Laboratory, German Primate Centre).

To analyse the interaction between KIR and AcGFP-tagged Mamu class I proteins 5×10^5 cells were transferred to FACS tubes and washed twice with 1 x PBS (10 min, 300 x g). Four different experimental set-ups were performed:

1) The negative control was carried out without adding any protein
2) Biotinylated protein A multimerised with PeCy5-labelled streptavidin
3) The purified and multimerised supernatant of mock transfected HEK-293 cells
4) The multimerised KIR fusion proteins (2.4.3)

The cells were incubated for 1 hour at 4 °C with the different protein preparations and were washed twice with 1 X PBS afterwards. The FACS analyses were performed in a FACScan Flowcytometer and analysed with the FlowJo 5.7.1 software.

2.4 Biochemical methods

2.4.1 Purification of KIR-Fc fusion proteins

The HEK-293 cells were stably transfected with *KIR* gene constructs, which allow for secretion of KIR-Fc fusion proteins, as well as a mock control (2.3.6). Cells were incubated in ultraCHO serum-free medium. After 3 days the supernatant was centrifuged (3,000 x g, 15 min) and filtered (0.45 µm). The recombinant KIR-Fc fusion protein was purified using the MApTrap Kit according to the supplier's recommendations. The purificated proteins were concentrated by Amicon Ultra-30 columns used as described in the user manual. The integrity of the fusion proteins was determined by SDS gel electrophoresis (2.4.2).

2.4.2 SDS-PAGE

To determine the concentration of the purified proteins, the protein preparations were analysed together with a dilution series of human IgG. The proteins were mixed with 1 volume Laemmli buffer and 1 volume DDT and boiled for 5 min at 95 °C. Afterwards, the samples were cooled on ice and electrophoresed under reducing conditions in a 10 % SDS gel. Proteins were transferred to a nitrocellulose membrane in a semi-dry electro-blotter for 1 h at 2 mA / cm^2. After the transfer, the membrane was incubated with blocking solution for 1 h at RT. The anti-human IgG antibody, coupled with a horseradish peroxidase, was added and incubated over night at 4 °C. Prior to visualization the blots were washed five times for 5 min in washing buffer, and once more in 1 x TBS. The developing solutions 1 and 2 were mixed in 1:1 ratio and the

membrane was incubated in this mixture for 1 min. Membranes were exposed to hyperfilm for 20 to 40 seconds and films were developed subsequently.

2.4.3 Multimerisation of KIR-Fc fusion proteins

Twenty µg of KIR-Fc recombinant protein were mixed with 1.4 µl of biotinylated protein A and left for 30 min at RT. As negative control, the same volume of protein-G purified 293 mock transfected supernatant was used and treated like the other fusion proteins. Afterwards PeCy5-labelled streptavidin was added in a proportion of 1:100 and incubated for 15 min at 4 °C to multimerise the proteins. Afterwards, the multimerised protein was used for FACS analyses (2.3.9).

2.5 Establishment of a cDNA library

2.5.1 Isolation of PBMC from rhesus macaque whole blood

Ficoll-Hypaque centrifugation was used to isolate peripheral blood mononuclear cells (PBMC) from whole blood. 4 ml Ficoll-Hypaque 1.077 was overlaid with whole blood mixed with RPMI medium in 1:1 ratio and centrifuged for 40 min at 600 x g without using brakes. The lymphocyte-containing interphase was transferred to a new tube, centrifuged for 10 min at 300 x g, and the supernatant was discarded. The cell pellet was washed twice with 1 x PBS, resolved in 50 ml MACS buffer, and the viable cell number was determined (2.3.5). After a centrifugation step (10 min, 300 x g), 1.2 x 10^8 cells were resolved in 1 ml MACS buffer.

2.5.2 Isolation of $CD16^+$ cells

The CD16 molecule was used as an established and known marker for NK cells in rhesus macaques. 240 ml CD16 MicroBeads (non-human primate) were mixed with 1.2 x 10^8 PBMC dissolved in MACS buffer. The mixture was incubated for 15 min at 4 °C, afterwards washed with 12 ml MACS buffer (10 min 300 x g) and the supernatant was completely discarded. A MS column was put in the MACS separator and activated by adding 500 µl MACS buffer. Afterwards the cells were resolved in 500 µl MACS buffer and added to the column. The column was washed afterwards three times with

500 µl MACS buffer. Both the cell suspension and the washing solution were used as negative control for the subsequent FACS analysis. Subsequently, the column was removed from the MACS separator and washed with 1 ml MACS buffer to obtain the $CD16^+$ cells.
The viable cell number was determined for all fractions (2.3.5) and 10^6 cells were used for FACS analysis to determine the efficiency of the enrichment of $CD16^+$ cells. The remaining fraction of cells was used to isolate total RNA (2.2.15).

2.5.3 FACS analysis of the cell fractions

The FACS analysis was performed in the department of Cellular and Molecular Immunology of the University Göttingen. Each cell fraction was distributed in three FACS tubes and washed with 1 x PBS (10 min, 300 x g) and resolved in 200 µl 1 x PBS. To the first tube, 1 µl of an TC-labelled mouse anti-human CD16 antibody was added. To the second tube, 1 µl of a FITC-labelled mouse IgG1 isotype control antibody was added. The third tube was not treated with any antibody. The incubation of the cells with the antibody was carried out by 4 °C for 1 hour. Before FACS analysis, the cells were washed with 1 x PBS (10 min, 300 x g) and resolved in 500 µl 1 x PBS.

2.5.4 Establishment of the cDNA library

The cDNA library was established by using the Creator SMART cDNA Library Kit and ElectroMAX DH10B cells. All steps of mRNA preparation, cDNA synthesis and cDNA library preparation are described in detail in the supplier's protocol and all recommendations were exactly followed.

2.6 cDNA library screening

2.6.1 Establishment of radioactive probes

To identify *KIR* gene-containing plasmids, a radioactive probe was produced. DNA hexamers were added to a PCR product spanning exons 3-5 of a *KIR* sequence. The Prime-a-Gene Labelling system with radioactive cytosine was used to label the PCR product.

25 ng DNA was added to H_2O to a total volume of 30 µl and denatured for 2 min at 100 °C. Afterwards, the reaction was cooled on ice and 10 µl 5 x labelling buffer, 2 µl dNTPs, 2 µl 1 x BSA, 50 µCi dCTP and 1 µl DNA polymerase (5 U/µl) were added. The reaction was incubated 1 hour at RT and again denatured at 100 °C and cooled on ice. Subsequently, 2 µl of 0.5 M EDTA (pH 8.0) was added. The probe could be used directly for hybridisation or stored at 4 °C for later use.

2.6.2 cDNA library screening with a radioactive probe

10^5 cells of the enriched cDNA library were incubated for 14 hours at 37 °C on a LB-CM plate. Subsequently, the plate was incubated for 30 min at 4 °C, a filter membrane was laid on the plate, and the position was marked on the plate. The agar plate was incubated for 1 hour at 37 °C. The filter membrane was first incubated for 5 min in 10 % SDS. Afterwards, it was incubated for 3 min in denaturation buffer, 2 min in neutralisation buffer, and finally for 10 min in 2 x SSC. The membrane was air-dried and the DNA was linked to the membrane in an UV-Stratalinker (12,000µJ).

The membrane was washed for 5 min at RT in 2 x SSC buffer and subsequently incubated for 30 min at 50 °C in pre-washing solution. Both incubation steps were carried out on a shaker to guarantee constant washing of the membrane.

A pre-hybridisation was carried out to block unspecific binding sites. The membrane was incubated in a hybridisation oven for 1 hour at 60 °C with 20 ml preheated hybridisation buffer with 400 µl salmon sperm as additive. After that, the membrane was incubated over night at 60 °C with the radioactive probe.

The membrane was washed at least two times with washing buffer until the radioactivity was no longer reduced. The membrane was wrapped in plastic wrap and a photographic film was exposed for 1 hour at -80 °C. Films were developed and fixed with a developer.

Clones which showed a clear signal were picked and incubated over night at 37 °C in 3 ml LB-Cm medium. Plasmid DNA was prepared (2.2.14) and the clones were completely sequenced (2.2.18).

3 Results

3.1 *Isolation of KIR genes*

To analyse the diversity of *KIR* genes, a cDNA library was established. Compared with a standard PCR amplification approach, screening of a cDNA library has significant advantages. PCR primers are always specifically designed for already-known sequences; therefore, sequences which differ from known sequences might escape detection. In contrast, screening of a cDNA library with radioactive probes should allow detection of novel *KIR* sequences. Another advantage of this method is that the 5' and 3' untranslated regions (UTRs) of the cDNA are also isolated.

3.1.1 Establishment of a cDNA library from enriched NK cells

For the establishment of the cDNA library, whole blood was pooled from 30 unrelated rhesus macaques. Since most studies use rhesus macaques of Indian origins and data suggest an evolutionary separation of rhesus macaques originating from India and China (Smith, 2005), all animals used in this study were of Indian origin exclusively. The pooled peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll separation gradient. From 90 ml of total blood, 3.88 x 10^8 PBMCs were isolated. The NK cells were enriched using CD16 non-human primate MicroBeads. The percentage of NK cells in the PBMCs and the enriched cells were analysed by FACS analysis. Therefore, NK cells were stained with a TC-labelled anti-human CD16 antibody and all probes were used for FACS analysis. In contrast to the untreated PBMCs (with 9.3 % NK cells), the percentage of NK cells in the enriched probe was raised to >90 % (Figure 9). Thus, a considerable enrichment of NK cells was achieved.

Total RNA was isolated from the enriched NK cells and used for cDNA library generation. The correct cDNA synthesis was checked by gel electrophoresis. As expected, the gel shows a smear of cDNA ranging from 0.1 to >3 kb with two significant bands (Figure 10), indicating that the cDNA synthesis was successful.

The presence of genomic DNA was checked by performing a PCR with *KIR*-specific primers that bind to genomic DNA and cDNA encompassing two adjacent exons. Therefore, products obtained from cDNA and genomic DNA would differ in size due to amplification of intronic sequence between the two adjacent exons. No product of the

size expected for genomic DNA was detected, whereas the use of the cDNA as template resulted in a specific PCR product of 200 bp (data not shown).

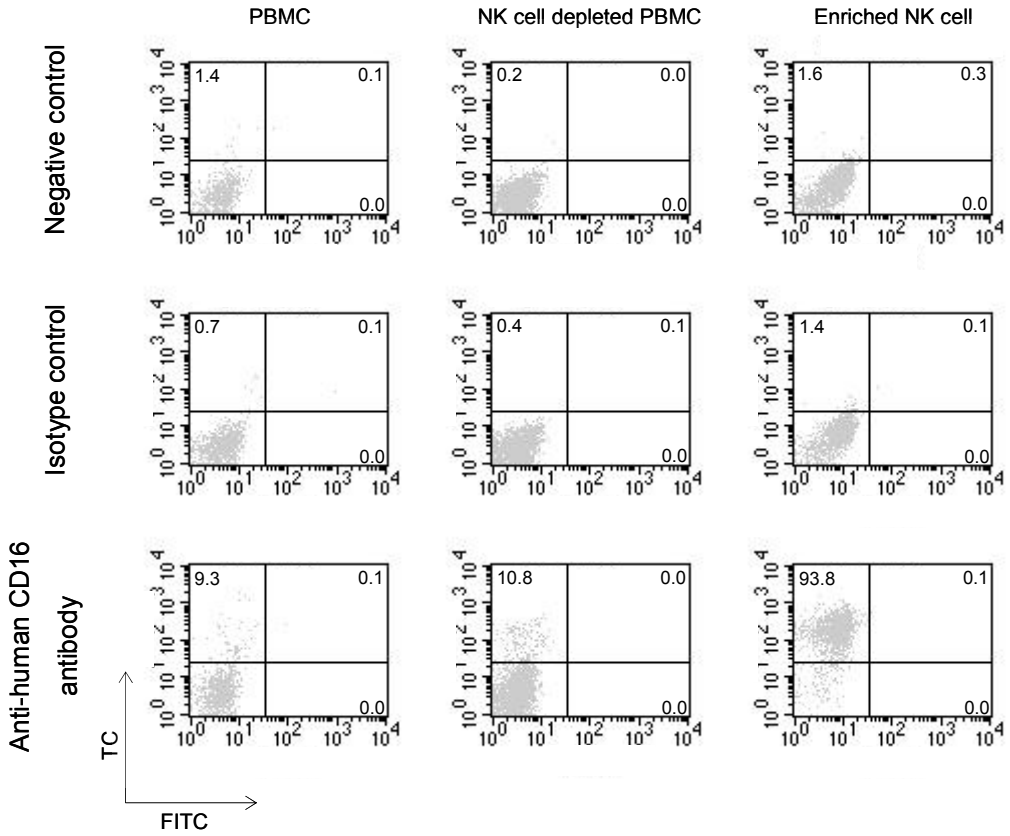

Figure 9: FACS analyses of enriched NK cells. The FACS analysis of the PBMCs, the NK cell depleted PBMC and the enriched NK cells were shown as dotblots. The TC fluorescence is shown with the FITC fluorescence. The diagrams show the FACS analyses without an antibody (negative control), with the FITC-labelled isotype control and the TC-labelled anti-human CD16 antibody. The percentage of CD16-positive cells (mainly NK cells) are indicated in the upper left quadrant.

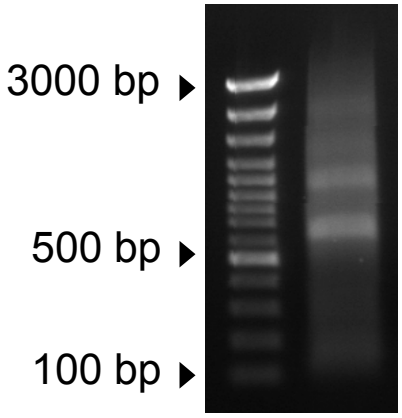

Figure 10: Analysis of cDNA synthesis. 5 µl of obtained cDNA was checked by agarose gel electrophoresis. A smear between 0.1 and >3 kb can be seen with two distinct bands at about 700 and 1000 bp.

The cDNA was separated by size using a CHROMA SPIN-400 column (Figure 11). Fractions 2 to 6 contained fragments of the size of *KIR* genes and were therefore used for the further procedure. The remaining fractions were discarded due to inadequate size of the cDNA fragments.

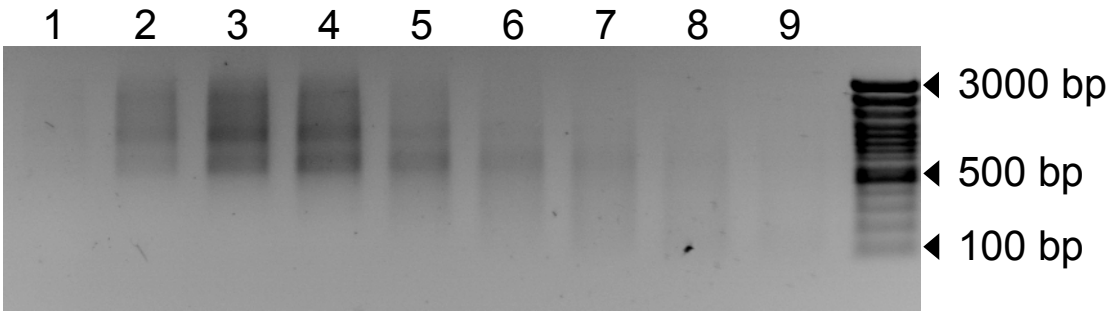

Figure 11: Analysis of the cDNA size from eluted fractions. 3µl of the fractions separated with CHROMA SPIN-400 column was checked for the size of containing cDNA by agarose gel electrophoresis.

The fractions were pooled, ligated in the pDNR-LIB vector and transformed in ElectroMAX DH10B cells. The amount of independent clones in the cDNA library was checked by titration. The unamplified library contained 1.15 x 10^7 cDNA clones.
To determine the size of inserts, a colony PCR of 90 independent clones was performed with M13 insert-flanking vector primers (Table 1). The insert sizes ranged from 400 to 1,500 bp, and the distribution approximately matched a normal distribution curve with a maximum at 800 bp (Figure 12).

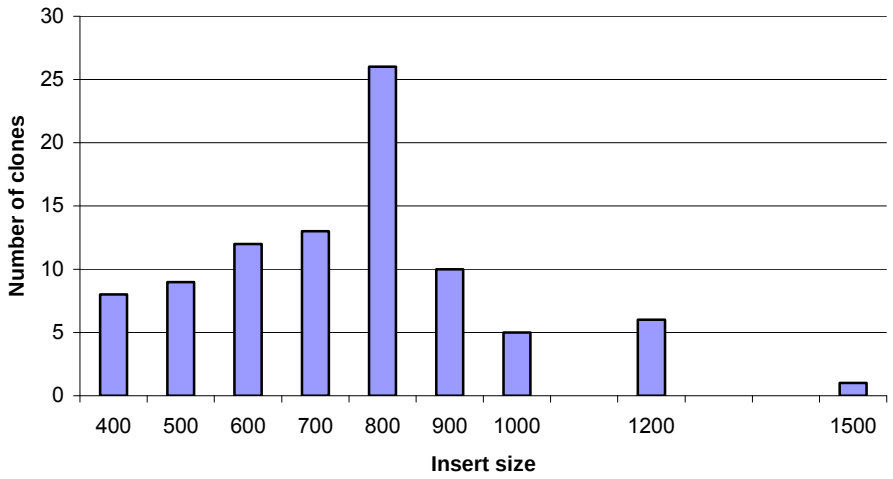

Figure 12: Distribution of insert size of the cDNA library. A colony PCR of 90 independent clones was performed with M13 insert-flanking vector primers. The size of obtained PCR products was determined by gel electrophoresis and shown as a bar chart.

3.1.2 Isolation of *KIR* genes from cDNA library

The detection of *KIR* gene-containing clones was carried out with a radioactive probe, which was synthesized as a PCR product of the exons encoding the Ig domains of a *KIR* gene. By this method, 14 different *KIR* sequences were isolated. All *KIR* sequences were completely sequenced (Table 1) and analysed. Six *KIR* sequences were not isolated in full-length. In some cases, the sequence showed a deletion at the 5' end. Other clones represented a splice variant where only the first Ig domain was present either with or without the transmembrane and intracellular part of the *KIR* sequence, as previously characterised by Sambrook and colleagues (2005). Eight different full-length *KIR* sequences could be isolated which were named by the rhesus macaque KIR Gene Nomenclature Committee and were used for further analyses. All isolated *KIR* sequences code for KIR proteins with three Ig domains (Figure 13). Three *KIR* sequences code for inhibitory and five for activating KIR molecules (7.1). Four of the activating KIRs belong to receptors previously named KIR3DH (Hershberger et al., 2001); one belongs to a group of activating receptors previously designated KIR3DM (Blokhuis et al., 2009a). According to novel nomenclature guidelines, these two types are now collectively named KIR3DS.

Characteristic features of primate KIR3D molecules were found for the isolated *KIR* clones: inhibitor KIRs have two ITIMs in their cytoplasmic region whereas activating KIRs lack these motifs and display a charged amino acid in the transmembrane region, which is in all cases an arginine residue (Figure 13). Thus, these data confirm previous findings by others that activating KIR molecules of the rhesus macaque contain an arginine instead of a lysine residue (Hershberger et al., 2001; Sambrook et al., 2005; Bimber et al., 2008; Blokhuis et al., 2009a). With phylogenetic analyses, it was shown that all identified sequences represent up to now unknown *KIR* sequences (Figure 14). Nevertheless, all sequences cluster together with already-known *KIR* sequences.

The deduced amino acid sequences of some KIRs, KIR3DL11*003 and *004 as well as KIR3DSW08*006 and *007, are nearly identical and differ only at a few positions (Figure 13 and figure 14). KIR3DL11*003 and KIR3DL11*004 differ only at two amino acid positions, whereas KIR3DLW08*006 and KIR3DLW08*007 differ by six amino acid residues. Therefore, both pairs of cDNA sequences might represent different alleles of a distinct *KIR* gene. This was also assumed by the rhesus macaque KIR Gene Nomenclature Committee and were therefore described as alleles of the *KIR3DL11* loci (alleles **003* and **004*) and the *KIR3DSW08* loci (alleles **006* and **007*).

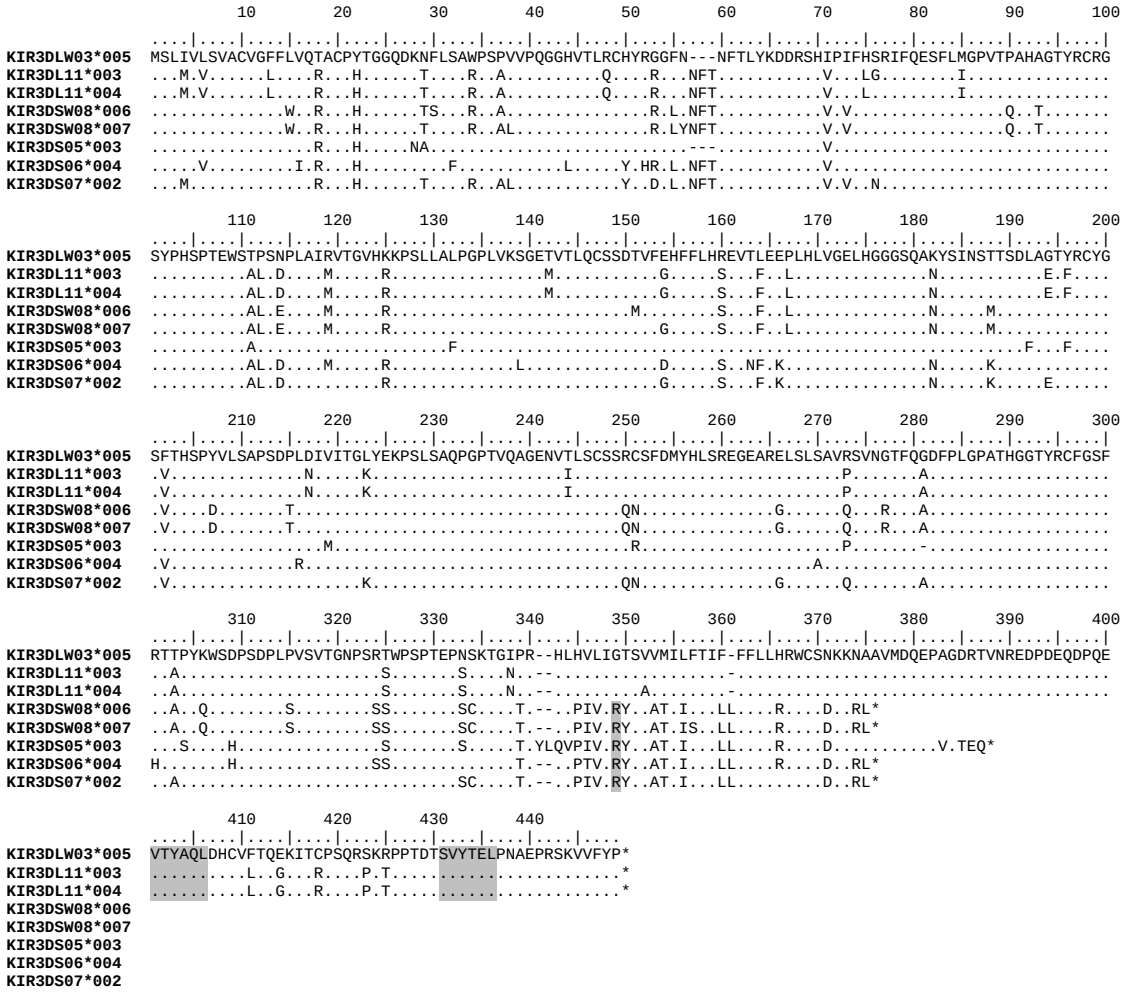

Figure 13: Comparison of deduced amino acid sequences of the newly identified *KIR3D* cDNA sequences. Identical amino acids residues are indicated by a dot, dashes denote introduced gaps to maximise homology. ITIMs of inhibitory KIRs as well as positive charged amino acid residues in the transmembrane region of activating KIRs are highlighted in grey. * indicates the end of translation.

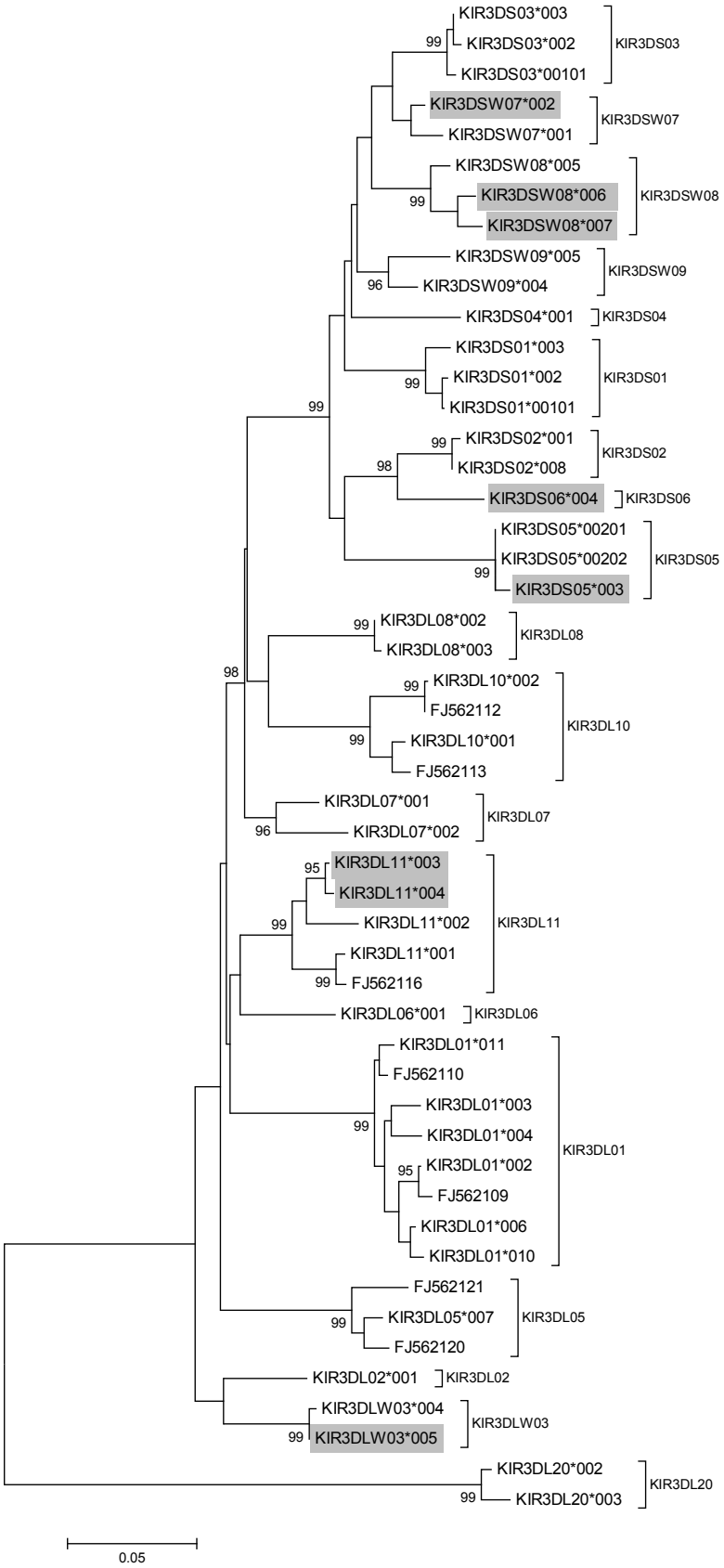

Figure 14: Phylogenetic tree of KIR amino acid sequences. The phylogenetic relationships among sequences were calculated using the Neighbor-Joining algorithm as implemented in MEGA 3.1 with 10,000 bootstrap replications. Newly described KIR sequences are highlighted in grey. Numbers on branches indicate bootstrap support values. Values above 95 % exclusively are shown, which indicates statistically significant branching.

3.2 *KIR genotyping*

In order to determine *KIR* genotypes and haplotypes, a *KIR* genotyping was established for the rhesus macaque. Based on multiple alignments of the newly detected full-length *KIR* sequences as well as sequences available in public databases, sequence-specific substitutions were identified and used to establish sequence-specific primers (7.2). Some primer pairs allow the detection of alleles, e.g. primer pairs 9 and 10. However, due to a high degree of sequence homology, it was not possible to design sequence-specific primers for all sequences. Therefore, group-specific primers were designed for some sequences, e.g. primer pairs 6 and 12. Altogether, a set of 31 primer pairs was established (Table 2). Negative results were evaluated as an absence of the analysed gene. *β-actin* primers were used as internal positive control to exclude false negative results and were added to the same PCR reactions (Table 1). A negative result of the *KIR* typing PCR was accepted only if the PCR was positive for β-actin.

3.2.1 Determination of *KIR* genotypes

The 31 *KIR* genotyping primers were used to type a panel of four families with a total number of 70 animals. In these families, 25 *KIR* genotypes were identified (Table 4). The gene number of the genotypes varied from 10 to 16 *KIR* genes per genotype. Four *KIR* sequences (*2DL4*, *3DL11*, *3DL20*, and *3DSW08*) were found in all identified *KIR* genotypes, whereas *3DL06*, *3DL07*, *3DS06*, and *3DSW07* were absent in all analysed animals.

The primer sets designed for this study were used only for analysis of presence/absence polymorphisms of *KIR* genes. However, these sets can also be exploited for transcription studies of individual *KIR* genes because all primer pairs were located in exons, with the exception of primer pairs 2 and 11 that are specific for *KIR3DL01* and *KIR3DL08*, respectively, and are encompassing exon-intron boundaries.

Table 4: *KIR* genotypes

Genotype No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
KIR2DL04 *001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KIR3DL01 *002, FJ562109	+	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+	+	+	+	+		+
KIR3DL01 *003, *004, *006, *008N, *009N, *010									+		+		+												
KIR3DL01 *011, FJ562110	+	+	+	+	+	+	+	+		+	+			+	+	+		+	+	+	+	+			
KIR3DL02 *001				+	+	+	+	+						+	+		+				+			+	+
KIR3DLW03 *004, *005																	+		+	+	+	+	+	+	+
KIR3DL05 *007, FJ562120, FJ562121	+	+					+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KIR3DL06 *001																									
KIR3DL07 *001																									
KIR3DL07 *002																									
KIR3DL08 *001	+	+			+	+	+	+						+											
KIR3DL08 *002, *003									+		+		+		+										
KIR3DL10 *001, FJ562113	+	+	+	+		+	+	+	+	+	+	+	+	+			+	+	+	+	+	+	+	+	+
KIR3DL10 *002, FJ562112	+	+	+	+		+	+	+		+		+	+	+			+	+	+	+	+	+	+	+	+
KIR3DL11 *001, FJ562116									+			+				+									
KIR3DL11 *002	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KIR3DL20 *002, *003																									
KIR3DL20 AF334646, AF334647	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KIR3DS01 *00101, *002, *003					+	+	+	+						+											
KIR3DS02 *001, *008	+	+	+	+		+	+	+						+			+	+	+	+	+		+	+	+
KIR3DS03 *00101, *002, *003									+		+		+				+	+	+	+			+		
KIR3DS04 *001		+	+	+		+								+											
KIR3DS05 *00201, *00202, *003	+	+	+	+		+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+		+
KIR3DS06 *004																									
KIR3DSW07 *001																									
KIR3DSW07 *002																									
KIR3DSW08 *005									+		+		+				+	+	+	+	+	+	+	+	+
KIR3DSW08 *006	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KIR3DSW08 *007	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+									
KIR3DSW09 *004																		+		+	+	+			
KIR3DSW09 *005																									

3.2.2 Determination of *KIR* haplotypes

Following the segregation of *KIR* sequences in the offspring of the four analysed families, it was possible to determine several haplotypes (Figure 15 and 7.3). Altogether 21 haplotypes were identified (Table 5), which vary in the number of *KIR* genes between 5 and 11. Only two haplotypes (haplotype 15 and 16) were found in more than one family (7.3), which confirms the considerable diversity of rhesus macaque *KIR* genes reported by others (Hershberger et al., 2001; Sambrook et al., 2005; Blokhuis et al., 2009a). Nevertheless, it was possible to identify some common features. Four *KIR* genes (*2DL4*, *3DL11*, *3DL20* and *3DSW08*) were found in all analysed genotypes (Table 4). Segregation of these genes in offspring could not be observed. Consequently, it is concluded that these genes are present in all haplotypes. Three of these genes are three domain-*KIRs*, further emphasizing the diverse nature of lineage II *KIR* genes in rhesus macaques.

As an additional tool for the determination of *KIR* haplotypes, microsatellite analyses were used. Therefore, the completely sequenced haplotype (Sambrook et al., 2005) was scanned for microsatellites. Nine microsatellites were identified on this haplotype and were analysed for their polymorphisms (Table 1). Two out of the nine microsatellites turned out to be polymorphic in the analysed animals and were used to confirm the determination of *KIR* haplotypes.

Microsatellite I is located 6 kb 5' of *KIR2DL4*, while microsatellite II maps outside of the *KIR* region approximately 30 kb 3' of the neighborius *FCAR* gene and is located in the *NALP7* gene (Figure 16). Both of these microsatellites show a remarkable degree of polymorphism, microsatellite II in particular (Table 5).

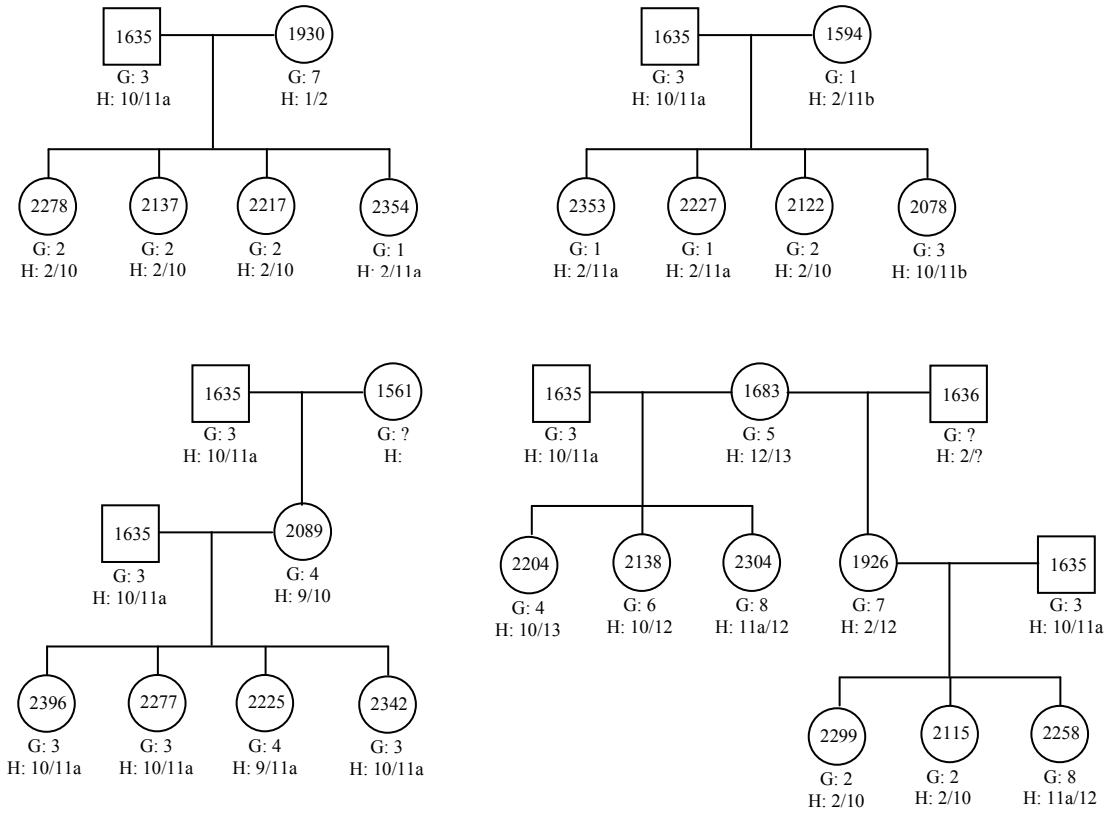

Figure 15: Pedigree of one of the analysed rhesus macaque families. Animal identification numbers are indicated. G and H denote *KIR* genotype and *KIR* haplotype, respectively. DNA of rhesus macaques 1561 and 1636 is not available and their *KIR* haplotypes were partially inferred from offspring.

With these two microsatellites, it was not only possible to confirm the KIR haplotypes, but to make a finer distinction. Subsequently, it turns out that the haplotypes (15 and 16), which are present in more than one family, as well as haplotype 11, have to be divided in subgroups. Although these haplotypes contain identical number and types of *KIR* genes, they differ in their microsatellites. Haplotype 11 and 16 were divided in two subgroups; haplotype 15 was divided in three subgroups. Hence, haplotypes 15 and 16 appeared in two families, but every subgroup was present only in one family.

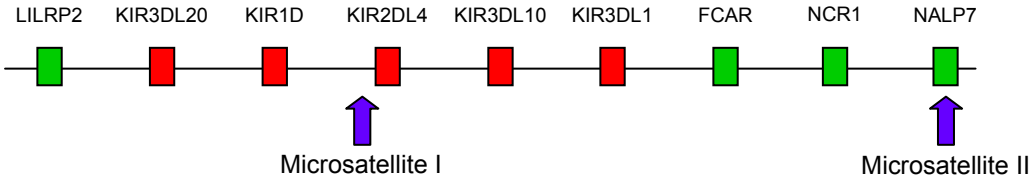

Figure 16: Scheme of the sequenced rhesus macaque *KIR* haplotype with the position of the analysed microsatellites.

Table 5: *KIR* haplotypes

Haplotype No.	KIR2DL04	KIR3DL01		KIR3DL02	KIR3DLW03	KIR3DL05	KIR3DL06	KIR3DL07		KIR3DL08		KIR3DL10		KIR3DL11		KIR3DL20		KIR3DS01	KIR3DS02	KIR3DS03	KIR3DS04	KIR3DS05	KIR3DS06	KIR3DSW07		KIR3DSW08	KIR3DSW09		Microsatellite I	Microsatellite II
	*001	*002, FJ562109	*003, *004, *006, *008N, *009N, *010	*011, FJ562110	*001	*004, *005	*001	*001	*002	*001	*002, *003	*001, FJ562113	*002, FJ562112	*001, FJ562116	*002	*002, *003	AF334646, AF334647	*00101, *002, *003	*001, *008	*00101, *002, *003	*001	*00201, *00202, *003	*004	*001	*002	*006	*007	*004	*005		
1	+	?		?	+					?		?	?	+		+		+	?			?				+	?			128	174
2	+	+		?		?				+		+	+	+		+			+			+				+	?			128	174
3	+	+		+		?						?		+		+			+			+				+	?			134	162
4	+				+	?				+		?				+										+	?			128	208
5	+	+				?						+				+						+				+	?			128	192
6	+			+	+	?				+		?				+		+	+		+					+	?			134	172
7	+	+	+			?					+	?				+				+			+				+	?			130	168
8	+					?						?		+		+										+	?			130	170
9	+	+		?	+							?	?	+		+		+	?		+	?				+	?			128	194
10	+			?								+	+	+		+		+	+			+				+	?			134	162
11a	+	+		?								+	+	+		+			+			+				+	?			134	172
11b	+	+		?								?	?	+		+			?			?				+	?			128	162
12	+	+		?	+					+				+		+		+								+	?			122	174
13	+	+		?	+									+		+										+	?			128	194
14	+	+		+	?							?	?	+		+						?				+	?			128	168
15a	+	?			+	?						?	?	+		+			+	+		?				+	?			128	172
15b	+	+			?	+						+	?	+		+			+	+		?				+	?			128	212
15c	+	+			?	+						+	?	+		+			+	+		?				+	?			128	194
16a	+	?			+	?						?	?	+		+			?	+		?				+	?			130	212
16b	+	+			?	+						+	?	+		+			?	+		?				+	?			128	210
17	+	?		+		?						?	?	+		+			+	+		?				+	?			128	170
18	+	?		+		?						?	?	+		+			+			?				+	?	+			128	192
19	+	?		+	+	?						?	?	+		+						?				+	?	+			128	168
20	+				?									+		+			?							+	?				128	210
21	+				?	+						+	?	+		+			?							+	?				128	210

3.3 *Specific interaction between KIR and MHC class I molecules*

HLA-C molecules are the main ligands for KIRs in humans. Considering that rhesus macaques are missing *MHC-C* type of class I genes, the first aim was to analyse whether Mamu-A or -B molecules represent the main ligands in rhesus macaques. There are four *Mamu-A* genes in rhesus macaques with Indian origin (Otting et al., 2005). At least one representative of all four genes was included in this analysis. The Mamu-A1 gene is the most polymorphic of the Mamu-A genes. Accordingly, three alleles of this gene, namely *A1*00101*, *A1*00801*, and *A1*01101*, have been analysed. *A1*00101* represents an important allele of *Mamu-A1* because it is known to be associated with long-term survival and low viral load upon experimental SIV infection (Miller et al., 1991; Mühl et al., 2002; Zhang et al., 2002; O'Connor et al., 2003). The *Mamu-A2*, *-A3,* and *-A4* genes were represented by *Mamu-A2*050402*, *Mamu-A3*1311*, and *Mamu-A4*1403*, respectively. These genes are less polymorphic than the Mamu-A1 gene and are characterised by low level of mRNA transcription. From the *Mamu-B* region, nine genes were analysed, namely *B*01202*, *B*01704*, *B*02101*, *B*02804*, *B*03002*, *B*04801*, *B*06002*, *I*010201,* and *I*0121*. The so-called *Mamu-I* genes were previously designated *Mamu-B*09* (Urvater et al., 2000) and are members of the *Mamu-B* gene family. Expression constructs of AcGFP-tagged Mamu-A and -B/-I were transiently transfected in K562 cells. Both the constructs and transfected K562 cells were kindly obtained from Dr. Cornelia Rosner. Their expression patterns have already been described (Rosner et al., 2010).

For the establishment of soluble recombinant KIR proteins, constructs encoding the Ig domains of rhesus macaque *KIR* genes were fused to the Fc fragment of human IgG1 (KIR-Fc). Primers were designed (Table 1) that allowed amplification of exons spanning the Ig domains of five inhibitory KIRs with three-Ig domain structure, namely KIR3DLW03*004, KIR3DL05*007, KIR3DL11*002, KIR3DL11*003, and KIR3DL11*004. The obtained products were cloned into the PCR cloning vector pGem-TEasy; their inserts were subsequently isolated by EcoRI restriction and ligated into the dephosphorylated pFUSE-hIgG1-Fc2 vector. To obtain stably transfected cells, the vectors were linearized with NotI and stably transfected in HEK-293 cells. The KIR-Fc fusion proteins were harvested from the supernatant of stably transfected cells purified by affinity chromatography using protein G columns. The fusion proteins were

then multimerized to increase the threshold of detection of specific interactions. Binding of KIR-Fc multimers to Mamu-A, Mamu-B, and Mamu-I-transfected K562 cells was examined by flow cytometry. In parallel experiments, the expression of Mamu-A, and -B/-I was checked by flow cytometry as well. As a control experiment, mock transfected K562 cells were used and the supernatant of mock transfected HEK-293 cells was added to MHC class I-transfected K562 cells.

In order to compare the different measurements, data were calculated as relative binding intensity in the following manner: the mean fluorescence intensity (MFI) of transfected MHC molecule with purified KIR protein / MFI of mock transfected cells with purified KIR protein (Figure 17). The relative cell surface expression of the MHC class I molecules was calculated in the following manner: the MFI of transfected MHC molecule with W6/32 antibody / MFI of mock transfected cells with W6/32 antibody (Figure 18). At least three independent experiments were performed. Statistically significant differences were calculated using Student's *t* test and results with p values below 0.05 were considered statistically significant (Figure 17).

Significant interactions were found for Mamu-A1*00101, Mamu-A1*00801, Mamu-A1*01101, and Mamu-A3*1311 with three inhibitory KIR3D molecules (Figure 17A). The strongest interaction was detected between Mamu-A3*1311 and KIR3DL05*007 ($p<0.05$). The same KIR also binds Mamu-A1*00101 ($p<0.01$), but to a lower extent. KIR3DLW03*004 specifically binds to Mamu-A1 alleles A1*00101 ($p<0.005$), A1*00801 ($p<0.05$) and A1*01101 ($p<0.0005$). Significant binding was also found between KIR3DL11*003 and Mamu-A1*00801 ($p<0.01$). No significant interactions between any KIR and Mamu-B or -I molecules were found (Figure 17B).

MHC class I expression might have an effect on the binding intensity of the KIR-Fc fusion proteins. Therefore, cell surface expression was analysed parallel to the binding intensity. The intensities of cell surface expression of the MHC class I molecules showed different values between the different Mamu-A and -B molecules. Most of the Mamu-A molecules show a high cell surface expression, whereas some Mamu-B molecules show a very low cell surface expression (Figure 18). This was also demonstrated in other studies (Rosner et al., 2010) where the cell surface expression of the MHC class I molecules was, in addition to W6/32, checked with an antibody against β2 microglobulin and showed a similar pattern.

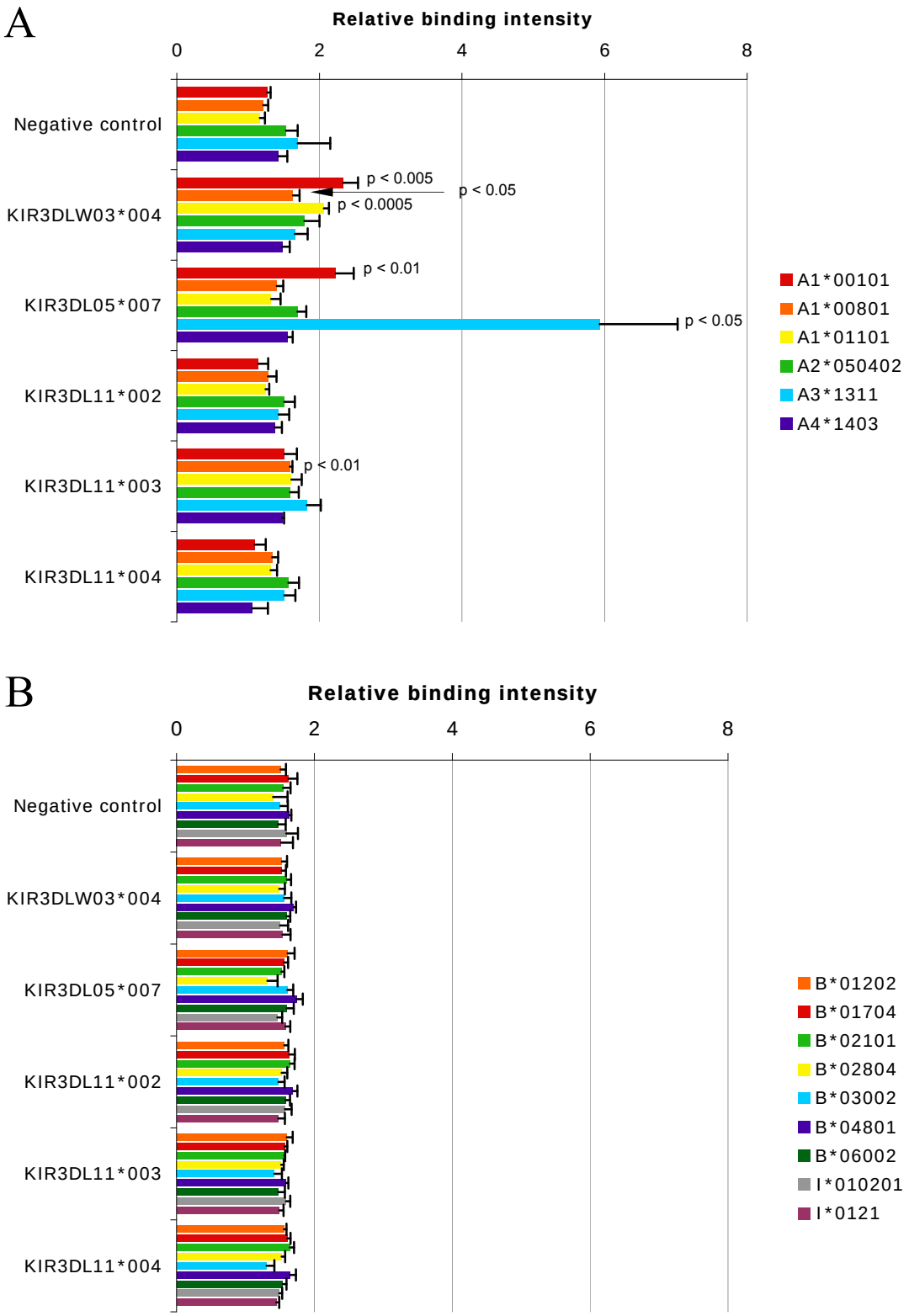

Figure 17: Interaction between KIR and Mamu-A, -B and -I molecules. Relative binding intensity between Mamu-A (A), and Mamu-B and -I molecules (B) is shown. Relative binding intensity is calculated from the mean value of at least three experiments: MFI of transfected MHC molecule with purified KIR protein / MFI of mock transfected cells with purified KIR protein. p values <0.05 are indicated.

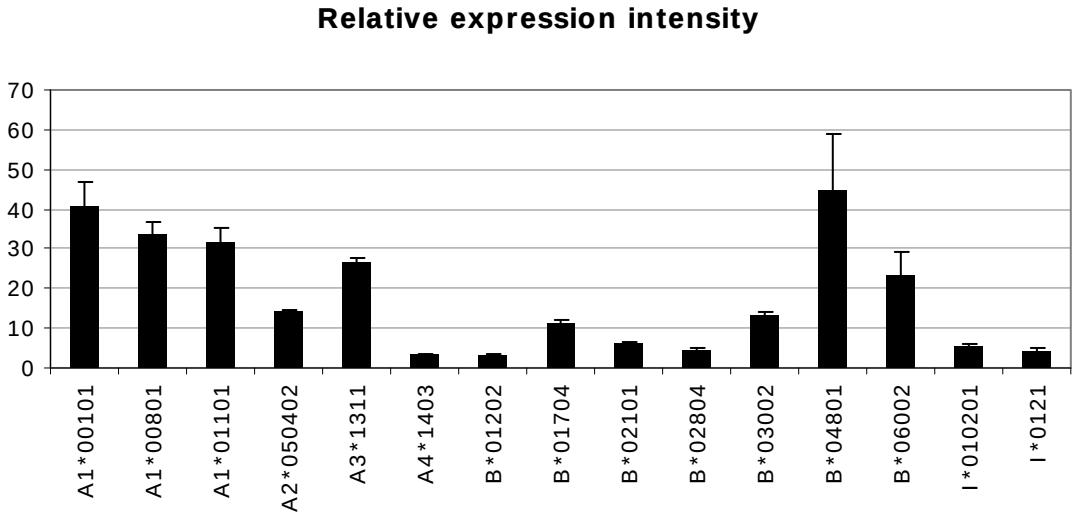

Figure 18: Relative cell surface expression of Mamu-A, -B and -I molecules. Relative cell surface expression is calculated of the mean value of at least three experiments: MFI of transfected MHC molecules with W6/32 / MFI of mock transfected cells with W6/32.

3.3.1 Mapping of specific binding sites of MHC class I molecules

To analyse the interaction site of Mamu-A molecules with KIR receptors, chimeric constructs and mutated MHC class I molecules were designed. Initially, the identity of the domains of the Mamu-A molecule that are responsible for KIR binding were studied. Therefore, two pairs of chimeric constructs were established of both the "high-binder" Mamu-A1*00101 and "low-" or "non-binder" Mamu-A1*00801 (Figure 19) as well as another one of both the "binder" Mamu-A3*1311 and "non-binder" Mamu-A2*050402 (kindly provided by Dr. C. Rosner). In these constructs, the α domains of the Mamu-A molecules were swapped (either the α1 or α2 domain or both domains).

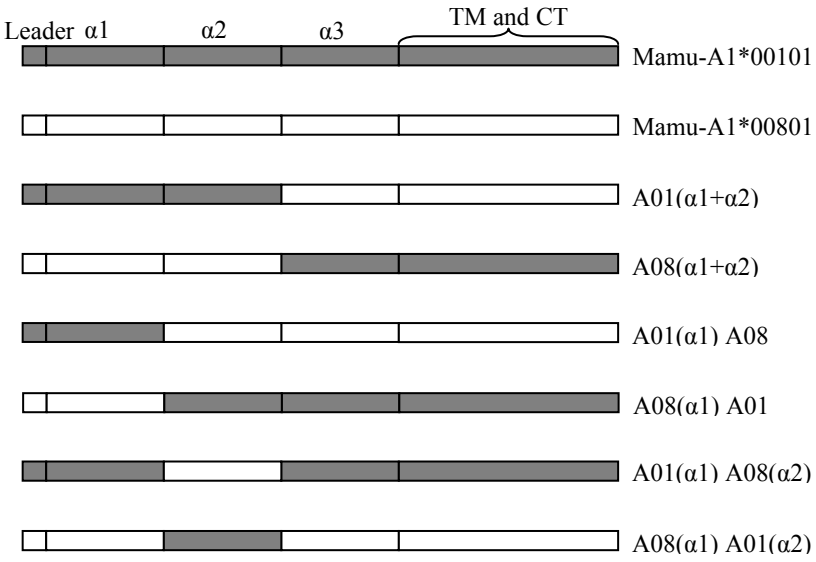

Figure 19: Scheme of chimeric constructs of Mamu-A1*00101 and Mamu-A1*00801. The α1, α2, and α3 domains as well as transmembrane and cytoplasmic regions (TM and CT) are indicated.

Whenever the α1 domain of the "high-binder" Mamu-A1*00101 was present, an interaction with KIR3DLW03 was detected (Figure 20A). Therefore, the binding between KIR3DLW03 and Mamu-A1*00101 is mainly influenced by the α1 domain. The α2 domain plays a subordinated role in binding (if at all). On the other hand, binding between KIR3DL05 and chimeric constructs was only detected in cases where both the α1 and α2 domains of the "binder" Mamu-A1*00101 were present (Figure 20A). The same result occurred with the chimeric constructs of "binder" Mamu-A3*1311 and "non-binder" Mamu-A2*050402 (Figure 20B) Therefore, the binding of KIR3DL05 with its ligands is influenced by the α1 as well as α2 domain. This is in contrast to the results of binding between KIR3DLW03 and Mamu-A1*00101.

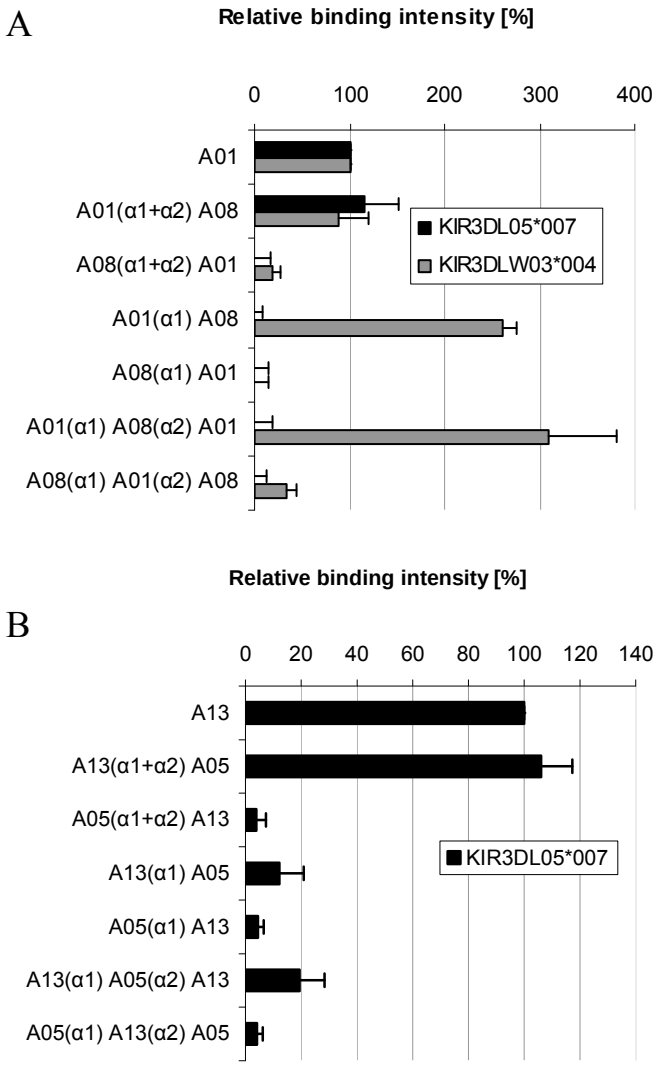

Figure 20: Interaction between KIR and Mamu-A chimeric constructs. (A) Relative binding intensity of KIR3DL05*007 as well as KIR3DLW03*004 and Mamu-A1*00101/Mamu-A1*00801 chimeric constructs are shown. (B) Relative binding intensity of KIR3DL05*007 and Mamu-A3*01311/Mamu-A2*050402 chimeric constructs is shown. The relative binding intensity of the wildtype of the Mamu-A molecule is set to 100 %. Mean value of relative cell surface expression of three independent experiments is displayed in percentage of relative binding of the wildtype.

In humans, the HLA-A and -B molecules can be distinguished on the basis of whether they possess a Bw4 or a Bw6 epitope. This serological epitope is located in the α1 domain (positions 77, 80-83). It is known that the binding of KIR3DL1 depends on the presence of the Bw4 epitope (Gumperz et al., 1997; Sanjanwala et al., 2008). Analyses of the Mamu-A and -B molecules in the rhesus macaque show that, as in humans, most molecules can be divided into $Bw4^+$ and $Bw6^+$ molecules (Table 6). This finding suggests that binding between KIR and Mamu-A molecules in the rhesus macaque is possibly also influenced by the Bw.

Table 6: Bw4 and Bw6 epitopes of rhesus macaque MHC class I molecules

Molecule	Sequence at position 70, 80-83	Epitope	Binding of KIR3DLW03*004	Binding of KIR3DL05*007	Binding of KIR3DL11*003
A1*00101	NTLLR	Bw4	**yes**	**yes**	no
A1*00801	GNLRG	Bw6	**yes**	no	**yes**
A1*01101	NTALR	Bw4	**yes**	no	no
A2*050402	NTLLR	Bw4	no	no	no
A3*1311	ANLRG	Bw6	no	**yes**	no
A4*1403	ANLLR	mixed	no	no	no
B*01201	SNLRR	Bw6	no	no	no
B*01704	NTALR	Bw4	no	no	no
B*02101	NTLLR	Bw4	no	no	no
B*02804	DTLRG	Bw6	no	no	no
B*04801	GILRG	Bw6	no	no	no
B*06002	GNLRG	Bw6	no	no	no
I*010201	NTALR	Bw4	no	no	no
I*0121	NTALR	Bw4	no	no	no

Analyses of the Bw epitope of the Mamu-A ligands (A1*00101, A1*00801, A1*01101, and A3*1311) that interact with KIR molecules and the non-binding Mamu-A and Mamu-B molecules show that the binding of the KIR molecules is not limited by the presence of a specific Bw epitope. On the one hand, KIR3DLW03 shows weak binding with Mamu-A1*00801 (which possesses a Bw6 epitope). On the other hand, KIR3DLW03 shows strong binding with two $Bw4^+$ molecules (Mamu-A1*00101 and -A1*01101), but no binding with other molecules that share the same epitope (e.g. Mamu-A2*050402, -B*06002, and -B*01704, which are identical to Mamu-A1*00101, -A1*00801, and -A1*01101, respectively) (Table 6 and figure 17). KIR3DL05 also binds to molecules which possess either the Bw4 or the Bw6 epitope, whereas KIR3DL11*003 bind only to a $Bw6^+$ molecule. Nevertheless, due to the restricted binding of KIR3DL1 with $Bw4^+$ molecules in humans the influence of the Bw epitope in rhesus macaques was analysed. The interaction of Mamu-A1*00101 and -A3*1311 molecules with a high binding affinity to the binding KIR molecules were analysed with mutated Bw epitopes (construct kindly provided by Dr. C. Rosner).

Besides mutants in which the whole Bw epitope was replaced (A1(^{77}NTLLR^{83}>^{77}ANLRG^{83}), A3(^{77}ANLRG^{83}>^{77}NTLLR^{83})), mutants which show

only one amino acid exchange at position 77 (A1(N77A), A3(A77N)) or position 83 (A1(R83G), A3(G83R)) as well as mutants with several amino acid exchanges (A1(^{80}TLLR^{83}>^{80}NLRG^{83}), A3(^{80}NLRG^{83}>^{80}TLLR^{83})) were established (Table 7).

Table 7: Mutations of the Bw epitopes of Mamu-A1*00101 and -A3*1311

Molecule	Sequence at position 70, 80-83	Epitope	Binding of KIR3DLW03*004	Binding of KIR3DL05*007
A1*00101	NTLLR	Bw4	yes	yes
A3*1311	ANLRG	Bw6	no	yes
A1(^{77}ANLRG^{83})	ANLRG	Bw6	lost[a]	slightly reduced[a]
A3(^{77}NTLLR^{83})	NTLLR	Bw4	gained[a]	enhanced[a]
A1(^{80}NLRG^{83})	NNLRG	mixed	lost[a]	enhanced[a]
A3(^{80}TLLR^{83})	ATLLR	mixed	gained[a]	no change[a]
A1(^{77}A)	ATLLR	mixed	reduced[a]	lost[a]
A3(^{77}N)	NNLRG	mixed	enhanced[a]	enhanced[a]
A1(^{83}G)	NTLLG	mixed	lost[a]	reduced[a]
A3(^{83}R)	ANLRR	mixed	enhanced[a]	enhanced[a]

[a]reduced, lost, enhanced, gained and no change refer to parental binding to Mamu-A1*00101 or Mama-A3*1311

Similar to the analyses with the chimeric constructs, KIR3DLW03 and KIR3DL05 show differences in binding pattern with the constructs of the mutated Bw epitope. KIR3DLW03 shows a complete loss of interaction with Mamu-A1*00101 molecules in which the Bw4 epitope was replaced by Bw6 (Figure 21A and table 7). KIR3DLW03 also shows a gain of interaction with Mamu-A3*1311 molecules in which the Bw6 epitope was replaced by Bw4 (Figure 21B and table 7). Mutations of only parts of the Bw4 or Bw6 epitopes show either a reduction or a gain of interaction but only with a reduced impact (Figure 21A and B).

On the other hand, binding between KIR3DL05 and Mamu-A1*00101 and -A3*1311 did not show such clear results. There was almost no influence on the interaction when the Bw4 epitope was replaced by the Bw6 epitope (Figure 21C and table 7) but a gain of interaction when the Bw6 epitope was replaced by the Bw4 epitope (Figure 21D and table 7). Mutations at amino acid position 80-83 showed that the amino acid sequence asparagine, leucine, arginine and glycine (NLRG) at these positions confer a stronger

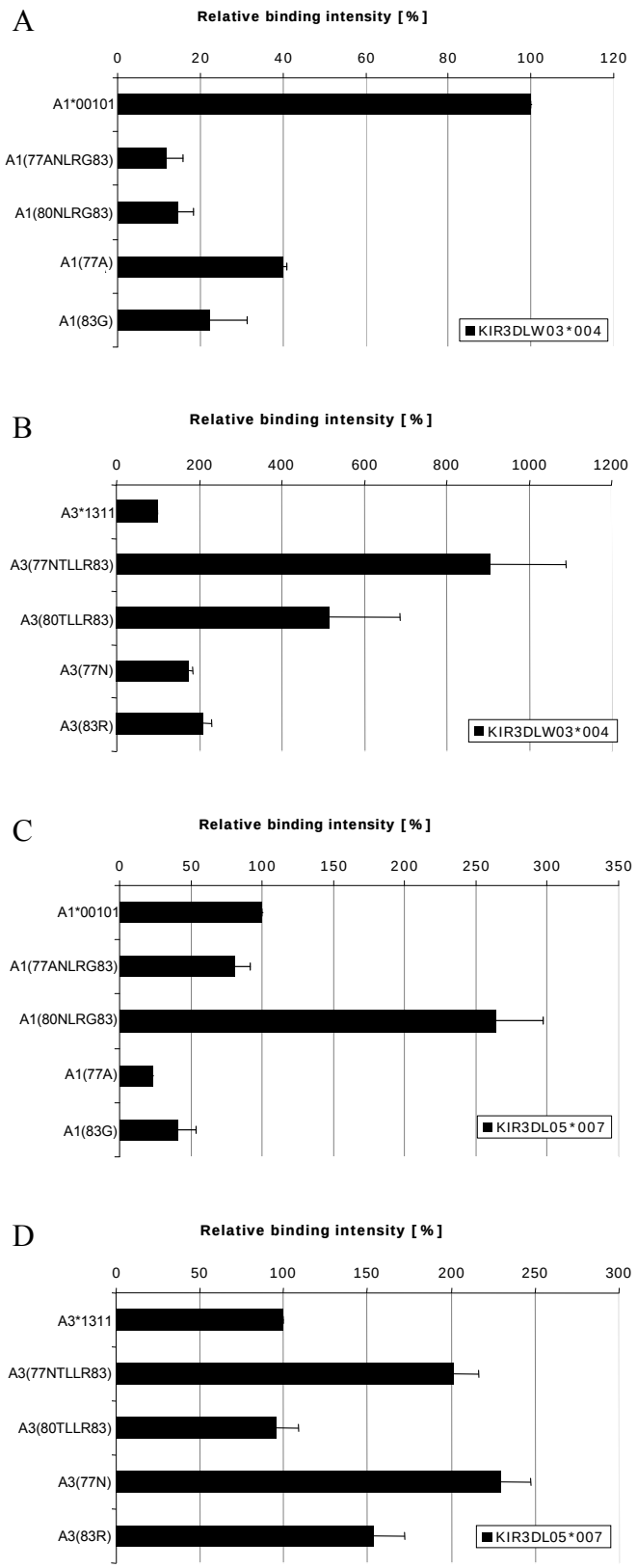

Figure 21: Binding between KIR and Mamu-A molecules with mutated Bw epitopes. AA position 77, 80, 81, 82, and 83 are part of the Bw epitope, which is important for KIR interaction in humans. Relative binding intensity of KIR3DLW03*004 with Mamu-A1*00101 (A) and -A3*01311 (B) with mutated Bw epitope as well as KIR3DL05*007 with Mamu-A1*00101 (C) and -A3*01311 (D) is shown. Relative binding intensity of the wildtype of the Mamu-A molecule is set to 100 %. Mean value of relative binding intensity of three independent experiments is displayed in percentage of relative binding of the wildtype.

interaction as compared with threonine, leucine, leucine and arginine (TLLR). On the other hand, single mutations of the amino acid position suggest that an arginine at position 83 increases the interaction with KIR3DL05 as well as an asparagine at amino position 77 (Figure 21C and D). Hence, a mix between the Bw4 and Bw6 epitope with asparagines at position 77 and asparagine, leucine, arginine and arginine (NLRR) at position 80-83 seems to raise the binding affinity for KIR3DL05. These results show that in contrast to humans, the Bw epitope is important for the binding between KIR and Mamu-A molecules in rhesus macaques, but does not limit it.

3.3.2 Mapping of specific binding sites of KIR3DL05*007

To determine the specific binding sites of KIR3DL05*007, mutants of this KIR were established. Specifically, an amino acid residue at position 230 that has been shown to be important for the binding between KIR and MHC molecules in humans was mutated (Khakoo et al., 2002). On the other hand, the amino acid position 44 of human KIR2DL2/3 molecules interacts with the amino acid at position 80 of the HLA-C (Winter and Long, 1997; Boyington et al., 2001). The corresponding amino acid position in rhesus macaque KIR3DL molecules is amino acid position 144. Interestingly, analyses of the amino acid sequences of the rhesus macaque KIR molecule have shown that almost all molecules possess a glutamate at this position, with the exception of KIR3DL05*007 (which has a lysine at this position). Therefore, this amino acid was a good mutation candidate for determining whether it is important for the binding between KIR3DL05*007 and Mamu-A1*00101 as well as Mamu-A3*1311. In addition to these two amino acids the amino acid residue at position 204 was choosen to mutate because this residue seperates KIR3DL05*007 from all other analysed KIR molecules. The amino acid residues at positions 203 and 233 were chosen as controls because these positions are known to be important for the formation of molecular structure, but not for specific binding in humans (Khakoo et al., 2002). Mutations at these positions should therefore prevent any interaction of the mutants with Mamu-A molecules, as the KIR molecule is unable to form its normal structure and thus becomes unreactive.

As shown in figure 22, mutations at amino acid positions 203 (Y203A) and 233 (D233H), which are important for the formation of the structure of KIR molecules, lead to a complete loss of interaction between the KIR and both Mamu-A molecules. In addition, mutations at the amino acid positions 204 (K204E) and 230 (R230C) also led to a complete reduction of the interaction between KIR and Mamu-A molecules.

Although a slight interaction can be seen between Mamu-A3*1311 and KIR3DL05*007 (K204E), as well as (R230C), these interactions were below 20 % of the binding capacity of the wildtype and can hence be neglected. Similar to humans, these results indicate that amino acid 230 is important for the binding between KIR and MHC-A molecules. Additional to this, the amino acid at position 204 seems to play an important role in the binding in rhesus macaques, too. A difference can be seen with respect to the mutants of amino acid at position 144: while the mutation at amino acid position 144 seems to have no influence on the binding between KIR3DL05*007 and Mamu-A01*00101, it reduces the interaction between KIR3DL05*007 and Mamu-A3*1311 to 60 % as compared with the wildtype. This provides evidence for the difference in binding between KIR3DL05*007 and Mamu-A1*00101 on the one hand, and KIR3DL05*007 and Mamu-A3*1311 on the other hand.

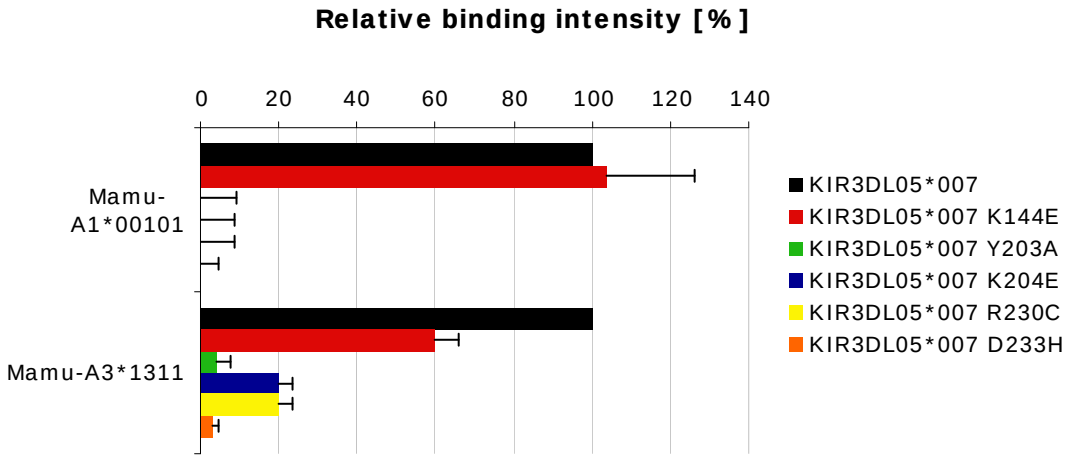

Figure 22: **Binding between KIR3DL03*007 mutants and Mamu-A1*00101 as well as Mamu-A3*1311.** AA positions 203 and 233 are important for the formation of the KIR molecule structure and are used as negative control. AA position 230 is shown to be important for the binding between KIR and MHC molecules in humans, whereas aa position 144 and 204 separate KIR3DL03*007 from all other KIR molecules analysed. The relative binding intensity of the KIR3DL05*007 wildtype is set to 100 %. The mean value of relative cell surface expression of three independent experiments is displayed in percentage of relative binding of the wildtype.

4 Discussion

Rhesus macaques serve as an important non-human primate model of human infectious and autoimmune diseases as well as for transplantation studies. Combinations of *KIR* and *MHC class I* genotypes are known to influence some human diseases belonging to the abovementioned categories. However, the knowledge of rhesus macaque *KIR* genotypes was lacking and methods to determine them were also not available. Furthermore, no information about the specific interactions between KIR and MHC class I molecules were available up to this point. This study reports on the establishment of a PCR-based sequence-specific *KIR* genotyping in the rhesus macaque and its usage for determination of *KIR* haplotypes in family studies. Furthermore, this study demonstrated specific interaction between inhibitory KIR molecules with Mamu-A molecules and established the first fine mapping of the interaction sites of KIR and Mamu-A molecules which has not been described before in the rhesus macaque.

4.1 *Diversity of KIR genes*

In humans, the diversity of *KIR2D* genes is higher than the diversity of *KIR3D* genes. In contrast to this, previous studies of the *KIR* genes in the rhesus macaque have shown that the diversity of *KIR2D* genes is very low (Grendell et al., 2001; Hershberger et al., 2001). *KIR2DL4* is the only known *KIR2D* gene in the rhesus macaque. One reason for this low diversity of *KIR2D* genes might be that in humans, KIR2D molecules (except KIR2DL4) exclusively interact with HLA-C molecules (Yawata et al., 2006; Moesta et al., 2008). KIR2DL4, however, interacts with HLA-G molecules (Rajagopalan and Long, 1999; Rajagopalan et al., 2006). The rhesus macaque lacks *MHC-C* but possesses *MHC-G-like* genes (Boyson et al., 1996; Parham, 2005b). Therefore, since the possible interaction partner for KIR2D molecules (with the exception of KIR2DL4) is absent in rhesus macaques, the diversity of the *KIR2D* genes is accordingly low.

It has been shown that the diversity of *KIR3D* genes is very high in rhesus macaques (Grendell et al., 2001; Hershberger et al., 2001; Sambrook et al., 2005). The identification by Blokhuis and colleagues (2009a) of a novel group of activating KIR molecules that differ (in the stem and transmembrane region, as well as the cytoplasmic tail) from the already-known activating receptors further underscores the diversity of rhesus macaque *KIR3D* genes. This high diversity might also be associated with the

ligands for the KIR3D molecules. In humans, the ligands for KIR3D molecules are HLA-A and -B molecules (Litwin et al., 1994; Vitale et al., 1996). Therefore, Mamu-A and -B molecules might be the interaction partners for KIR molecules in the rhesus macaque. The diversity of *Mamu-A* and *-B* genes is quite considerable. There are four known *Mamu-A* loci and up to seventeen *Mamu-B* loci (Daza-Vamenta et al., 2004; Otting et al., 2007; Bonhomme et al., 2008). Consequently, it is likely that the diversity of their interaction partners shows a similar diversity. The evolution of *KIR* and *MHC class I* genes is further discussed in chapter 4.4.

In this study, eight *KIR* sequences were isolated. All newly-detected sequences represent previously unknown *KIR* sequences that code for KIR3D molecules. Therefore, this study underscores the previously observed diversity of *KIR3D* genes in the rhesus macaque. This indicates that the *KIR* genes of rhesus macaques have evolved as rapidly as their human and great apes counterparts (Hershberger et al., 2001; Sambrook et al., 2005).

4.2 *Genotyping*

The genotyping resulted in identification of 25 *KIR* genotypes (Table 4) among 70 rhesus macaques from four families, which emphasizes the considerable diversity of *KIR* genes in rhesus macaques. Based on family studies, 21 *KIR* haplotypes could be identified (Table 5), again highlighting the *KIR* diversity in these non-human primates.

The gene number per haplotype varied between 5 and 11 in the analysed families. Differential *KIR* gene contents and duplication of *KIR2DL4* were previously observed in rhesus macaques (Sambrook et al., 2005; Blokhuis et al., 2009a) and duplications were also found in cynomolgus macaques (Bimber et al., 2008). This study extends these findings on differential gene content and suggests that inhibitory and activating *KIR3D* genes might be duplicated in some haplotypes. In particular, recent duplications can result in complicated genetic situations, and it is difficult to define whether the same *KIR* sequence is derived either from an allele or from a distinct gene. In humans several studies have shown that due to unequal crossing over or non-allelic recombinations, haplotypes with duplicated *KIR* genes have emerged (Figure 23) (Martin et al., 2003; Williams et al., 2003; Norman et al., 2009). This makes sequence-specific genotypings technically demanding. Future studies involving complete sequencing of rhesus macaque *KIR* haplotypes will significantly contribute to the identification of

recombinant *KIR* genes and recent duplications as well as *KIR* gene fusions that can result from deletions (Abi-Rached et al., 2010; Traherne et al., 2010).

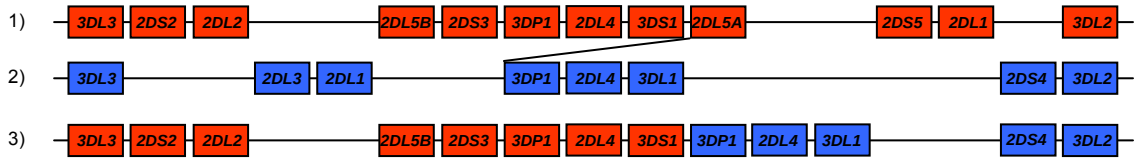

Figure 23: Schematic representation of duplication of KIR genes in humans. The names of KIR loci are indicated in the boxes. The donor haplotypes 1 and 2 are indicated in red and blue, respectively. The compound duplication haplotype 3 is coloured accordingly. The line indicates the non-allelic homologous recombination (Figure modified from Norman et al., 2009).

Four identified *KIR* genes (*2DL4, 3DL11, 3DL20,* and *3DSW08*) were present in all genotypes of the four analysed rhesus macaque families. Therefore, these genes represent framework genes as is common in humans (*KIR2DL4*, *KIR3DL2*, *KIR3DL3,* and *KIR3DP1)* (Hsu et al., 2002a; Hao and Nei, 2005). Nevertheless, it cannot be excluded that there are rare haplotypes that lack these genes similar to what was found for human *KIR* haplotypes. In humans, for example, the framework genes *KIR3DP1*, *KIR2DL4* and *KIR3DL1/S1* may be deleted in a haplotype (Traherne et al., 2010). These rare haplotypes can only be detected by measuring the gene dosage with a real-time PCR or by sequencing complete haplotypes. By genotyping, these rare haplotypes can only be detected in an animal that is homozygous for the haplotype.

The completely sequenced rhesus macaque *KIR* haplotype carries only five *KIR* genes: *3DL20*, *1D*, *2DL4*, *3DL10*, and *3DL01* (Sambrook et al., 2005). Interestingly, this haplotype does not contain typical activating *KIR* genes, which are present in all haplotypes identified in this study and by others (Blokhuis et al., 2009a). Additionally, it contains two *KIR* genes (*KIR2DL4* and *KIR3DL20*) that are identified as possible framework genes, and lacks the other two framework genes (*3DL11* and *3DSW08*). Therefore, the sequenced *KIR* haplotype might be one of the rather uncommon haplotypes mentioned above.

Human *KIR* haplotypes are assigned to either group A or group B, which differ considerably in both number and type of *KIR* genes (Uhrberg et al., 1997; Hsu et al., 2002a). Although rhesus macaque *KIR* haplotypes vary strikingly in gene content of both inhibitory and activating *KIR* genes, no clear differences could be detected in the analysed rhesus macaque cohort that would allow for a clear-cut discrimination similar to human group A and B haplotypes.

Five *KIR* haplotypes also show the presence of two sequences which are designated by the official KIR Gene Nomenclature Committee to be alleles of one gene. *KIR* haplotypes 2, 10 and 11a possess two "allelic" sequences of *KIR3DL10*; haplotype 8 contains different sequences of *KIR3DL11* whereas haplotype 7 possesses two sequences of the *KIR* genes *3DL01* and *3DSW08* (Table 5). The rhesus macaque KIR Gene Nomenclature Committee only distinguished the *KIR* sequences into genes and alleles based on their sequence similarities, without including any data on *KIR* haplotypes. Therefore, it is possible that these sequences belong to different *KIR* genes that co-segregate in the offspring. In this case, the nomenclature of genes *3DL01*, *3DL10*, *3DL11*, and *3DSW08* demands reconsideration. However, another explanation with similar probability is that these *KIR* genes have undergone duplication and represent two independent *KIR* loci. In any case, further haplotype analyses of additional rhesus macaque families are needed to shed light on this matter.

The method described here is a quick and cost-efficient way to analyse the genotypes. Therefore, it is also ideal for high-throughput screening of large rhesus macaque cohorts. Another advantage of this method is the short sizes of PCR products (between 150 and 300 bp), which means that obtaining DNA is not restricted to blood samples. Due to the short size of the PCR products, it might even be possible to genotype faecal or fur samples of free-living monkeys. On the other hand, with this method, it is not possible to detect novel alleles or genes. In addition, recombinant genes might lead to misinterpretation of the obtained genotyping data. Nevertheless, this is the first approach of a *KIR* genotyping for the rhesus macaque. However, additionally detected *KIR* sequences will make constant updates necessary.

4.3 *Specific interactions between KIR and MHC class I molecules*

Information about the specificity of interactions between KIR molecules and their MHC class I ligands have not published until now. Therefore, the specific interactions between five KIR and several Mamu-A as well as -B/-I molecules were analysed. Some of these MHC class I molecules are known to be associated with prolonged survival after experimental SIV infection such as Mamu-A1*01, Mamu-A3*13, or Mamu-B*17 (Mothe et al., 2003; Yant et al., 2006).

In this study, significant interactions could be detected for Mamu-A1*00101, Mamu-A1*00801, Mamu-A1*01101 and Mamu-A3*1311 with analysed inhibitory KIR3D molecules (Figure 17A). The interaction between KIR3DL05*007 and Mamu-A1*00101 as well as Mamu-A3*1311 is not only significant ($p<0.01$ and $p<0.05$, respectively), but also shows a high value of relative binding intensity (2.23 and 5.93, respectively), whereas the negative control shows only values between 1.16 and 1.7. KIR3DLW03*004 also shows highly significant binding with Mamu-A1*00101 and Mamu-A1*01101 ($p<0.005$ and $p<0.0005$, respectively) with relative binding intensity values of 2.34 and 2.06, respectively, which are also above the values of the negative control. Therefore, these two KIR molecules show clear interaction with the respective Mamu-A molecules.

Significant binding was also found between Mamu-A1*00801 and KIR3DLW03*004 ($p<0.05$) as well as KIR3DL11*003 ($p<0.01$), but with a very low value of relative binding intensity (1.63 and 1.59, respectively). These values are not higher than some of the values found for negative controls (which vary between 1.16 and 1.7). Therefore, the gain of interaction was so small that it is unlikely that these represent strong interactions. It is more likely that the very small standard error of the mean (SEM) of the experiments lead to a significant result, although the amount of binding is just above the negative control. Therefore, it might be possible that this represents a background signal. Nevertheless, if there is an interaction between KIR3DLW03*004 as well as KIR3DL11*003 and Mamu-A1*00801, it might be so weak that it cannot be clearly identified by this approach. Thus, these interactions have to be studied with a more sensitive method to check whether these KIR molecules interact with Mamu-A1*00801.

The results show that the binding of KIR3DL molecules in the rhesus macaque is both allele- and locus-specific. For example, KIR3DL05*007 shows strong interaction with Mamu-A1*00101 and Mamu-A3*1311, but not with Mamu-A1*00801 or Mamu-A1*01101. On the other hand, KIR3DLW03*004 shows significant binding with Mamu-A1*00101 and Mamu-A1*01101 but only low binding with Mamu-A1*00801. KIR3DL molecules show a binding pattern similar to its human counterparts; this indicates that at least some conserved features of these otherwise rapidly evolving families of receptors and ligands.

In this study, nine Mamu-B/-I molecules and only six Mamu-A molecules were analysed. However, only significant interactions between Mamu-A and KIR molecules

were detected. In previous studies, it was shown that the cell surface expression, especially of Mamu-B*01202, -B*02804, -I*010201, and -I*0121, was very low (Rosner et al., 2010). These results were also confirmed in this study, where the cell surface expression of all MHC class I molecules was measured in addition to the interactions (Figure 18). This low cell surface expression might be one reason for the lack of interaction between KIR and Mamu-B/-I molecules because specific interactions fall below the threshold of detection. Another explanation might be that the Mamu-B molecules are not suitable ligands for KIR molecules due to their high genomic plasticity. Still, it needs to be shown whether analyses with further Mamu-B and KIR molecules will lead to a detection of a specific interaction between KIR and Mamu-B molecules.

4.3.1 Mapping of specific binding sites of KIR and MHC class I molecules

Although KIR3DLW03 and KIR3DL05 share the same ligand, several differences in binding patterns were found during the fine mapping of the interaction sites of the Mamu-A molecules. While the interaction of KIR3DLW03 is mainly influenced by the α1 domain, the interaction between KIR3DL05 and Mamu-A molecules is additionally influenced by the α2 domain (Figure 20). Another difference was found by analysing the interaction between the KIR molecules with Mamu-A mutants of the Bw epitope. The binding of KIR3DLW03 is restricted to $Bw4^+$ Mamu-A molecules, whereas the binding of KIR3DL05 is influenced by the Bw epitope, but does not seem to be restricted by it (Figure 21).

The findings that KIR3DLW03 binds only to $Bw4^+$ molecules and KIR3DL05 is not restricted to any Bw epitope confirm the results seen with the chimeric constructs. Since the binding of KIR3DLW03 is mainly limited by the α1 domain, which includes the Bw epitope, the binding of KIR3DL5 seems to be influenced by other amino acids that also reside in the α2 domain. On the other hand, the presence of the Bw6 epitope might be responsible for Mamu-A1*00801 being a weak ligand. It might be that the binding between Mamu-A1*00801 and KIR3DL5 would be increased if the Bw6 epitope would be replaced by a Bw4 epitope. If the binding would not be increased by replacement of the Bw epitope, it would be even more unlikely that Mamu-A1*00801 represents a possible ligand for KIR3DLW03.

The influence of the Bw epitope and of the amino acid at position 83 in binding of human KIR3D molecules has also been demonstrated in humans (Sanjanwala et al., 2008; Sharma et al., 2009). Therefore, the binding to $Bw4^+$ molecules and the influence of the amino acid at position 83 in the MHC class I ligand are properties shared between rhesus macaques and humans. Nevertheless, it could be shown that the presence of the Bw4 epitope and especially the specific residue at position 83 are not the only sites influencing binding between KIR and Mamu-A molecules. Other analysed MHC class I molecules share the same Bw epitope (e.g. Mamu-A2*050402 or -B*01202) (Table 6), but neither bind to KIR3DLW03 nor to KIR3DL05. Therefore, it is obvious that other amino acids in α1 and α2 exert an influence on binding specificity as well. This is comparable to findings in humans, whereby residues in the α2 domain of $Bw4^+$ HLA molecules were shown to influence binding with KIR3DL1 (Sanjanwala et al., 2008).

The fine mapping of the interaction sites of rhesus macaque KIR3DL05 demonstrates that, as in humans, amino acid position 230 is important for binding. Additionally, it could be demonstrated that the amino acid position 204 has an influence on binding of KIR and MHC molecules in rhesus macaques, whereas amino acid position 144 has only an influence on binding of KIR3DL05*007 to Mamu-A3*1311, but not to Mamu-A1*00101 (Figure 22). This indicates differences in binding between KIR3DL05 and its two ligands. Further mutations of KIR molecules will be needed to show if amino acid position 144 is the only amino acid residue that exerts different influences on the binding between KIR3DL05 and the Mamu-A molecules. It would also be useful to analyse the same mutations in KIR3DLW03. However, it seems that there are at least two different binding patterns between KIR and Mamu-A molecules in the rhesus macaques. These two types of interactions might have evolved due to a more complex diversity of *KIR* and *MHC class I* genes in the rhesus macaques compared to humans.
Therefore, these data show that, besides the similarities in the structure of human and rhesus macaque KIR molecules, multiple differences in the binding pattern can be found. Still, notwithstanding the rapid evolution of these genes (Abi-Rached et al., 2010), some characteristics in binding pattern of KIR and MHC class I molecules have been conserved between humans and rhesus macaques, which demerged from each other about 25 million years ago (Steiper et al., 2004).

4.4 *Evolution of KIR and MHC class I genes*

The *Mamu-A* and *-B* genes show a high diversity in the rhesus macaque due to several duplication events (Daza-Vamenta et al., 2004; Otting et al., 2007), whereas *MHC-C* gene is missing (Parham, 2005a). In humans, it was also shown that the HLA-A and -B molecules interact with KIR3D molecules (Cella et al., 1994; Hansasuta et al., 2004), whereas the HLA-C acts as a ligand for KIR2D molecules (Yawata et al., 2006; Moesta et al., 2008). Therefore, the diversity of *KIR3D* genes should be higher in rhesus macaques than the diversity of *KIR2D* genes. This discrepancy between the diversity of *KIR3D* and *KIR2D* genes has been shown previously by different groups (Hershberger et al., 2001; Sambrook et al., 2005; Blokhuis et al., 2009a) and was confirmed by this study, as well.

The driving force of the rapid evolution of *KIR* and *MHC class I* genes presumably are pathogens that mainly influence the diversity of the *MHC class I* genes. On the other hand, to maintain the possibility to interact with their ligands, the *KIR* genes have to react to changes in *MHC class I* genes. But in contrast to *MHC class I* genes, the *KIR* genes show only a moderate level of sequence polymorphism (Kelley et al., 2005b). Therefore, the adaptation of the *KIR* genes to the *MHC class I* genes has not happened on the basis of high polymorphism of these genes. Instead, it rather happened more recently by duplication of genes , as was observed in rodents (Hao and Nei, 2004).

One reason for this is the location of *KIR* and *MHC* genes on different chromosomes. The unlinked heredity of the receptor and ligand genes makes the co-evolution of both gene families more difficult. This allows for different combinations of receptors and ligands, which leads to a better immune response to different pathogens. However, it has to be warranted that every individual possesses at least some receptors that show specific interactions with MHC class I molecules. This can be warranted by a wide spectrum of specific interactions of receptors or by an increased number of receptor genes.

In this study, it was shown that the two KIR molecules show only strong binding to two MHC ligands. This shows that the KIR genes do not have a wide binding spectrum. Otherwise, a higher number of strong interactions between KIR and MHC molecules would have been detected. Therefore, a wide spectrum of interactions specificity can be excluded. However, the *KIR* gene number per haplotype was higher than previously assumed. The completely sequenced haplotype possesses only five *KIR* genes (Sambrook et al., 2005), whereas this study has shown that the gene number per

haplotype can vary between 5 and 11 genes. Therefore, the adaptation of *KIR* genes to *MHC* genes in the rhesus macaque has happened by gene duplication, as was seen in rodents (Hao and Nei, 2004)

It was also shown that primates and rodents show almost the same number (about ten) of functional *KIR* and *Ly49* genes, respectively (Hsu et al., 2002b), although only a few classical *MHC class I* genes (one to four) are present in a single individual (Klein and Figueroa, 1986; Anzai et al., 2003). This might be a proof for a limitation in the number of functional *KIR* and *Ly49* genes - their interactions with MHC class I molecules may be a limiting factor (Hao and Nei, 2004). In the rhesus macaques, the number of genes coding for possible interaction partners is relatively high due to duplications of *Mamu-A* and *-B* genes, while the number of *KIR* genes shows almost the same diversity as in other species. This would be contradictory to the findings in other primates and rodents. Yet, in this study, only interactions between KIR and Mamu-A molecules were found. Therefore, it is possible that the only ligands for KIR3D molecules in rhesus macaques are Mamu-A molecules. In that case, the ratio between *KIR* genes and genes coding for potential ligands would fit to findings in other species.

4.5 *Outlook*

It was shown in humans that specific combinations of *KIR* and *HLA* genes have an impact on the outcome of diseases (i.e. susceptibility and resistance). However, detailed knowledge about *KIR* haplotypes, genotyping tools and specific KIR/MHC interactions of rhesus macaques have been missing up to now. The elucidation of this information is important for evaluation of rhesus macaques as non-human primate animal models for human diseases.

Specific interactions between KIR and Mamu-A have been shown in this study. The interactions between the two KIR molecules and Mamu-A*00101 in particular are of great interest, as Mamu-A1*01 is associated with lower virus load and prolonged survival of rhesus macaques after experimental SIV infection (Mühl et al., 2002; Mothe et al., 2003; Sauermann et al., 2008). Additionally, an interaction between Mamu-A3*1311 and KIR3DL05*007 was identified. Mamu-A3*1311 differs only by 7 amino acids from Mamu-A3*1303, which is also associated with longer survival time after SIV infection (Mühl et al., 2002).

In humans, it has been shown that the progression of AIDS is influenced by the presence of distinct combination of KIR3DL1 and its specific ligand (Martin et al., 2007). Therefore, the presence of a possible binding partner for Mamu-A1*01 and Mamu-A3*1303 might have an influence on survival time after experimental SIV infection. Consequently, it should be analysed whether the presence of *KIR3DLW03*004* and *KIR3DL05*007* has an influence of the progression on AIDS in *Mamu-A1*01* or *Mamu-A3*1303*-positive rhesus macaques experimentally infected with SIV.

The knowledge of the established *KIR* genotyping and the interactions found will lead to association studies of *KIR* and *MHC* genotypes in rhesus macaque disease models. Consequently, further knowledge of the influence of distinct *KIR* and *M*HC *class I* haplotypes on different diseases can be gained. This knowledge can be used to select rhesus macaques for disease studies which do not differ due to their genetic background on the outcome of this disease. This will lead to achieve both comparable data and higher significance of the results.

Additionally, the *KIR* genotyping can be used to bread rhesus macaques with a certain haplotypes which are advantageous for specific disease studies. On the other hand the *KIR* genotyping can serve to prevent inbreeding in a rhesus macaque colony. Therefore, the *KIR* genotyping can be used to conserve rare haplotypes and prevent their loss with a view to maintaining the KIR diversity in the breading colony. As it can be seen in the mouse model, for some studies inbreeding have numerous advantages and for other genetic diversity is needed. Hence the knowledge gained in this study will constantly improve the power of the rhesus macaque as animal model and will decrease the number of rhesus macaques needed for disease studies.

5 Summary

This PhD thesis reports the characterisation of the genetic diversity of killer cell immunoglobulin-like receptors (KIR) as well as interactions between KIR and MHC class I molecules in the rhesus macaque (*Macaca mulatta*). In humans, it has been shown that *KIR* haplotypes have an influence on the resistance against various diseases. The rhesus macaque is a well established non-human primate model for several diseases such as AIDS. The diversity of *KIR* genes has been analysed in previous studies; yet, a genotyping tool for rhesus macaque KIR genes has not been described so far and detailed information of distinct *KIR* genotypes and haplotypes was previously unavailable. Furthermore, specific interactions between KIR and MHC class I molecules have not been characterised so far.

One aim of this study was to establish a *KIR* genotyping assay for the rhesus macaque and to determine distinct *KIR* genotypes and haplotypes. Eight new *KIR* cDNA sequences were isolated from a NK cell cDNA library. All these sequences code for KIR3D molecules, confirming the high diversity of lineages II *KIR* genes in rhesus macaques. Using these sequences and those available in public database, a set of 31 primers for *KIR* genotyping was established. 25 *KIR* genotypes could be identified in four families containing a total number of 70 animals. With segregation analyses in four rhesus macaque families, 21 haplotypes could be determined. The haplotypes varied in gene number between 5 and 11 *KIR* genes. The results show a comparable level of diversity and complexity between human and rhesus macaque *KIR* haplotypes.

The second aim of this study was to identify specific interactions between KIR and MHC class I molecules and to define the interaction sites between these molecules. KIR-Fc fusion proteins were used with different Mamu-A and -B transfectants and strong specific interactions were identified for two KIR molecules each of them with two Mamu-A molecules. No significant interaction was found with Mamu-B molecules. Fine mapping of the interaction sites was carried out for interacting KIRs (KIR3DLW03 and KIR3DL05) and revealed different binding patterns for these two KIR molecules.

While the binding specificity of KIR3DL05 molecule shows similarities to the binding specificity in humans and was mainly determined by the α1 domain of the Mamu-A molecule, especially by the Bw epitope, the binding specificity of KIR3DL05 was influenced by the α1 and α2 domain of the Mamu-A molecule. The interaction pattern

differs also in the amino acids of the KIR molecules that are involved in the interaction with the Mamu-A molecules.

The knowledge of the established *KIR* genotyping and the newly discovered interactions between KIR and Mamu-A molecules will improve the power of the rhesus macaque as animal model and will reduce the number of animals needed for disease studies.

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7 Appendix

7.1 DNA sequences of isolated *KIR* clones

The 5'- and 3'-UTR are shown in small letters, the coding DNA region is shown in capital letters. The start codon (ATG), stop codon (TGA) and the poly(A) signal are highlighted in grey.

```
                    10        20        30        40        50        60        70
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
KIR3DLW03*005       gggggcggccgcctgtctgcaccggcagcaccATGTCGCTCATAGTCCTCAGCGTGGCGTGTGTTGGGTT
KIR3DL11*003        ...c.....................................G..TG....T.................
KIR3DL11*004        ...c.....................................G..TG....T.................
KIR3DS05*003        ----------.ggg.....................................................
KIR3DS06*004        ..ac.....................................A.........G..........A.....
KIR3DSW07*002       ........................t.....................G.....................
KIR3DSW08*006       ...c.....................................................................
KIR3DSW08*007       ...c.....................................................................

                             80        90       100       110       120       130       140
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
KIR3DLW03*005       CTTCTTGGTCCAGACGGCCTGTCCATACACGGGTGGTCAGGACAAGAACTTCCTGTCTGCCTGGCCCAGC
KIR3DL11*003        G.............G..........C.....................C.............C........
KIR3DL11*004        G.............G..........C.....................C.............C........
KIR3DS05*003        ..............G..........C.....................CGC...................
KIR3DS06*004        .......A......G..........C.............................T.............
KIR3DSW07*002       ..............G..........C.....................C.............C........
KIR3DSW08*006       .....G........G..........C.....................C..C..........C........
KIR3DSW08*007       .....G........G..........C.....................C.............C........

                           150       160       170       180       190       200       210
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
KIR3DLW03*005       CCTGTGGTGCCCCAGGGAGGACATGTGACTCTTCGGTGTCACTATCGTGGTGGGTTTAACAACTT-----
KIR3DL11*003        G..........T...........C..........A.............C................TACCA
KIR3DL11*004        G..........T...........C..........A.............C................TACCA
KIR3DS05*003        ...........T.....................................................------
KIR3DS06*004        ...........T..........TC..............T......A.C.A..AC..........TACCA
KIR3DSW07*002       G..C.......T...........C..............T.........A...AC..........TACCA
KIR3DSW08*006       G..........T...........C.......................C.A..AC..........TACCA
KIR3DSW08*007       G..C.......T...........C.......................C.....C..T.......TACCA

                           220       230       240       250       260       270       280
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
KIR3DLW03*005       ----CACCCTGTACAAAGACGACAGAAGCCACATTCCCATCTTCCACAGCAGAATATTCCAGGAGAGCTT
KIR3DL11*003        ACTT...T.........................G............T.G.....................
KIR3DL11*004        ACTT...T.........................G............T.......................
KIR3DS05*003        ---T...T.........................G.......................................
KIR3DS06*004        ACTT...T.........................G..........T...........................
KIR3DSW07*002       ACTT...T...........T............G.....G.........A.....................
KIR3DSW08*006       ACTT...T.........................G.....G...........C...................
KIR3DSW08*007       ACTT...T.........................G.....G...........C...................

                           290       300       310       320       330       340       350
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
KIR3DLW03*005       CCTCATGGGCCCTGTGACCCCGGCACACGCAGGGACCTACAGATGTCGGGGTTCATACCCGCACTCCCCC
KIR3DL11*003        ......A.................................................................
KIR3DL11*004        ......A.................................................................
KIR3DS05*003        .........................................................................
KIR3DS06*004        ...........................................................A.............
KIR3DSW07*002       ....................A.....................................C.............
KIR3DSW08*006       ...................A.......A.............................................
KIR3DSW08*007       ...................A.......A.............................T...........
```

```
                    360       370       380       390       400       410       420
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|
KIR3DLW03*005      ACTGAGTGGTCGACTCCCAGCAACCCCCTGGCGATCAGGGTCACAGGAGTCCACAAAAAGCCTTCCCTCC
KIR3DL11*003       ............G.A.T...TG..............T.................G...A.........
KIR3DL11*004       ............G.A.T...TG..............T.................G...A.........
KIR3DS05*003       ............G.....................................................
KIR3DS06*004       ............G.A.T....G..............T.................G...A.........
KIR3DSW07*002      ............G.A.T...TG..............................G...A.........
KIR3DSW08*006      ............G.A.T...TG.A............T.................G...A.........
KIR3DSW08*007      ............G.A.T...TG.A............T.................G...A.........

                    430       440       450       460       470       480       490
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|
KIR3DLW03*005      TGGCCCTCCCAGGTCCCCTGGTGAAATCAGGAGAGACGGTCACCCTACAATGTTCATCAGATACCGTGTT
KIR3DL11*003       ..................................T.................G..............
KIR3DL11*004       ..................................T.................G..............
KIR3DS05*003       .....T.............................................................
KIR3DS06*004       ..............T.........T.........................C..............
KIR3DSW07*002      ........................................A..........G..............
KIR3DSW08*006      ..................................................T.......TG.....
KIR3DSW08*007      ..................................................C..............

                    500       510       520       530       540       550       560
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|
KIR3DLW03*005      TGAGCACTTCTTTCTGCACCGTGAGGTGACCCTTGAGGAGCCCTTGCACCTTGTTGGAGAGCTCCATGGT
KIR3DL11*003       ..G..............A...........T.........T...........................
KIR3DL11*004       ..G..............A...........T.........T...........................
KIR3DS05*003       .....................................................................
KIR3DS06*004       ...C.............A.........A.T.....A...............................
KIR3DSW07*002      ..G..............A...........T.....A.............................C...
KIR3DSW08*006      ................A...........T.........T...........................
KIR3DSW08*007      ..G..............A...........T.........T...........................

                    570       580       590       600       610       620       630
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|
KIR3DLW03*005      GGGGGATCCCAGGCCAAGTATTCCATCAATTCCACGACATCTGACCTTGCAGGGACCTACAGATGCTATG
KIR3DL11*003       .....T........A..C..C..................G.............A.....T.........C.
KIR3DL11*004       .....T........A..C..C..................G.............A.....T.........C.
KIR3DS05*003       .....T.....................................T...........T.........C.
KIR3DS06*004       ..A..T...........C................A...G.............................C.
KIR3DSW07*002      .....T...........C................A...G.............A..............C.
KIR3DSW08*006      .....T...........C..C.............T...G.............................C.
KIR3DSW08*007      .....T...........C..C.............T...G.............................C.

                    640       650       660       670       680       690       700
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|
KIR3DLW03*005      GTTCTTTTACTCACTCCCCCTATGTGTTGTCAGCTCCCAGTGACCCCCTGGACATCGTGATCACAGGTCT
KIR3DL11*003       .....G.C.........................................AA.................AA
KIR3DL11*004       .....G.C.........................................AA.................AA
KIR3DS05*003       .....................................................A...............
KIR3DS06*004       .C...G.C.....................A...................G...................
KIR3DSW07*002      .....G.C.....................................................AA
KIR3DSW08*006      .....G.C............G.......................A.....................
KIR3DSW08*007      .....G.C............G.......................A.....................

                    710       720       730       740       750       760       770
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|
KIR3DLW03*005      ATATGAGAAACCTTCTCTCTCAGCCCAGCCGGGCCCCACGGTTCAAGCAGGAGAGAACGTGACCTTGTCC
KIR3DL11*003       ..........................................G.................T.......
KIR3DL11*004       ..........................................G.................T.......
KIR3DS05*003       .....................................................................
KIR3DS06*004       ............................A.............G.........................
KIR3DSW07*002      ..........................................G.........................
KIR3DSW08*006      ..........................................G.........................
KIR3DSW08*007      ..........................................G.........................

                    780       790       800       810       820       830       840
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|
KIR3DLW03*005      TGCAGCTCCCGGTGCTCCTTTGACATGTACCATCTATCCAGGGAGGGGGAGGCTCGTGAACTTAGTCTCT
KIR3DL11*003       ................G.....................................................
KIR3DL11*004       ................G.....................................................
KIR3DS05*003       ............C.....................................C.................
KIR3DS06*004       .....................................................C..............G
KIR3DSW07*002      ..........A.AA...G.....................................G...........
KIR3DSW08*006      ..........A.AA...G.....................................C....G......
KIR3DSW08*007      ..........A.AA...G.....................................C....G......
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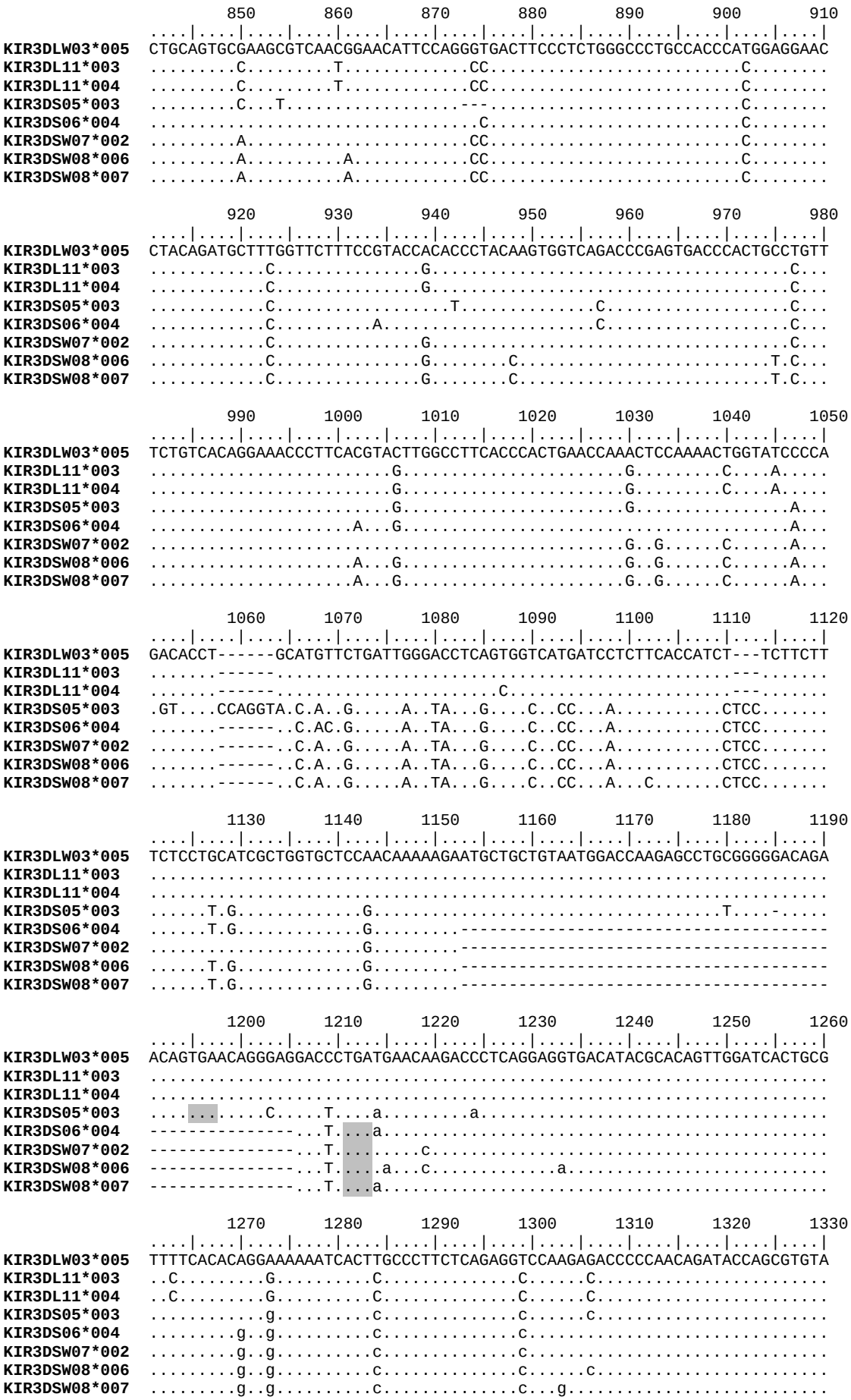
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KIR3DL11*003 .....C.....T.....CC.....C.....
KIR3DL11*004 .....C.....T.....CC.....C.....
KIR3DS05*003 .....C...T.....---.....C.....
KIR3DS06*004 .....C.....C.....
KIR3DSW07*002 .....A.....CC.....C.....
KIR3DSW08*006 .....A.....A.....CC.....C.....
KIR3DSW08*007 .....A.....A.....CC.....C.....
920 930 940 950 960 970 980
KIR3DLW03*005 CTACAGATGCTTTGGTTCTTTCCGTACCACACCCTACAAGTGGTCAGACCCGAGTGACCCACTGCCTGTT
KIR3DL11*003 .....C.....G.....C...
KIR3DL11*004 .....C.....G.....C...
KIR3DS05*003 .....C.....T.....C.....C...
KIR3DS06*004 .....C.....A.....C.....C...
KIR3DSW07*002 .....C.....G.....C...
KIR3DSW08*006 .....C.....G.....C.....T.C...
KIR3DSW08*007 .....C.....G.....C.....T.C...
990 1000 1010 1020 1030 1040 1050
KIR3DLW03*005 TCTGTCACAGGAAACCCTTCACGTACTTGGCCTTCACCCACTGAACCAAACTCCAAAACTGGTATCCCCA
KIR3DL11*003 .....G.....G.....C....A.....
KIR3DL11*004 .....G.....G.....C....A.....
KIR3DS05*003 .....G.....G.....A...
KIR3DS06*004 .....A...G.....A...
KIR3DSW07*002 .....G..G.....C.....A...
KIR3DSW08*006 .....A...G.....G..G.....C.....A...
KIR3DSW08*007 .....A...G.....G..G.....C.....A...
1060 1070 1080 1090 1100 1110 1120
KIR3DLW03*005 GACACCT------GCATGTTCTGATTGGGACCTCAGTGGTCATGATCCTCTTCACCATCT---TCTTCTT
KIR3DL11*003 .....------.....---.....
KIR3DL11*004 .....------.....C.....---.....
KIR3DS05*003 .GT....CCAGGTA.C.A..G.....A..TA...G....C..CC...A.....CTCC.....
KIR3DS06*004 .....-------..C.AC.G.....A..TA...G....C..CC...A.....CTCC.....
KIR3DSW07*002 .....-------..C.A..G.....A..TA...G....C..CC...A.....CTCC.....
KIR3DSW08*006 .....-------..C.A..G.....A..TA...G....C..CC...A.....CTCC.....
KIR3DSW08*007 .....-------..C.A..G.....A..TA...G....C..CC...A...C.....CTCC.....
1130 1140 1150 1160 1170 1180 1190
KIR3DLW03*005 TCTCCTGCATCGCTGGTGCTCCAACAAAAAGAATGCTGCTGTAATGGACCAAGAGCCTGCGGGGGACAGA
KIR3DL11*003 .....
KIR3DL11*004 .....
KIR3DS05*003 .....T.G.....G.....T....-.....
KIR3DS06*004 .....T.G.....G.....----------
KIR3DSW07*002 .....G.....----------
KIR3DSW08*006 .....T.G.....G.....----------
KIR3DSW08*007 .....T.G.....G.....----------
1200 1210 1220 1230 1240 1250 1260
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KIR3DL11*003 .....
KIR3DL11*004 .....
KIR3DS05*003 .....C.....T...a.....a.....
KIR3DS06*004 ----------...T...a.....
KIR3DSW07*002 ----------...T.....c.....
KIR3DSW08*006 ----------...T....a...c.....a.....
KIR3DSW08*007 ----------...T...a.....
1270 1280 1290 1300 1310 1320 1330
KIR3DLW03*005 TTTTCACACAGGAAAAAATCACTTGCCCTTCTCAGAGGTCCAAGAGACCCCCAACAGATACCAGCGTGTA
KIR3DL11*003 ..C.....G.....C.....C.....C.....
KIR3DL11*004 ..C.....G.....C.....C.....C.....
KIR3DS05*003 .....g.....c.....c.....c.....
KIR3DS06*004 .....g..g.....c.....c.....
KIR3DSW07*002 .....g..g.....c.....c.....
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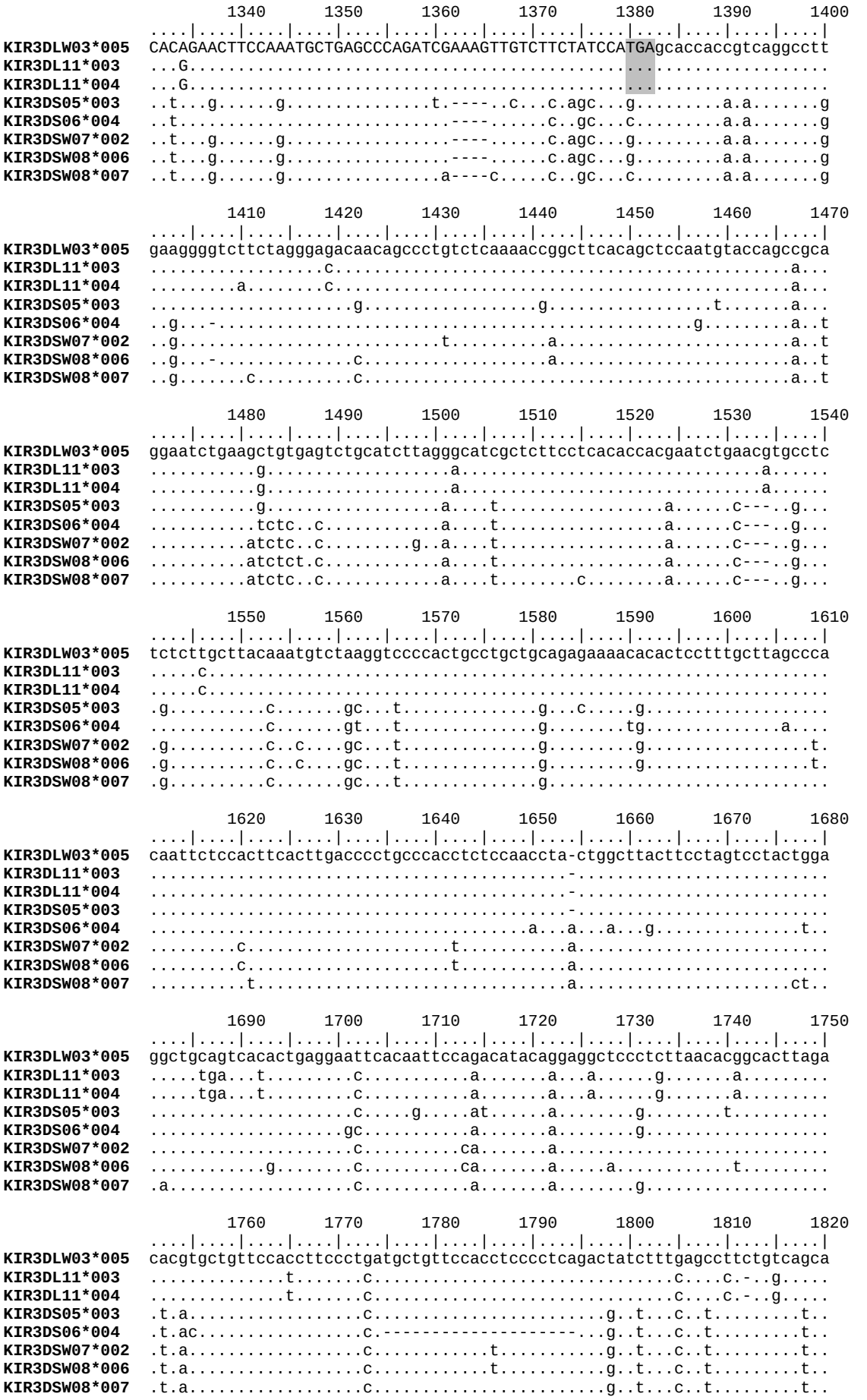
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KIR3DL11*004 ...G.....
KIR3DS05*003 ..t...g.....g.....t.----..c...c.agc...g.....a.a.....g
KIR3DS06*004 ..t.....----.....c..gc...c.....a.a.....g
KIR3DSW07*002 ..t...g.....g.....----.....c.agc...g.....a.a.....g
KIR3DSW08*006 ..t...g.....g.....----.....c.agc...g.....a.a.....g
KIR3DSW08*007 ..t...g.....g.....a----c.....c..gc...c.....a.a.....g
1410 1420 1430 1440 1450 1460 1470
KIR3DLW03*005 gaaggggtcttctagggagacaacagccctgtctcaaaaccggcttcacagctccaatgtaccagccgca
KIR3DL11*003 .....c.....a...
KIR3DL11*004 .....a.....c.....a...
KIR3DS05*003 .....g.....g.....t.....a...
KIR3DS06*004 ..g...-.....g.....a..t
KIR3DSW07*002 ..g.....t.....a.....a..t
KIR3DSW08*006 ..g...-.....c.....a.....a..t
KIR3DSW08*007 ..g.....c.....c.....a..t
1480 1490 1500 1510 1520 1530 1540
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KIR3DL11*003 .....g.....a.....a.....
KIR3DL11*004 .....g.....a.....a.....
KIR3DS05*003 .....g.....a...t.....a.....c---..g...
KIR3DS06*004 .....tctc..c.....a...t.....a.....c---..g...
KIR3DSW07*002 .....atctc..c.....g..a...t.....a.....c---..g...
KIR3DSW08*006 .....atctct.c.....a...t.....a.....c---..g...
KIR3DSW08*007 .....atctc..c.....a...t.....c.....a.....c---..g...
1550 1560 1570 1580 1590 1600 1610
KIR3DLW03*005 tctcttgcttacaaatgtctaaggtccccactgcctgctgcagagaaaacacactcctttgcttagccca
KIR3DL11*003 .....c.....
KIR3DL11*004 .....c.....
KIR3DS05*003 .g.....c.....gc...t.....g...c.....g.....
KIR3DS06*004 .....c.....gt...t.....g.....tg.....a....
KIR3DSW07*002 .g.....c..c....gc...t.....g.....g.....t.
KIR3DSW08*006 .g.....c..c....gc...t.....g.....g.....t.
KIR3DSW08*007 .g.....c.....gc...t.....g.....
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KIR3DL11*003 .....-.....
KIR3DL11*004 .....-.....
KIR3DS05*003 .....-.....
KIR3DS06*004 .....a...a...a...g.....t..
KIR3DSW07*002 .....c.....t.....a.....
KIR3DSW08*006 .....c.....t.....a.....
KIR3DSW08*007 .....t.....a.....ct..
1690 1700 1710 1720 1730 1740 1750
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KIR3DL11*003 .....tga...t.....c.....a.....a...a.....g.....a.....
KIR3DL11*004 .....tga...t.....c.....a.....a...a.....g.....a.....
KIR3DS05*003 .....c.....g.....at.....a.....g.....t.....
KIR3DS06*004 .....gc.....a.....a.....g.....
KIR3DSW07*002 .....c.....ca.....a.....
KIR3DSW08*006 .....g.....c.....ca.....a.....a.....t.....
KIR3DSW08*007 .a.....c.....a.....a.....g.....
1760 1770 1780 1790 1800 1810 1820
KIR3DLW03*005 cacgtgctgttccaccttccctgatgctgttccacctcccctcagactatctttgagccttctgtcagca
KIR3DL11*003 .....t.....c.....c....c.-..g.....
KIR3DL11*004 .....t.....c.....c....c.-..g.....
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KIR3DSW07*002 .t.a.....c.....t.....g..t...c..t.....t..
KIR3DSW08*006 .t.a.....c.....t.....g..t...c..t.....t..
KIR3DSW08*007 .t.a.....c.....g..t...c..t.....t..

```
                      1830      1840      1850      1860      1870      1880      1890
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
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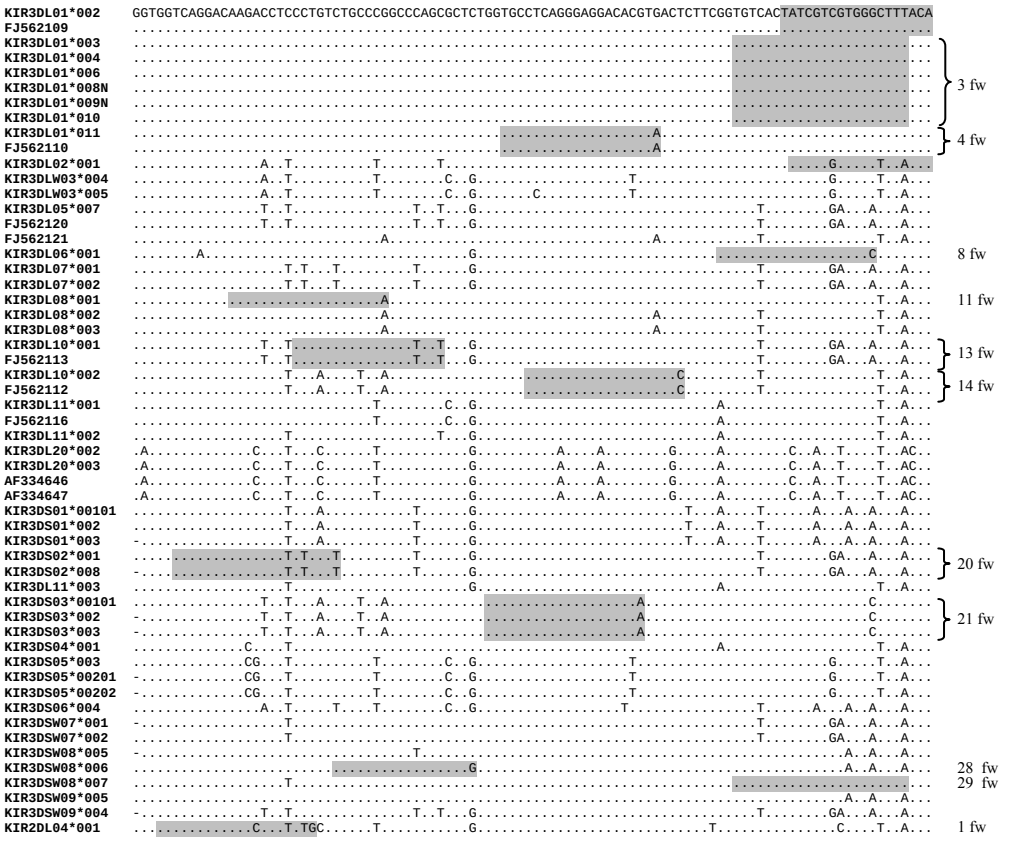
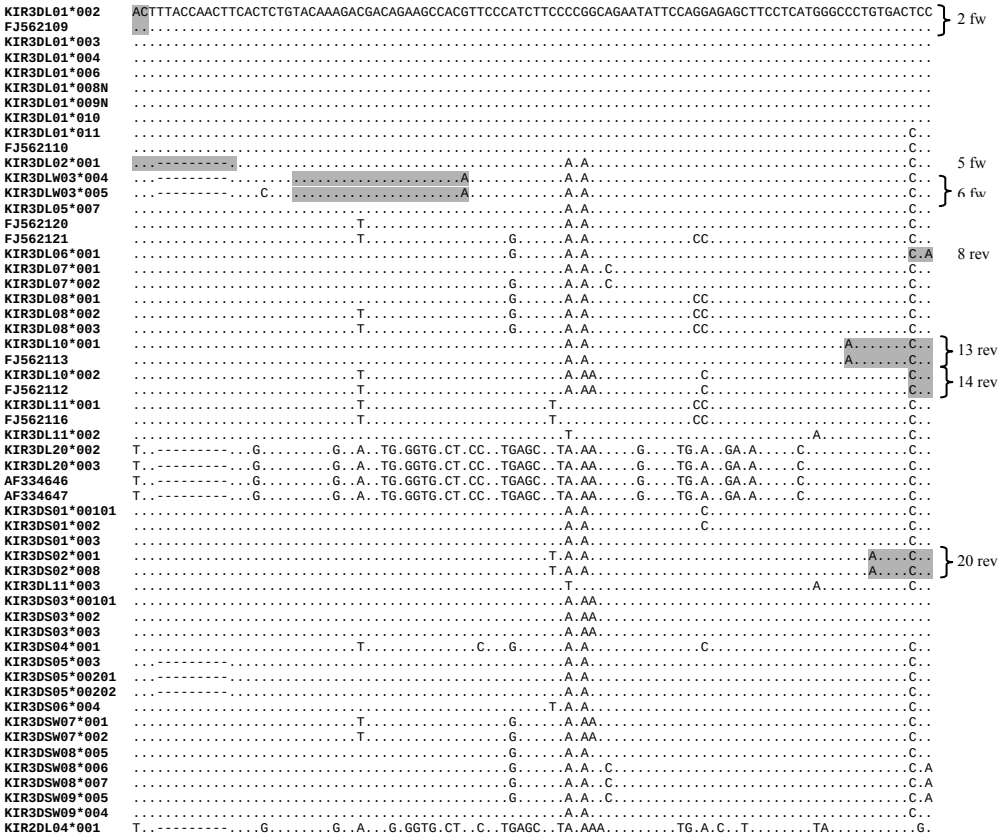
KIR3DL11*003    ..................tt.--.c.....................................g....
KIR3DL11*004    ..................tt.--.c.....................................g...a
KIR3DS05*003    ..a..c...t....aa...gttg-.g.....t.....................gt.a..g....
KIR3DS06*004    ..a..c........aa...gttg-.g.....t.............g.......gt.a..gt...
KIR3DSW07*002   ..a..c........aa...gttg-.g...a.t...................t......tgt....g...g
KIR3DSW08*006   ..a..c........aa...gttg-.g.....t.....................tgt....g....
KIR3DSW08*007   ..a..c........aa...gttg-.g.....t.....................gt.a..g....

                       1900      1910      1920
                ....|....|....|....|....|....|....|.
KIR3DLW03*005   ta----aaaaaaaaaaaaaaaaaaaaaaaaaa---
KIR3DL11*003    c.tccc.........................aaa
KIR3DL11*004    c.----.........................-----
KIR3DS05*003    c.ttca.........................aa-
KIR3DS06*004    c.ttca.........................aa-
KIR3DSW07*002   c.aaaa.........................-----
KIR3DSW08*006   c.tgaa.........................---
KIR3DSW08*007   c.ttct.........................aaa
```

7.2 *Multiple alignment of KIR DNA sequences*

The exons coding for the Ig domains of the newly detected *KIR* sequences and those available in public databases were aligned to identify sequence-specific substitutions. Locations of primers for the *KIR* genotyping were highlighted in grey. The primer numberes are indicated as in table 2. The exon boundaries are shown.

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KIR3DSW08*007
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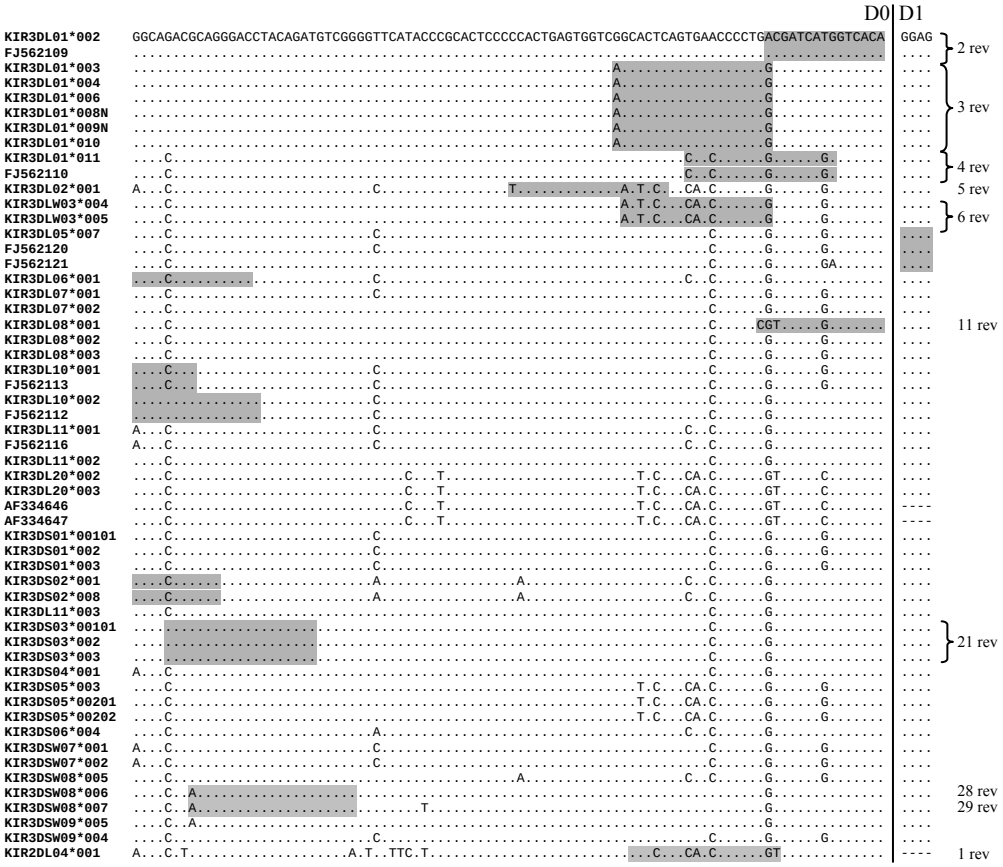
2 fw
5 fw
6 fw
8 rev
13 rev
14 rev
20 rev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KIR3DSW08*007
KIR3DSW09*005
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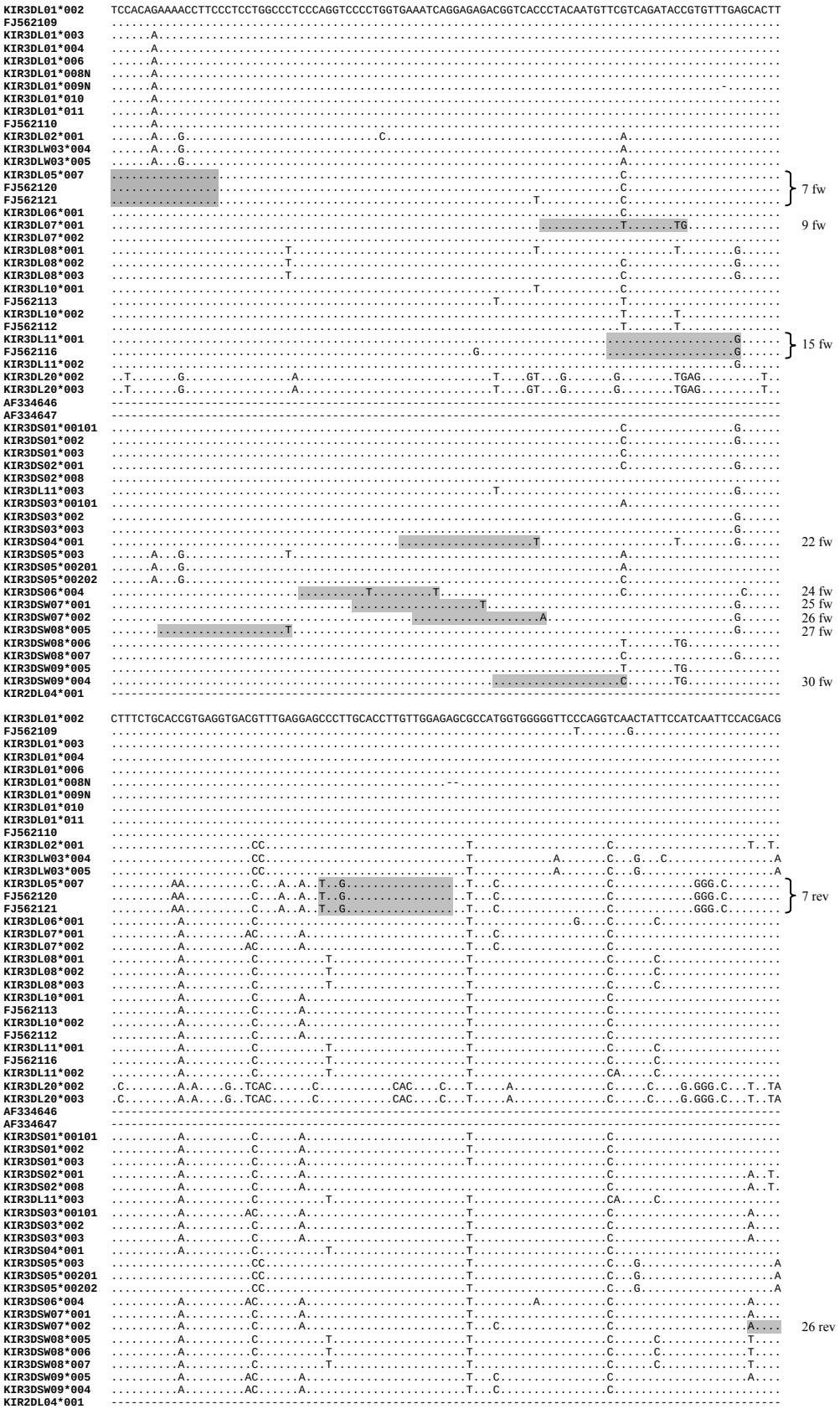
2 rev
3 rev
4 rev
5 rev
6 rev
11 rev
21 rev
28 rev
29 rev
1 rev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FJ562109
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FJ562121
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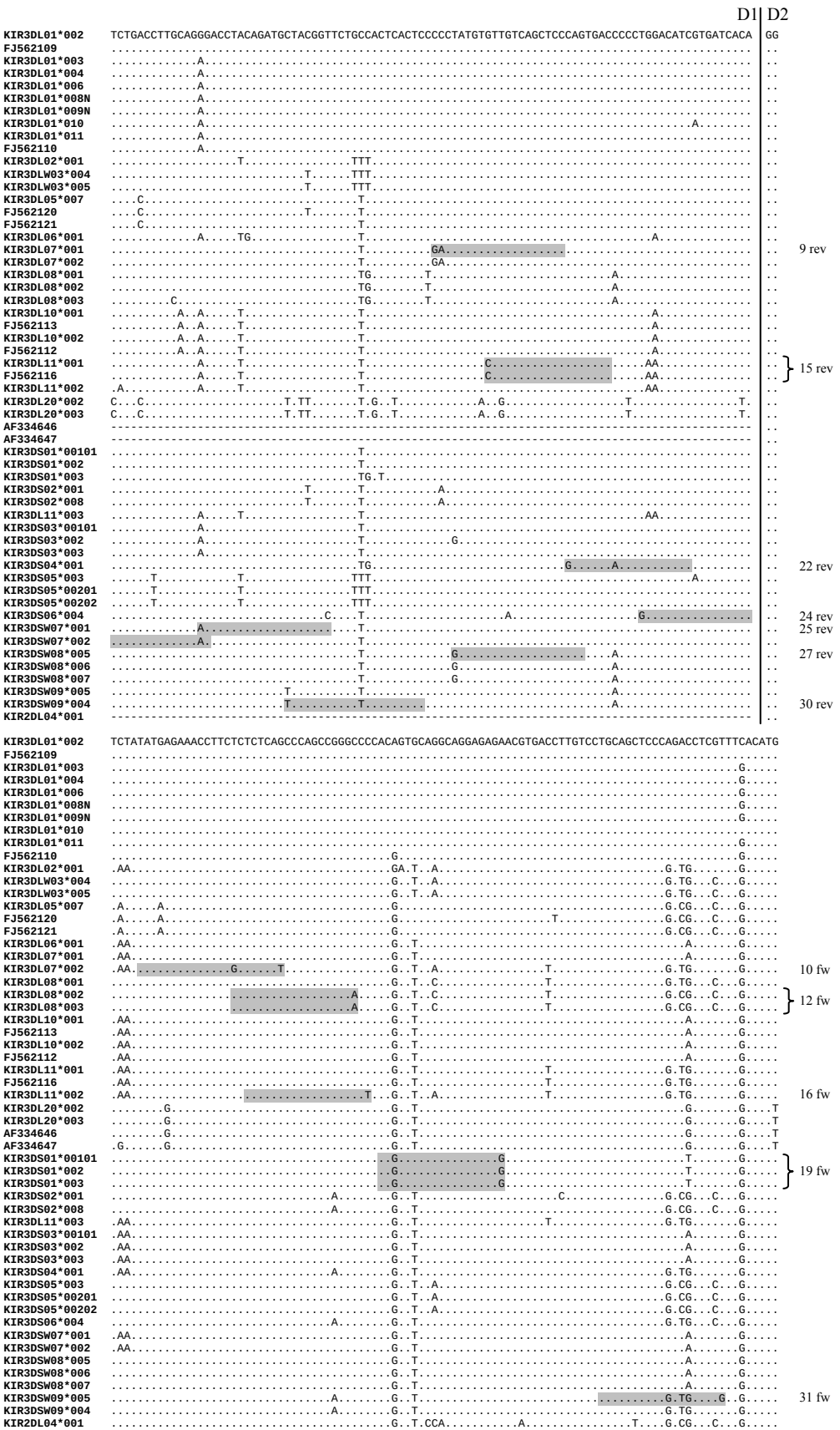
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KIR3DL01*006
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KIR3DL01*009N
KIR3DL01*010
KIR3DL01*011
FJ562110
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KIR3DLW03*004
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KIR3DSW08*005
KIR3DSW08*006
KIR3DSW08*007
KIR3DSW09*005
KIR3DSW09*004
KIR2DL04*001
9 rev
15 rev
22 rev
24 rev
25 rev
27 rev
30 rev
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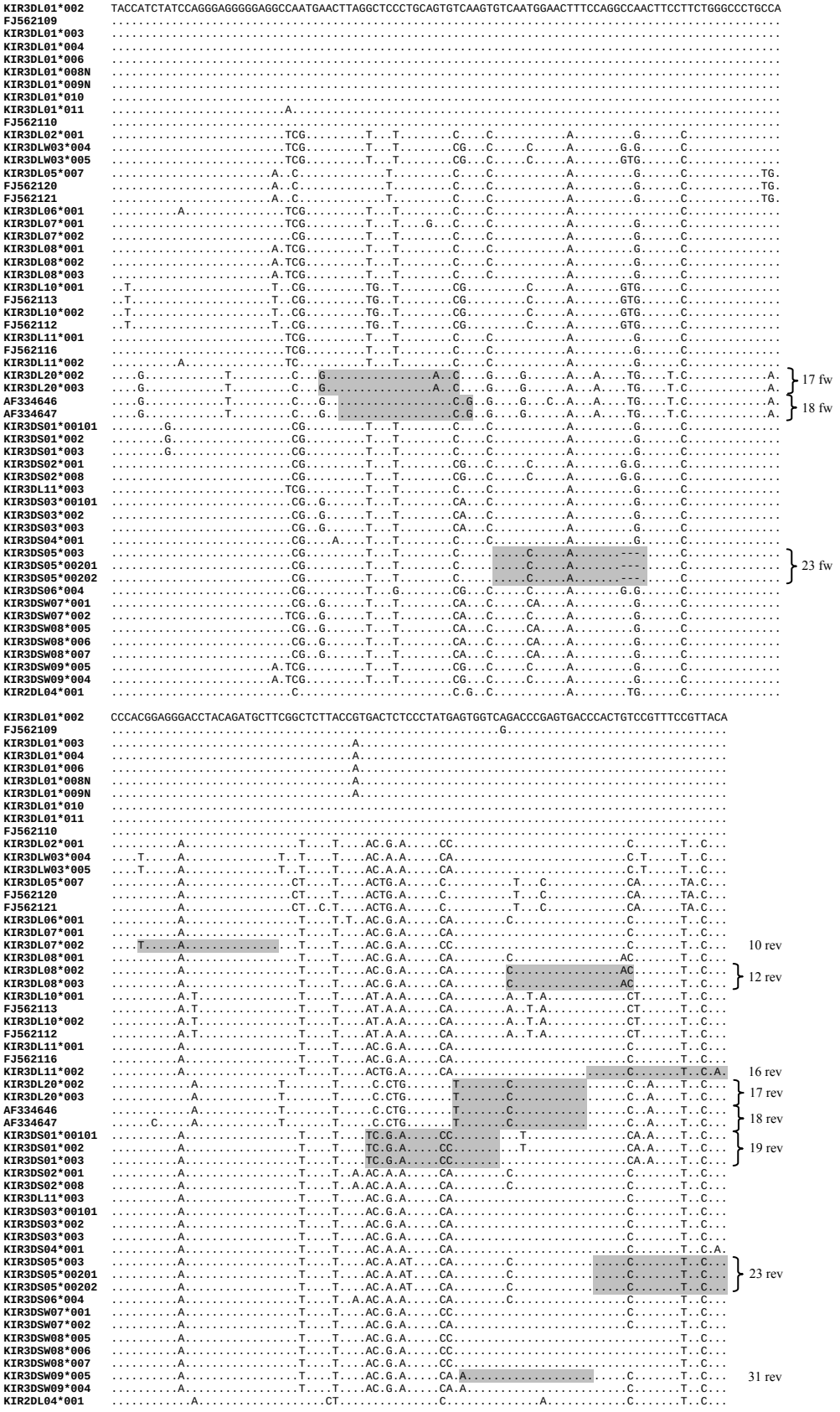
10 fw
12 fw
16 fw
19 fw
31 fw


KIR3DL01*002 TACCATCTATCCAGGGAGGGGGAGGCCAATGAACTTAGGCTCCCTGCAGTGTCAAGTGTCAATGGAACTTTCCAGGCCAACTTCCTTCTGGGCCCTGCCA
KIR3DL01*002 CCCACGGAGGGACCTACAGATGCTTCGGCTCTTACCGTGACTCTCCCTATGAGTGGTCAGACCCGAGTGACCCACTGTCCGTTTCCGTTACA
17 fw
18 fw
23 fw
10 rev
12 rev
16 rev
17 rev
18 rev
19 rev
23 rev
31 rev

7.3 *Pedigree of the analysed rhesus macaque families*

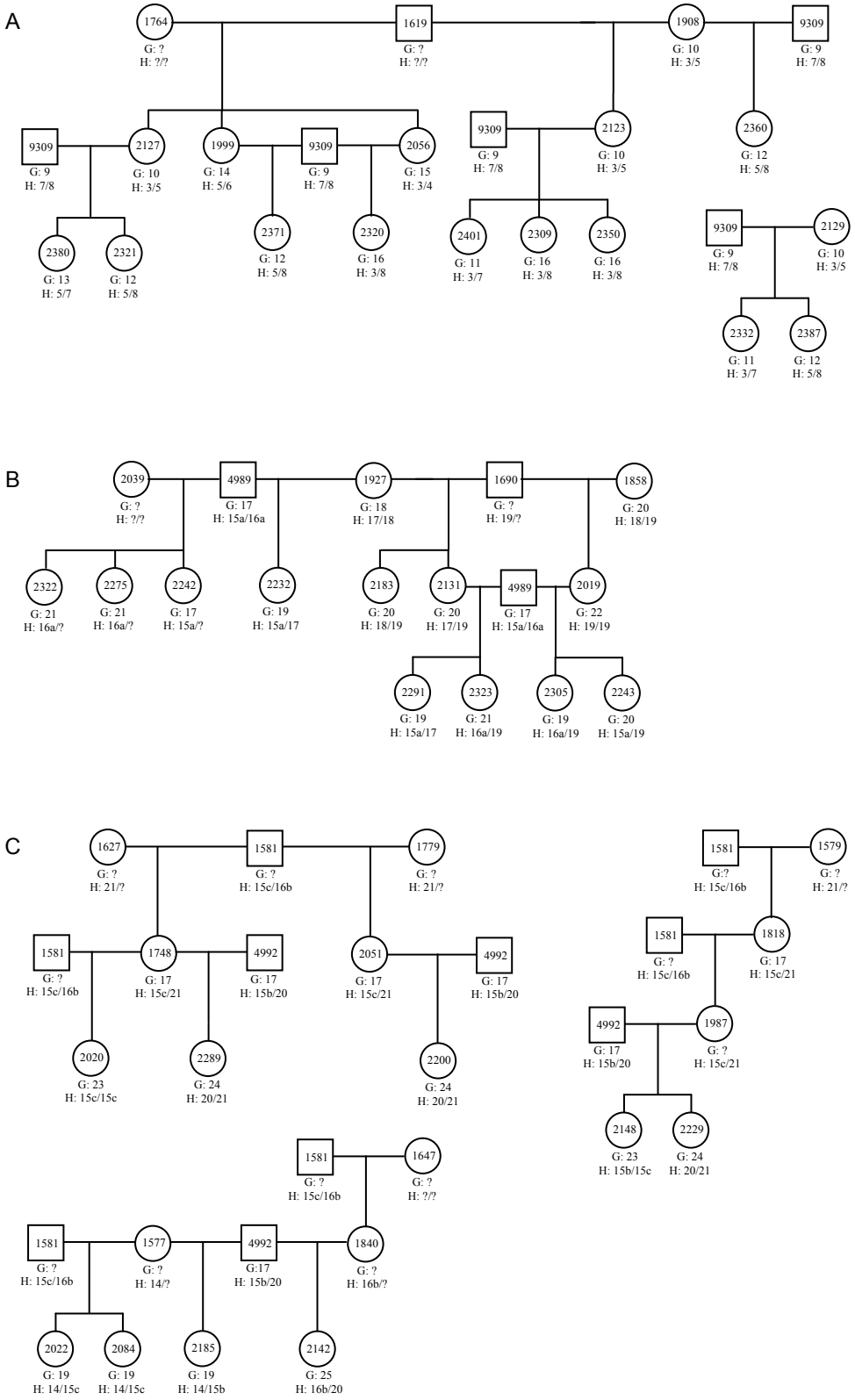


Figure 24: Pedigree of three analysed rhesus macaque families. Animal identification numbers are indicated. G and H denote *KIR* genotype and *KIR* haplotype, respectively. DNA of rhesus macaques 1764, 1619, 2039, 1690, 1627, 1518, 1779, 1579, 1987, 1647, is not available and their *KIR* haplotypes were partially inferred from offspring.

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