Complexity and evolution of KIR genes in rhesus macaques

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Complexiteit en evolutie van KIR genen
in rhesus makaken

(met een samenvatting in het Nederlands)

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Voor mijn ouders, Lisette en Julian

Love to doubt as well as know

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Introduction

GENERAL INTRODUCTION

Killer cell immunoglobulin-like receptors (KIR) are members of the immunoglobulin superfamily, and are expressed on natural killer (NK) cells and particular subsets of T cells. NK cells in particular play a pivotal role in determining immune responses, in maintaining immune homeostasis, and in remodelling blood vessels during pregnancy. In this manner, they have an impact on the outcome of infectious diseases, on the erradication of malignancies, on autoimmune disorders, and on reproductive success. From a functional point of view, KIR molecules play a key role in determining the activation state of these cells. Because of their broad medical relevance, KIR genes are of interest in many biomedical fields.

In order to transduce a signal into the cell, it is necessary for a KIR molecule to interact with its corresponding ligand. Ligands for KIR include major histocompatibility complex (MHC) class I molecules. In humans, the classical MHC class I loci are designated as human leukocyte antigens (*HLA*)-*A*, -*B* and -*C*, and are considered to be the most polymorphic genes known to date. The primary function of the MHC class I is to present intracellular peptides to effector T cells, and as such they constantly report the health status of a cell to the host's immune system. During a complex process, referred to as thymic education, T cells and their receptors are selected for their abilities to discern MHC molecules loaded with self-peptide from those with non-self peptides, respectively. Non-self peptides may originate from pathogens, and certain populations of T cells monitor the presence of MHC molecules loaded with foreign peptides and, if these molecules are present they react by eliminating such infected cells. As can be expected, certain pathogens try to evade and escape the immune response; for instance, by down modulating MHC class I cell surface expression. As a response, NK cells scan continuously for the presence and absence of MHC class I molecules by means of their KIR molecules. In humans the *KIR* genes are characterised by copy number variation and a high degree of polymorphism. Within any given human population, copious numbers of KIR genotypes are observed, which implies that the epistatic interactions of KIR and MHC, respectively, can vary significantly between individuals.

The individuals that are the main subject of this thesis belong to the species rhesus macaque (Macaca mulatta). In biomedical science, this species is widely used as an important animal model to study the fundamental biology of immunology. It is also employed to test new vaccines and drugs as well as treatments for human and animal disorders. Comparative genetic studies are extremely helpful in shedding light on the evolutionary history of a particular gene system, but they may also aid in studying and understanding its functions in depth. The rhesus macaque MHC class I system has been thoroughly investigated over a considerable period of time. However, still little is known about the KIR gene system. Because of its biomedical relevance, its close relationship with the MHC system, and the lack of knowledge involving this

species, the KIR gene system was investigated in rhesus macaques.

PRIMATE TAXONOMY AND EVOLUTIONARY RELATIONSHIPS

Like humans, the species of rhesus macaques (*Macaca mulatta*) belongs to the order of primates or *Primata*. Rhesus macaques belong to the suborder of Haplorrhini or 'dry-nosed' primates. They further belong to the parvorder of simians, *Catarrhini*, which also includes the different ape species (Fig. 1).

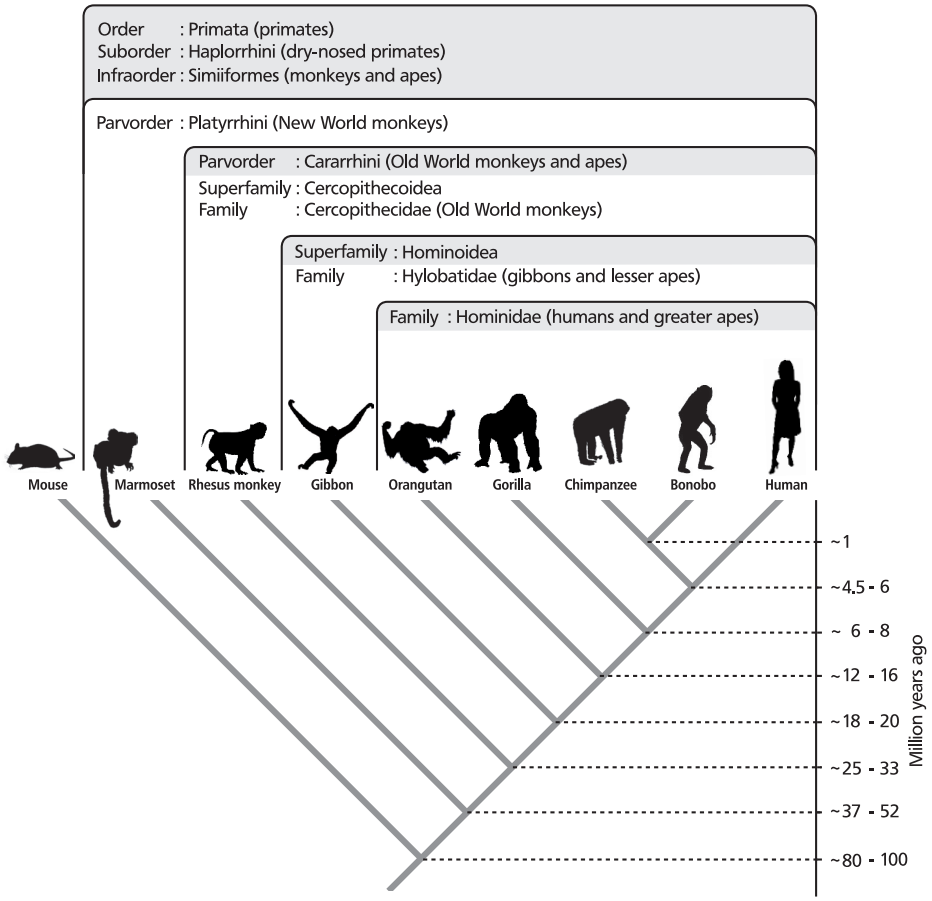

Figure 1 Evolutionary relationships and taxonomy of rhesus macaques and other monkeys and apes. Fossil records have yielded estimations when common ancestors have had to be present (1). Genetic distances have been determined by a variety of techniques, for instance, DNA crosshybridization (2), sequencing of mitochondrial (3) or nuclear DNA (4, 5), and whole genome analysis (6, 7). An approximation of the time frame when a common ancestor of Human (*Homo sapiens*) was shared with Common Marmoset (*Callithrix jacchus*), Rhesus macaque (*Macaca mulatta*), Gibbon (northern white cheeked; *Nomascus leucogenys*), Bornean Orangutan (*Pongo pygmaeus*), Western Gorilla (*Gorilla gorilla*), and Common Chimpanzee (*Pan troglodytes*) and Bonobo (*Pan paniscus*). Mouse (*Mus musculus*) has been added to illustrate the timepoint (~80-100 MYA) when primates have diverged from other mammals.

More precisely, they are part of the family of *Cercopithecidae* or Old World monkeys, and cluster into the genus *Macaca*. An Old World monkey species such as the rhesus macaque shares a common ancestor with the Great ape/human lineage approximately 25-33 million years ago (Fig. 1). The genetic separation of the different macaque species is of a more recent origin and is estimated to have happened approximately 1 million years ago (8). Most rhesus macaques used for biomedical research are usually obtained from populations that originate from either India or China (9). Genetic analysis has revealed that the separation of these two populations has occured approximately 160,000 years ago (10).

HISTORICAL INTRODUCTION OF THE NK CELL

The discovery of natural killer cells occurred, more or less simultaneously, in the fields of tumour immunology, transplantation and virology. Initially, however, divergent phenomena were observed that were not immediately understood correctly, nor were they thereupon attributed to this specialised set of lymphocytes. For instance, in 1964, a phenomenon called hybrid resistance was described in mice (11). A bone marrow transplant of homozygous donors was tested for take and growth in irradiated recipient animals, which were either heterozygous F1 or F1 hybrids from congenic parents, thus differing only for selected alleles of the histocompatibility loci. All heterozygous animals rejected transplantation of parental marrow cells, whereas only individuals homozygous for particular H-2 (12)[1] loci were non-resistant, despite the heterozygosity for one or several other histocompatibility loci. At that time, it was thought that the H-2 complex functioned as a recessive factor in the graft, which might be responsible for the recognition of donor cells by recipient effector cells. Subsequent work to define the nature of the effector cells revealed that they were of hematopoietic origin, but their development was independent of the thymus (13).

Another line of evidence, observed in the field of tumour immunology, and hinting at this natural reactivity, revealed that cells from non-immunised mice as well as normal human donors exhibited reactivity levels comparable with those observed in immunised animals and cancer patients (14, 15). Subsequent investigations employing target cells infected with different viruses also suggested the presence of preexisting cytotoxic lymphocytes in the absence of immunisation (16).

In those days, spontaneous cytotoxicity existing prior to immunisation was considered to contradict contemporary knowledge concerning T- and B-cell-mediated immunology. Not only bone marrow-derived cells showed these effects; lymphocytes with this specific ability could also be isolated from mouse spleen and, based on morphological features, they were distinctive from T cells. Due to their cytotoxic capability, without prior priming, they were termed 'natural killer' cells (17, 18). The existence of NK cells was considered controversial for a long period, as they were neither B nor T cells, and specific receptors that would dissect them, had not yet been described.

[1]This is the murine equivalent of the MHC.

As such, they would sometimes be referred to as 'null' cells. However, more evidence that these NK cells possessed unique functional characteristics was accumulating (19). Morphologically, they were described as large granular lymphocytes (20), and with the help of antibodies against CD16, CD56, and CD57 their unique immunophenotypical characteristics could be distinguished (21). NK cells are now regarded as a distinct lineage of lymphocytes, although the mechanisms of their activation remained a mystery for some time.

MECHANISM OF ACTIVATION: THE MISSING-SELF HYPOTHESIS

Hybrid resistance experiments (22) elucidated an important role for MHC class I in the selective rejection of grafts. Re-evaluation of this data, and the observation that certain tumour cell lines had lost or downregulated their MHC class I, allowed Kärre to propose the following mechanism: NK cells are effector cells of the immune defence, and their biological function is to detect the reduced expression or absence of self-MHC class I antigens (23). An elaborated version of this explanation was subsequently termed the 'missing-self hypothesis' (24), and was based on the premise that NK cells are capable of recognising and killing cells that lack MHC-class I (Fig. 2).

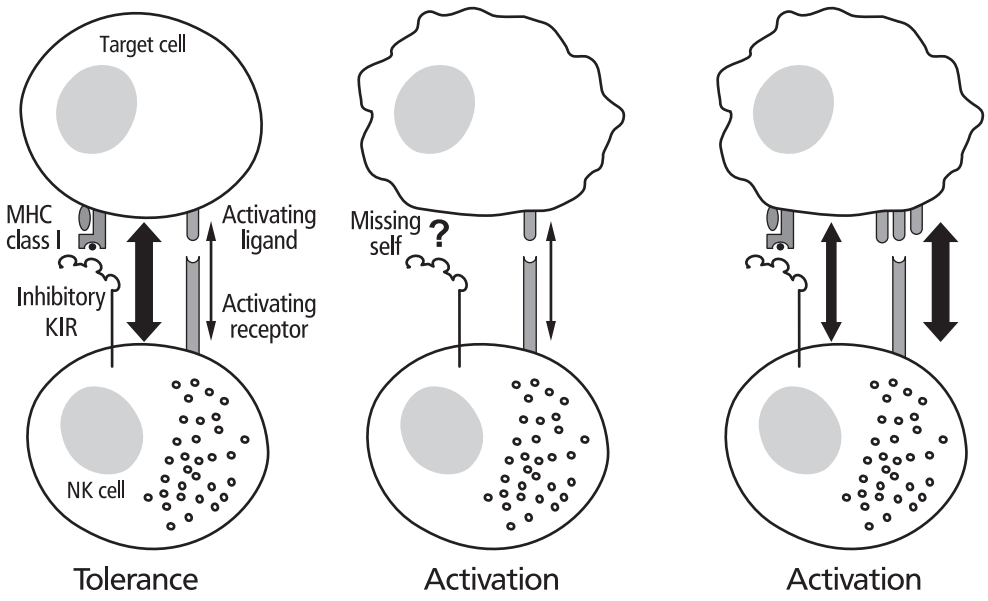

Figure 2 Schematic representation of NK cell activation. In the panel on the left, tolerance is maintained by the target cell that expresses inhibitory and activating ligands for NK cell receptors. In the middle panel the target cell has lost (missing-self) or altered its inhibitory ligands so that it no longer can be recognized and the NK cell is activated. On the panel on the right the NK cell is activated because the balance is shifted by an overstimulation of activating receptors.

A next level of sophistication was implemented by the notion that NK cells are being inhibited actively by interaction with self-MHC class I molecules. This receptor-inhibition model could be experimentally mimicked by transfecting target cells with MHC class I genes (25). Specific epitopes and residues of MHC class I molecules that were controlling these protective effects, were subsequently identified (26, 27). *In vivo*, this role of MHC class I was recorded in mice (28). More precisely, the murine Ly49 family of NK cell receptors appeared to be responsible for signalling through interactions with MHC class I (29). The 'missing-self hypothesis' also predicted the existence of inhibitory MHC class I-specific receptors in humans, which were later identified and mapped to the KIR family. Adoptive transfer studies on double transgenic mice that possess both a human *KIR* haplotype as well as the *HLA-C* locus (30) illustrated the, *in vivo*, importance of KIR molecules in detecting the missing MHC class I.

EDUCATION AND MEMORY

In line with the missing-self hypothesis, two additional criteria needed explanation. First, NK cells need to express at least one inhibitory receptor (at-least-one hypothesis) to prevent them from becoming reactive in an uncontrolled state (31). Second, target cells that lack all ligands for inhibitory receptors are expected to be killed. This latter hypothesis could be tested in mice that lack MHC class I expression. These animals were viable (under SPF[2] conditions) but showed no signs of auto-reactive NK cells (32, 33). On the contrary, their NK cells showed a reduced functional activity against target cells (34). A similar phenotype was observed in NK cells from human patients with a TAP-deficiency disorder, who have lost their MHC class I peptide-loading capacity (35). These observations illustrated that the environment of MHC class I expression, in which the NK cell develops, influences its capacity to be activated. This confirms an observation - made in 1987 - that NK cell activity against the known target cell line K562 was determined to be influenced by whether donors were homozygous or heterozygous for their HLA (36).

To explain the aforementioned phenomena, a model of 'NK cell licensing' was proposed. By engaging a self-MHC ligand with its cognate receptor a NK cell would be rendered functionally competent (37).

The at-least-one hypothesis could also be tested because, in healthy mice, subsets of NK cells were detected that lacked their inhibitory receptors for MHC class I. These cells were not overly auto-reactive but instead displayed a hyporesponsive phenotype (38). A similar phenotype was observed in human NK cells lacking all known inhibitory receptors (39). In line with this, human NK cells expressing only a specific activating receptor for self-MHC would in theory be auto-reactive; however, a mechanism was shown to exist that 'down-tunes' this unwanted reactivity (40). A rheostat model of functional maturation proposes that each NK cell is educated based on the type of receptors it expresses and on the ligands it encounters (41). In this manner,

[2]Specific pathogen free.

the collective of NK cells can detect very subtle differences in receptor-ligand interactions. The flexibility of this education system was highlighted by the adoptive transfer of NK cells to a new MHC setting, where after time they would become re-accustomed (42). Moreover, functional gain-of-function is observed for MHC class I-deficient NK cells that have been adoptively transfered to wild-type recipients. This suggests that neither exposure to MHC class I ligands during development in the bone marrow nor endogenous MHC class I expression is absolutely required for licensing (43). The fluidity of this system opens up new prospects for therapeutic approaches, and indeed some lines of evidence suggest that the education of NK cells can be influenced using antibodies directed against inhibitory receptors (44). The fact that similar observations on the functional maturation of both murine and human NK cells have been done suggests that education is an important control mechanism in their functional maturation.

Recently, it was recorded that NK cells possess some of the features that are normally associated with cells that belong to the adaptive compartment of the immune system (45). For instance, antigen recognition, subsequent expansion, contraction, and faster reactivation after re-challenge of NK cells in a receptor-specific manner has been reported. This adaptive character was shown by hapten-induced immune activation (46), but can also be provoked using viral pathogens (47). Recently, the longevity of these natural killer cells with a 'memory-like' phenotype upon viral infection was recorded as well (48).

BIOLOGICAL FUNCTION OF NK CELLS

NK cells protect the host from infection, and control the unwanted growth of malignant cells. Indeed, humans who lack natural killer cells suffer from severe infections (49, 50). Accordingly, in NK cell-deficient mice an increased susceptibility to viral infection along with greater tumour incidence and severity is also documented (51). The mechanism by which NK cells bestow their function is driven either by the direct killing of infected and transformed cells or by the secreting of chemokines/cytokines that influence the immune response. The mechanism of target-cell killing is mediated by death receptor interactions (FAS-FASL, TRAIL) or by the release of granzyme and perforin (52). The cytokine most associated with NK cell performance is interferon-γ and its production shapes: for instance, the 'cell-mediated' response by priming CD4+ T-helper type 1 cells (Th1). Interferon-γ also activates antigen presenting cells (APCs) to upregulate their MHC class I expression and produce cytokines, activates macrophages to kill intracellular pathogens, and has an anti-proliferative effect on virally and malignantly transformed cells (53). Moreover, NK cell responses in turn can be enhanced by particular cytokines (IL-12, IL-15, IL-18, and type I interferons) produced by APCs (54).

As well as these important functions, NK cells can regulate or exacerbate in-

flammatory responses (55). Immature dendritic cells (DC) are susceptible to NK cell-mediated autologous cytolysis, whereas mature DCs are protected (56). NK cells can positively - by promoting CD8+ T cell and humoral responses (57, 58) - or negatively (59, 60) influence T and B cell immunity. Furthermore, a possible subset known as NK-22 cells - found in mucosa associated lymphoid tissues - is specifically triggered by IL-23 to produce IL-22 (61). This niche of cells may play an important role in anti-bacterial responses and in the protection of epithelial barriers.

Finally, a very special role for NK cells is observed during pregnancy. A particular subset, referred to as decidual uterine NK cells (62), is the dominant lymphocyte population present in the maternal endometrial tissue (63). Implantation might predominantly involve an allogeneic recognition system based on NK cells (64). These cells secrete proangiogenic factors, such as vascular endothelial growth factor (VEGF), placental growth factor (PLGF), and NKG5. These factors propagate the remodelling of the feeding spiral arteries that support maternal endometrial tissues at sites of implantation and during subsequent placental development (63). In this way, NK cells promote reproductive success.

NK CELL RECEPTORS

Natural killer cells express different types of immunoreceptors (Table 1).
These receptors usually come in two functionally distinctive forms: namely, activating and inhibitory. The signal transduction of each receptor is largely determined by its transmembrane and cytoplasmic domains. Generally, inhibitory receptors possess one or more immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tail (65). Activating receptors lack ITIM but possess particular positively charged amino acid residues in their transmembrane region, which can interact with negatively charged residues on adaptor molecules. These adaptor molecules, including DAP10, DAP12, and FcεRIγ, contain immunoreceptor tyrosine-based activation motifs (ITAM), required to transduce an activating signal into the cell (66).

At least three protein families have a prominent impact on the activation state of the NK cell. These are the immunoglobulin-like receptors, C-type lectin-like, and natural cytotoxicity (NCR) receptors (Table 1). The immunoglobulin-like receptors comprise members of the CD2-like, LILR, LAIR, and KIR proteins, which share typical beta sheets stabilised by disulfide bonds formed between conserved cysteine residues. The C-type lectin-like receptors comprise members of the NKG2 and CD94 (KLRD1) family of receptors. Finally, the NCR comprise proteins that are designated as 'NKp' followed by the particular molecular weight of that molecule: for instance, NKp46 (Table 1).

KIR STRUCTURE AND NOMENCLATURE

During their discovery, the various KIR receptors were given many different names

Table 1 Key receptors on human NK cells and their ligands.

	Inhibitory receptors	Ligands
Immunoglobulin-like receptors	KIR2DL1	HLA-C
	KIR2DL2 KIR2DL3	HLA-C
	KIR2DL4	HLA-G
	KIR2DL5A KIR2DL5B	unknown
	KIR3DL1	HLA-B
	KIR3DL2	HLA-A
	KIR3DL3	unknown
	KIR3DX1 (soluble)	unknown
	LIR-1 (ILT2, LILRB1)	HLA class I
	LAIR1	Collagen
	SIGLEC 3, 7, 9	Sialic acid
C-type lectin-like	CD94 - NKG2A,B,E	HLA-E
	KLRG1	Cadherins
	NKR-P1A (KLRB1)	LLT-1

	Activating receptors	Ligands
Immunoglobulin-like receptors	KIR2DS1	HLA-C (weak)
	KIR2DS2 KIR2DS3	HLA-C (very weak)
	KIR2DS4	HLA-A, -C
	KIR2DS5	unknown
	KIR3DS1	HLA-B (by association)
	CD16	Fcg
	DNAM1 (CD226)	Nectin2 NECL5
	CRTAM	NECL2
	CD27	CD70
	CEACAM1	CEACAM1
	NTB-A	NTB-A
	SLAMF7	SLAMF7
	2B4	CD48
NCR	NKp30	B7-H6, BAT3
	NKp44	Viral HA
	NKp46	Viral HA
C-type	CD94-NKG2C	HLA-E
	NKG2D-NKG2D	ULBP/RAET/MIC
	NKp80	AICL

by various research teams (Table 2), but a standard nomenclature was introduced as soon it was recognised that the research community was dealing with a multigene family controlling multiple related genes. Initially, all these NK cell receptors were termed killer inhibitory receptor (KIR) (67), but it became clear that *KIR* genes also encompass activating receptors. Therefore the acronym KIR was changed into killer cell immunoglobulin-like receptor (68, 69).

Depending on their size, human KIR proteins consist of up to three extracellular Ig-like domains, followed by a common organisation of stem, transmembrane, and cytoplasmic domain. A differential system to classify *KIR* genes based on their extracellular Ig-like domains, the D0-D1-D2 system, was introduced (70). Based on

the number of Ig-like domains that is present, the first part of the name is either designated 1D, 2D or 3D, and if the cytoplasmic domain is present (long) or truncated (short), the gene is called, for instance, KIR3DL or KIR3DS, respectively (Fig. 3).

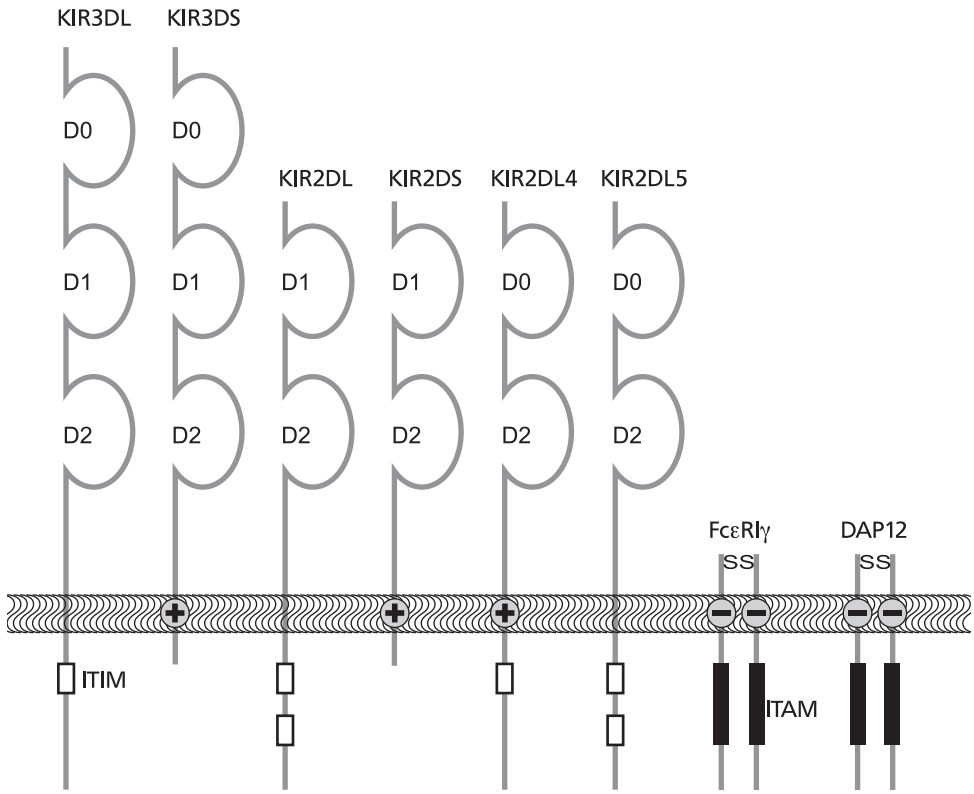

Figure 3 Schematic overview of KIR structure and nomenclature. A KIR molecule consists of extracellular immunoglobulin-like and stem domains, a transmembrane domain that may contain a positive charged amino acid, which can interact with different adaptor molecules that contain activating signal transducing ITAMs, and cytoplasmic domains that may contain inhibitory signal transducing ITIMs.

KIR DISCOVERY IN HUMAN

One of the mechanisms of recognition by human NK cells is associated with MHC class I. This was demonstrated by the transfection of HLA genes in target cells, which may inhibit killing depending on the combination of NK cell receptor and its MHC class I ligand (25, 71). In the first instance, the structures on NK cells responsible for this interaction were not known. NK cell clones that target cells with known HLA allotypes (72) or K562 cell lines transfected with particular HLA class I genes (73) were

discovered. Among the specificities identified were NK cell clones reacting against HLA-A (73), -B (74, 75) and -C (76-80). Fine mapping revealed that reactivity is associated with the presence of specific amino acid residues of the HLA molecules (81) (Table 2).

Table 2 KIR molecules and ligand specificity.

KIR	Signal	HLA ligand	HLA specificity	Alternative names	References
KIR2DL1	inhibitory	HLA-C	C2: Asn77, Lys80	nkat1, cl-42, p58.1, 47.11, nkat6, cl-43	(86, 87)
KIR2DL2	inhibitory	HLA-C	C1: Ser77, Asn80 HLA-B*4601	nkat2, nkat2a, nkat2b, cl-6, p58	(87, 88)
KIR2DL3	inhibitory	HLA-C	C1: Ser77, Asn80 HLA-B*4601		(86, 87)
KIR2DL4	inhibitory/ activating	HLA-G	HLA-G	103AS, 15.212	(89-91)
KIR2DL5A	inhibitory	unknown	unknown	KIR2DL5.1	(92)
KIR2DL5B	Inhibitory	unknown	unknown	KIR2DL5.2, KIR2DL5.3	(92)
KIR2DS1	activating	HLA-C	C2: Asn77, Lys80	Eb6ActI, Eb6ActII	(90)
KIR2DS2	activating	HLA-C (very weak)	C1: Ser77, Asn80	nkat5, cl-49	(87, 88, 93)
KIR2DS3	activating	HLA-C (very weak)	C1: Ser 77, Asn80	nkat7	(88, 93)
KIR2DS4	activating	HLA-A HLA-C	A*1102, A*1101 C1: C*1601, C*0101, C*1402 C2: C*0501, C*0202, C*0401	nkat8, cl-39, KKA3	(87, 88, 94, 95)
KIR2DS5	activating	unkown	unknown	nkat9	(88, 93)
KIR2DP1	pseudogene			KIRZ, KIRY, KIR15 KIR2DL6, AMB11	(92)
KIR3DL1	Inhibitory	HLA-B	Bw4: Ile80/Thr80	nkat3, cl-2, NKB1, NKB1B	(86, 87, 96)
KIR3DL2	Inhibitory	HLA-A HLA-B	A3, A11 B27	nkat4, nkat4a, nkat4b, cl-5	(86, 87, 97, 98)
KIR3DL3	Inhibitory	unkown	unknown	KIRC1, KIR3DL7, KIR44	(99)
KIR3DS1	activating	HLA-B (by association)	unknown	nkat10	(88)
KIR3DP1	pseudogene			KIRX, KIR48, KIR2DS6, KIR3DS2P	(92)

Remarkably, these epitopes correlated with the serological definitions (A3, A11, Bw4, Bw6, Cw3, Cw4) that had been defined long ago by allo-antisera (82).

Immunisation of mice with human NK cell clones yielded antibodies that could disrupt the interaction between NK and target cell, or could trigger the NK cell to become activated (83, 84). With these antibodies specific receptors on NK cells could be targeted and analysed biochemically. By means of immunoprecipitation assays the molecular weight could be determined at 50, 58, 70 kD, respectively (p50, p58, p70).

Some of these antibodies could either inhibit or trigger a NK cell clone, suggesting that multiple receptors with differential signalling profiles do exist (85).
To gain further insight into the genetics of all these different receptors, cDNA was isolated either from NK cell clones or T cell subsets. By screening cDNA libraries, the transcripts and corresponding genes that encode particular NK cell receptors were identified, and in this manner the genetic make-up of these molecules was elucidated (86-88, 96). All these NK cell receptors are members of the superfamily of immunoglobulin receptors, and were later designated as KIR. Moreover, cDNA sequencing resulted in the discovery of a fourth KIR family member with two Ig-like domains, of which the first D0-domain has high homology with the p70 (KIR3DL) genes, while the second D2-domain has homology with both p58 (KIR2DL) and p70 genes (89). The transmembrane and cytoplasmic domains of this KIR2DL4 differ from other known KIR (Fig. 3). Using primers that were derived from chimpanzee KIR genes, an additional human KIR was discovered, and designated KIR2DL5. Although this gene shares the D0-D2 structure of KIR2DL4, it differs and is situated on a separate locus (92).

With cloned receptors available, the specificity of individual receptors could now be defined (Table 1). For instance, the receptor interaction of KIR3DL2 with specific HLA-A allotypes was further revealed (100). Clones that contained this particular receptor, for instance, did not lyse target cells with the HLA-A3 or -A11 allotype, but effectively lysed target cells with the -A1, -A2, -A24 allotypes (101).

Similarly, the specific amino acid residues of the Bw4 motif that could inhibit particular subsets of NK cells expressing KIR3DL1 were discovered (102). Alternatively, amino acid residues 44, 45, and 70 determined the specificity of receptor KIR2DL1 for HLA-C group 2 antigens and of receptors KIR2DL2 and -3 for HLA-C group 1 (103).

KIR AND ITS RELATION TO REPRODUCTIVE BIOLOGY AND DISEASE

Given the important role of NK cells in both innate and adaptive immunity as well as in pregnancy, it can be extrapolated that KIR-ligand interactions are influential in these settings. Both MHC class I and KIR genes are highly polymorphic within a population. It is hypothesised that epistatic interaction between different combinations of alleles can influence the outcome of disease and immune disorders in their own manner. However, the chance that two unrelated individuals share a KIR genotype is very small. Because of this diversity of interactions and the incomplete understanding of the ligands for KIR, the interpretation of genetic studies is difficult. Hence, in order to draw meaningful conclusions, the manner in which KIR-HLA disease association studies are interpreted is at best a simplification of the complexity of KIR-MHC class I interactions (104). Despite this, however, expansive genetic studies with well-controlled populations have provided the first insight into the role of KIR in disease (Table 3) (105-107).

Table 3 Disease associations with combinations of KIR and HLA.

Disease and Response	Association	References
Infectious diseases		
HIV-1, delayed progression to AIDS rapid progression	Presence of KIR3DS1 and HLA-B (Ile80) Absence of HLA-B ligand	(108)
HIV-1, delayed progression to AIDS and lower plasma HIV RNA levels	Presence of KIR3DL1 and HLA-B (Bw4)	(109)
Hepatitis C resolution	Presence of KIR2DL3, HLA-C1	(110)
Plasmodium falciparum response *in vitro*	Presence of KIR3DL2*002 on NK cell	(111)
Ebola virus: fatal outcome	Presence of KIR2DS1 or KIR2DS3	(112)
Autoimmunity		
Crohn's disease increased susceptibility	Heterozygous for KIR2DL2/KIR2DL3 and presence of HLA-C2	(113)
Rheumatoid arthritis (positive response to anti-TNF-α therapy	Presence of KIR2DS2 and homozygous for HLA-C	(114)
Type 1 diabetes: increased risk	Presence of KIR2DS2 and HLA-C1	(115)
Multiple sclerosis: positive effect	Absence of HLA-C2 and HLA-Bw4	(116)
Cancer, hematopoietic stem cell transplantation against leukemia		
Myeloid leukemia: less relapse, increased graft vs. host disease	Presence of KIR2DS1 and HLA-C2	(117)
Outcome after transplantation in acute myelogenous leukemia improved	Recipient has less ligand for donor KIR Donor is homozygous for Cen-B KIR haplotype	(118)
Malignant melanoma: increased risk	Presence of KIR2DL2/KIR2DL3 and HLA-C1	(119)
Cervical Neoplasia (HPV) decreased risk increased risk	Presence of KIR3DL1 Presence of KIR3DS1	(120)
Pregnancy		
Pre-eclampsia: increased risk	Mother with AA KIR haplotype, fetus HLA-C2	(121)
Recurrent miscarriage: increased risk	Mother with AA KIR haplotype, fetus HLA-C2	(122)
Reproductive failure: increased risk decreased risk	Mother with AA KIR haplotype, fetus HLA-C2 Mother has Tel-B KIR haplotype with KIR2DS1	(123)

In HIV-1 infection, the presence of KIR3DS1 and its HLA-B ligand is associated with a delayed progression to AIDS (108). Alternatively, in the absence of its ligand, the presence of KIR3DS1 is associated with a more rapid progression. Moreover, KIR3DL1 alleles and various HLA-B alleles with a Bw4 motif influence the plasma HIV RNA levels and progression to AIDS. The pleiotrophic effects of KIR/HLA interaction have also been associated with a reduction in opportunistic infections in seropositive individuals (109). For hepatitis C virus infection it has been shown that the presence of the inhibitory KIR2DL3 and its HLA-C group 1 ligand directly influences resolution of the infection (110).

With regard to autoimmunity, certain KIR haplotypes have also been found to be associated with susceptibility or protection (113-116). In cancer research, therapeuti-

cal approaches involving KIR are being investigated. One approach to influence NK cells *in vivo* is to block inhibitory KIR receptors with antibodies to lower the threshold for NK cell activation (124). In the context of therapeutic hemopoietic stem cell transplant against myeloid leukemia, there is strong evidence that particular combinations of the donor's KIR genotype and the recipient's HLA type can influence beneficially the graft-versus-leukemia effects (117, 118).

In pregnancy, the immune system may act as a double-edged sword: and as early as in 1953, Medawar had proposed that pregnancy poses an immunological paradox (125). Although a full-blown immune response against the foetus has to be avoided, evidence has shown that particular alloreactivity of maternal uterine NK cells may be beneficial (64). Indeed, specific combinations of maternal KIR and foetal HLA are associated with reproductive success. The underlying manner of alloreactivity by maternal KIR and foetal HLA influences the risk of pre-eclampsia (121), recurrent miscarriage (122) and reproductive failure (123).

IMMUNOGENETICS OF THE KIR GENE CLUSTER

In humans, the *KIR* genes are located on the long arm of chromosome 19, and map to a 100-200 kilobase region of the leukocyte receptor complex (LRC) defined as 19q13.4 (126) (Fig. 4).

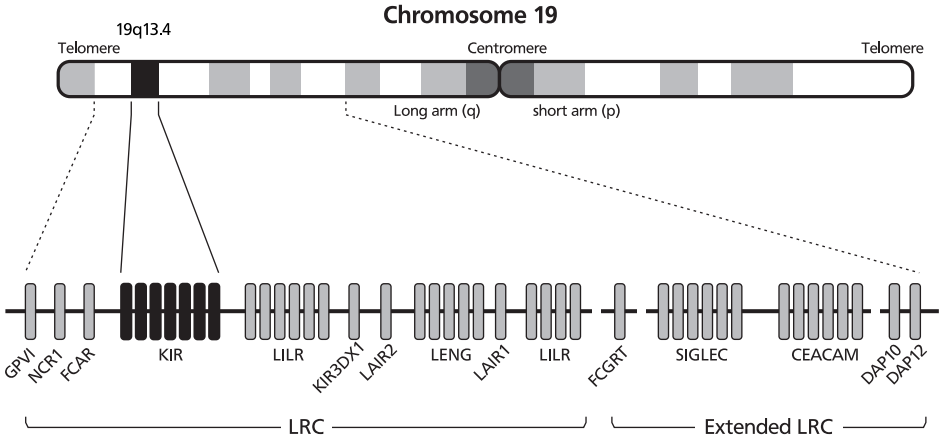

Figure 4 Genomic context of the KIR gene cluster located within the LRC on chromosome 19.

The LRC constitutes a large cluster with a high gene-density of immune-related genes. In addition to , this region consists of the leukocyte immunoglobulin-like receptors (*LILR*), the leukocyte-associated immunoglobulin-like receptors (*LAIR*), sial-

ic acid-binding immunoglobulin-like lectins (*SIGLEC*), the Fc-γ receptor transporter (*FCgRT*), the Fc-α receptor (*FCAR*), and the natural cytotoxicity triggering receptor 1 (*NCR1* also called Nkp46), as well as genes encoding for the DNAX activating proteins of 10kDa and 12kDa (*DAP10* and *DAP12*). The LAIR and LILR gene families are separated by one gene that resembles the structure of KIR and is phylogenetically most closely related to KIR. Therefore, this gene is designated as (127). The KIR gene complex is flanked centrometically by the gene and telomerically by (128) (Fig. 4). Within this region, KIR genes are arranged tandemly and display a high degree of sequence similarity, which predisposes the genes to evolve by non-allelic homologous recombination (129). This gives rise to a plastic system that shows expansion and contraction (99). In human populations many different KIR haplotypes have been observed, and generally a division can be made between group A (130) and group B haplotypes (131) (Fig. 5).

Figure 5 Gene content of human group A and B haplotypes. The group A haplotype contains the depicted genes or fewer, and group B is any haplotype which is not group A.

The group A haplotypes are characterised by the absence of the following genes: *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5*, and *KIR3DS1*, while the group B haplotypes are defined by the presence of one or more of these genes. Moreover, all haplotypes comprise the framework genes, *KIR3DL3*, *KIR2DL4*, and *KIR3DL2* and the pseudogene *KIR3DP1* (99).

Although recombination between different haplotypes can give rise to an enormous diversity, much of the observed haplotype diversity can be explained by dividing these haplotype groups into centromeric and telomeric parts, whereby new haplotypes may be formed by equal and unequal crossover events between these regions (132, 133). Within many populations, this diversity gives rise to a myriad of haplotypes and genotypes (134-136).

THE EVOLUTION OF THE KIR LIGANDS (MHC CLASS I) IN PRIMATES

The MHC class I molecules are divided into two subclasses designated Ia (classicals) and Ib (nonclassicals), respectively. In humans, there are three classicals - *HLA-A*, *-B*, and *-C* - whereas the *-E*, *-F*, and *-G* loci are considered to represent the nonclassicals. By definition, the classical proteins are expressed on all nucleated cells and display

abundant levels of polymorphism. The nonclassicals often exhibit differential tissue distribution and are characterised by the absence or low levels of polymorphism. The classical transplantation antigens are involved in the presentation of processed peptide fragments to cytotoxic T cells (CTL) (137), while the nonclassical MHC class I molecules display a complex variety of functions (138). Because KIR and its MHC ligands are functionally so closely connected, it is highly likely that co-evolution has occurred between these two gene systems. To facilitate an educated hypothesis on the organisation of the rhesus macaque KIR gene system, it is necessary to understand the extent of the species-specific evolution of both complexes. Indeed, the MHC class I genes of different primates have been studied extensively and have been assigned designations based on their Latin species name (e.g. *Macaca mulatta MHC-A* is named *Mamu-A*) (Fig. 6).

MHC class I

	shared					species specific
species	classicals			nonclassicals		
	A	B	C	E	G	
Human	*HLA-A*	*HLA-B*	*HLA-C*	*HLA-E*	*HLA-G*	
Chimpanzee	*Patr-A*	*Patr-B*	*Patr-C*	*Patr-E*	*Patr-G*	*Patr-AL*
Gorilla	*Gogo-A*	*Gogo-B*	*Gogo-C*	*Gogo-E*	*Gogo-G*	
Orangutan	*Popy-A*	*Popy-B*	*Popy-C*	*Popy-E*	*Popy-G*	
Gibbon (white-cheeked)	*Nole-A*	*Nole-B* expanded	absent	*Nole-E*	absent	
Rhesus monkey	*Mamu-A* expanded	*Mamu-B* expanded	absent	*Mamu-E*	inactive function taken over by→	*Mamu-I* *Mamu-AG*

Figure 6 Overview of MHC organisation in primates.

In chimpanzees, the evolutionary closest living relatives of humans, orthologues of the *HLA* genes are present and appear to be polymorphic as well (139, 140). Humans and chimpanzees share lineages but the sharing of alleles appears to be a rare event (141). Chimpanzees also possess a unique locus designated as *Patr-AL* that shows differential haplotype distribution and displays low levels of polymorphism, and of which the gene product has an HLA-A2-like peptide binding motif (142). The classical MHC class I molecules of the chimpanzee possess the signature of a repertoire reduction. This selective sweep may have been caused by SIV or a closely related retrovirus (143). Comparative analysis of the chimpanzee MHC orthologues has illustrated that structural motifs that may be recognised by KIR have been retained. As early as in 1981, it was shown that chimpanzee B cell lines are resistant to lysis by human NK

cells, which suggests that conserved inhibitory signals are present (144).

The MHC of gorillas seems to resemble the system observed in humans and chimpanzees. Class I loci are shared, and, moreover, humans and gorillas share the *A2* lineage, whereas the corresponding lineage is absent in chimpanzees (145). In orangutans, there is evidence for the presence of the *Popy-A* and *-B* genes, but they may not be directly orthologous to *HLA-A* and *HLA-B* (146, 147). On certain haplotypes the *Popy-B* gene may have been duplicated (148), while *Popy-C* is only present on 50% of the haplotypes, and thus a significant minority (25%) of individuals lack an *HLA-C*-like locus (146). In gibbons, the equivalents of *HLA-A* and *-B* have been detected (148), while those of *HLA-C* and *-G* loci are most likely absent (149).

The *A* and *B* loci of rhesus macaques have been subjected to several rounds of expansion (150). Moreover, the *C* locus is absent in this species (151). The orthologues of *HLA-E* (152) and *–F* (153) are highly conserved. The equivalent of *HLA-G* has been inactivated but its function has been taken over by *Mamu-AG* (154). In other macaque and Old World monkey species, a similar situation seems to exist.

COMPARATIVE GENETICS OF KIR IN PRIMATES

In recent years, comparative genetic studies have substantially increased the knowledge on the evolution of the KIR gene system. Complete KIR haplotypes of human, chimpanzee, orangutan, gibbon, and rhesus macaque have been sequenced (133, 149, 155, 156). In this manner, the evolutionary history of these regions could be deduced by analysing intergenic, intron and exon sequences. Moreover, comparison of ancient retroviral elements and repeat sequences within the introns yielded an insight into the time period when these had to be present in the genome (157). From this it could be deduced that an ancestral KIR gene had to be present approximately 60 to 100 million years ago, whereas duplication events range from around 30 to 45 million years ago (155).

The genetic relationships between different genes can be depicted by phylogenetic trees that portrait evolutionary distance between for instance KIR introns (Fig. 7). This led to the important discovery that four lineages of KIR are represented by gene members of the different primate species of which these sequences were available at the time. This subdivision has likely occured before speciation, and is therefore in accordance with the proposed duplication events. After speciation, genes of a certain lineage may have still undergone considerable divergence, which is, for instance, illustrated by the rhesus macaque KIR genes in lineage II. These genes cluster separately and, therefore, are sometimes assigned to the sub-lineage IV (Fig. 7). All human KIR genes can be assigned to particular lineages. The *KIR2DL4*, *KIR2DL5A* and *-B* genes belong to lineage I. Lineage II comprises *KIR3DL1*, *KIR3DL2*, and *KIR3DS1*. By far the most genes cluster in lineage III: *KIR2DL1-3*, *KIR2DS1-5*, as well as the pseudogenes *KIR2DP1*, and *KIR3DP1*. Finally, Lineage V comprises the framework *KIR3DL3*

gene. By comparing the coding regions of genes of specific lineages between species, it is possible to detect patterns of species specific evolution. Moreover, it is possible to discover which genes have remained orthologous to human KIR.

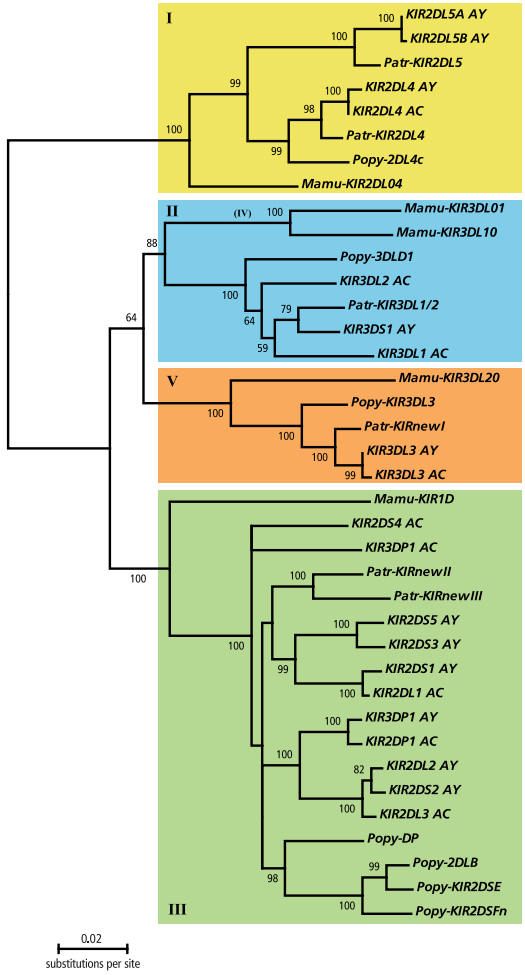

Figure 7 Phylogenetic tree depicting the four KIR lineages based on intron 3 sequences obtained from human (AY; AY320039, AC; AC006293), chimpanzee (*Pan troglodytes*; Patr, BX842589), orangutan (*Pongo pygmaeus*; Popy, EF014479), and rhesus macaque (*Macaca mulatta*; Mamu, BX842591). A neighbor-joining approach was used to construct the tree (midpoint rooted) with Tamura-Nei method and 1000 replicates. Bootstrap values that indicate confidence levels above 50 percent are shown.

The presence of KIR genes in the chimpanzee was first demonstrated by screening genomic DNA on southern blots with a probe directed against a conserved KIR structure (158). The first chimpanzee KIR cDNAs were obtained by using degenerate primers for amplification (159), and additional KIR genes were later identified in two more animals (155). Sequence analysis of these genes revealed that members of all KIR lineages are also present in chimpanzee. Lineage I and V comprise genes that are closely related to the human ones and therefore orthologous. Contrary, chim-

panzee lineage II genes have diverged considerably from human and are no longer considered to be orthologues. In humans, lineage III is represented by KIR with a D1-D2 conformation, while the chimpanzee is represented by orhologous genes that encode either two or three Ig-like domains. Although the human and chimpanzee species have diverged from a common ancestor relatively recently (Fig. 1), the KIR system shows considerable differences between species, which suggests that rapid evolution has occurred.

This rapid evolution was further confirmed by comparative genetics of KIR in the bonobo, a species which is closely related to the common chimpanzee (Fig. 1). Although genotyping showed that bonobo KIR resembles that of the chimpanzee, considerable differences were manifested (160). Bonobo KIR clusters within the four defined lineages, and orthologues of the human framework genes *KIR3DL3*, *KIR2DL4*, and *KIR3DL2* were detected. However, no lineage III genes were observed, while lineage II genes contain novel combinations of elements that are observed independently in humans and in chimpanzees. Since haplotypes in this studied cohort were characterised by a low gene content, it was proposed that these would embrace the minimal essential functions of KIR.

In the gorilla, all four lineages are present as well (161). However, the orthologue of KIR2DL4 (lineage I) lacks ITIMs, and members of lineage II display a chimeric structure and seem to have been formed by domain shuffling. Although lineage III genes are present, these are neither orthologous to chimpanzee nor to human KIR, again reflecting rapid evolution. This study confirmed the observations that new KIR genes may be formed by recombinations leading to exon shuffling.

These closely related hominids have more or less retained a similar MHC architecture, therefore, orangutans are informative to study the co-evolution of KIR with their MHC ligands (162), since there has been such a drastic divergence in MHC evolution as compared to humans and other great ape species. While all KIR lineages are represented in the orangutan, one common feature of all reported genes is that they exhibit a patchwork pattern of substitution reflecting their species-specific evolution by means of recombination. Since in orangutans only a C1 group MHC-C allotype may be present, it was observed that indeed lineage III genes displayed the characteristics of C1 specificity and not C2 specificity. This not only indicated that the lineage III KIR in hominids has co-evolved with the emergence of MHC-C, but also that species-specific co-evolution has occured with particular MHC-C allotypes (156, 163).

With regard to the gibbon MHC, differences from that of humans are even more drastic (Fig. 6). Therefore, it is hypothesised that the effect of co-evolution on the KIR system will be a significant divergence from other hominoids. KIR haplotypes of two species of gibbon have been described (149). One observation was that the haplotypes are smaller in comparison to other hominoids. Both species appear to have lost either their lineage I homologue of *KIR2DL4* or its inhibitory function. Lineage

II KIR genes are observed, although they have diverged considerably. Notably, lineage III genes are still present but they cluster separately, suggesting species-specific evolution. Furthermore, a novel activating KIR is detected, which was formed by recombination of a lineage I with a lineage III gene, highlighting the plasticity of this system. Finally, the lineage V homologue of *KIR3DL3* is the only gene present on all haplotypes, although it appears to be polymorphic and has preserved both ITIMs.

KIR IN THE RHESUS MACAQUE

The comparative genetic analyses of the KIR system in different primates highlighted certain evolutionary characteristics. The longer the time since an ancestor was shared with humans, the more differential evolution may have occurred. Moreover, because this evolution is possible at a rapid pace, large differences between KIR systems may occur. Co-evolution with changes in MHC compositions may also have had dramatic effects, leading in some cases to the loss of a KIR lineage. Because the evolutionary distance between humans and rhesus macaque is great, and significant changes in MHC composition have occurred (Fig. 6), this will likely have had an effect on the KIR architecture in this Old World monkey species. The extent of this species-specific evolution is for the most part unknown, since only few studies have investigated rhesus macaque KIR.

In 2005, the one rhesus macaque KIR haplotype was completely sequenced (155). This haplotype was constructed from two BAC clones that cover the KIR gene cluster, and, as in humans, it is flanked by LILR and FCAR genes, respectively. Within this cluster, five genes and one pseudogene, *DP*, were identified (Fig. 8).

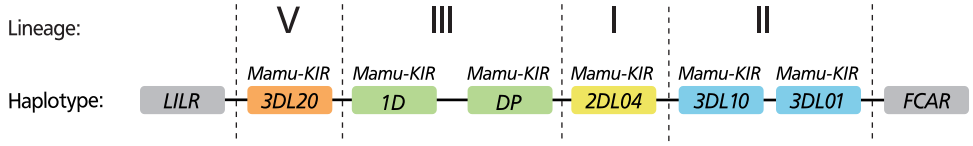

Figure 8 The architecture of the one rhesus macaque KIR haplotype (BX842591).

Notably, no genes that could encode activating KIR were detected on this haplotype. Subsequent analysis of the genomic sequences revealed that the genes are tightly clustered in a similar orientation with relatively short intergenic sequences, which is in line with the human situation. Some preliminary conclusions could be drawn; orthologues of lineage I and V genes exist in the rhesus macaque. Lineage II genes appeared to have diverged considerably and were assigned to a sub-lineage IV. Whereas, the only remnant of lineage III may be a gene, *Mamu-KIR1D*, that encodes a product

with one Ig-like domain and is possibly expressed in soluble form.

With regard to cDNA, the first rhesus macaque KIR sequences were obtained from five unrelated monkeys (164). Simultaneously, sequences involving one animal were reported from a decidual NK cell cDNA library (165), and subsequently also from two unrelated animals (166). In these eight animals, different activating and inhibitory genes were observed, which suggests that these genes are polymorphic and display copy number variation. However, the extent of this KIR diversity in a large population is still unknown, nor is anything known on variations in KIR repertoire between rhesus macaques from different geographic origins. Moreover, from these unrelated animals no haplotype information could be gathered.

THESIS AIM AND OVERVIEW

The aim of this thesis is to provide more insight into the KIR gene system of the rhesus macaque. By characterising and understanding the complexity of this system, the different types of selection that have acted on it may be deduced. Subsequently, hypotheses can be postulated as to which evolutionary forces are driving these types of selection. We wished to determine whether the species-specific expansion of MHC class I genes is reflected through co-evolution in their KIR genes. Furthermore, through increased knowledge of the differences and similarities of this system with that of humans, it may be possible to refine the selection of rhesus macaques for biomedical studies based on genetic factors.

In humans, *KIR2LD4* is considered to be a framework gene, and one of the most conserved KIR genes in primates. Although it displays limited allelelic variation, two forms exist, resulting in a long and a truncated gene product. Moreover, these forms appear to be equally distributed within a population. In the rhesus macaque, little was known about allelic variation of *2DL4*. Therefore, extensive sequence analyses of *Mamu-KIR2DL04* were performed to determine the degree of its polymorphism in a large population of Indian-origin animals. Furthermore, a comparison of synonymous versus non-synonymous polymorphisms in human and rhesus macaque KIR2DL4 revealed different modes of selection acting upon it (**Chapter 2**).

In rhesus macaques, it appeared that the activating genes were under represented in the reported KIR genes. Additionally, no activating gene had been reported on the published haplotype. Therefore, manners to detect activating KIR on a genomic and on a transcriptional level were investigated. In a panel of pedigreed animals, the allelic variation of activating KIR, as well as their haplotype composition, was investigated. Moreover, intron-exon boundaries were analysed to gain insights into the evolution of activating KIR (**Chapter 3**).

In human populations an enormous amount of genotypic and haplotypic KIR diversity can be observed. Because KIR neither in related animals nor in a large colony had ever been analysed, practically nothing was known of this diversity in rhesus

macaques. Therefore, KIR transcripts from four families were sequenced, and genotypes and haplotypes determined. For the first time it could be investigated whether the KIR system in rhesus macaque mirrors the type of haplotypic organisation and complexity observed in humans (**Chapter 4**).

Based on the data obtained and in a collaborative effort, an official rhesus macaque KIR nomenclature system has been devised. In humans the current standard for determining KIR genotypes is a sequence specific primer PCR assay. To further facilitate the analysis of KIR in rhesus macaques a typing system was developed and a large pedigreed cohort of animals from different geographical origins was screened. In this manner, genotypes and haplotypes could be defined, and for the first time an overview of the diversity that is present in a larger population could be obtained, and compared with that in the human population (**Chapter 5**).

In conclusion, the current KIR knowledge involving the rhesus macaque is summarized, differences and similarities between KIR in humans and in macaques are discussed and avenues for further research will be pointed out (**Chapter 6**).

REFERENCES

1. Benton MJ, Donoghue PCJ. 2007. Paleontological evidence to date the tree of life. *Mol Biol Evol* 24: 26-53
2. Sibley CG, Comstock JA, Ahlquist JE. 1990. DNA hybridization evidence of hominoid phylogeny: a reanalysis of the data. *J Mol Evol* 30: 202-36
3. Horai S, Satta Y, Hayasaka K, Kondo R, Inoue T, Ishida T, Hayashi S, et al. 1992. Man's place in Hominoidea revealed by mitochondrial DNA genealogy. *J Mol Evol* 35: 32-43
4. Glazko GV, Nei M. 2003. Estimation of divergence times for major lineages of primate species. *Mol Biol Evol* 20: 424-34
5. Perelman P, Johnson WE, Roos C, Seuánez HN, Horvath JE, Moreira MAM, Kessing B, et al. 2011. A molecular phylogeny of living primates. *PLoS Genet* 7: e1001342
6. Gibbs RA, Rogers J, Katze MG, Bumgarner R, Weinstock GM, Mardis ER, Remington KA, et al. 2007. Evolutionary and biomedical insights from the rhesus macaque genome. *Science* 316: 222-34
7. Locke DP, Hillier LW, Warren WC, Worley KC, Nazareth LV, Muzny DM, Yang S-P, et al. 2011. Comparative and demographic analysis of orang-utan genomes. *Nature* 469: 529-33
8. Osada N, Hashimoto K, Kameoka Y, Hirata M, Tanuma R, Uno Y, Inoue I, et al. 2008. Large-scale analysis of Macaca fascicularis transcripts and inference of genetic divergence between M. fascicularis and M. mulatta. *BMC Genomics* 9: 90
9. Ferguson B, Street SL, Wright H, Pearson C, Jia Y, Thompson SL, Allibone P, et al. 2007. Single nucleotide polymorphisms (SNPs) distinguish Indian-origin and Chinese-origin rhesus macaques (Macaca mulatta). *BMC Genomics* 8: 43
10. Hernandez RD, Hubisz MJ, Wheeler DA, Smith DG, Ferguson B, Rogers J, Nazareth L, et al. 2007. Demographic histories and patterns of linkage disequilibrium in Chinese and Indian rhesus macaques. *Science* 316: 240-3
11. Cudkowicz G, Stimpfling JH. 1964. Hybrid resistance to parental marrow grafts: association with the K region of H-2. *Science* 144: 1339-40
12. Snell GD, Russell E, Fekete E, Smith P. 1953. Resistance of various inbred strains of mice to tumor homoiotransplants, and its relation to the H-2 allele which each carries. *J Natl Cancer Inst* 14: 485-91
13. Cudkowicz G, Bennett M. 1971. Peculiar immunobiology of bone marrow allografts. I. Graft rejection by irradiated responder mice. *J Exp Med* 134: 83-102
14. Takasugi M, Mickey MR, Terasaki PI. 1973. Reactivity of lymphocytes from normal persons on cultured tumor cells. *Cancer Res* 33: 2898-902
15. Herberman RB, Nunn ME, Lavrin DH. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *Int J Cancer* 16: 216-29
16. Kiessling R, Klein E. 1973. Cytotoxic potential of mouse spleen cells on H-2 antibody-treated target cells. *J Exp Med* 137: 527-32
17. Kiessling R, Klein E, Wigzell H. 1975. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol* 5: 112-7

18. Kiessling R, Klein E, Pross H, Wigzell H. 1975. “Natural” killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol* 5: 117-21

19. Kiessling R, Petranyi G, Kärre K, Jondal M, Tracey D, Wigzell H. 1976. Killer cells: a functional comparison between natural, immune T-cell and antibody-dependent in vitro systems. *J Exp Med* 143: 772-80

20. Saksela E, Timonen T, Ranki A, Häyry P. 1979. Morphological and functional characterization of isolated effector cells responsible for human natural killer activity to fetal fibroblasts and to cultured cell line targets. *Immunol Rev* 44: 71-123

21. Robertson MJ, Caligiuri MA, Manley TJ, Levine H, Ritz J. 1990. Human natural killer cell adhesion molecules. Differential expression after activation and participation in cytolysis. *J Immunol* 145: 3194-201

22. Cudkowicz G, Bennett M. 1971. Peculiar immunobiology of bone marrow allografts. II. Rejection of parental grafts by resistant F 1 hybrid mice. *J Exp Med* 134: 1513-28

23. Kärre K, Ljunggren HG, Piontek G, Kiessling R. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 319: 675-8

24. Ljunggren HG, Kärre K. 1990. In search of the ‘missing self’: MHC molecules and NK cell recognition. *Immunol Today* 11: 237-44

25. Storkus WJ, Alexander J, Payne JA, Dawson JR, Cresswell P. 1989. Reversal of natural killing susceptibility in target cells expressing transfected class I HLA genes. *Proc Natl Acad Sci USA* 86: 2361-4

26. Storkus WJ, Alexander J, Payne JA, Cresswell P, Dawson JR. 1989. The alpha 1/alpha 2 domains of class I HLA molecules confer resistance to natural killing. *J Immunol* 143: 3853-7

27. Storkus WJ, Salter RD, Alexander J, Ward FE, Ruiz RE, Cresswell P, Dawson JR. 1991. Class I-induced resistance to natural killing: identification of nonpermissive residues in HLA-A2. *Proc Natl Acad Sci USA* 88: 5989-92

28. Bix M, Liao NS, Zijlstra M, Loring J, Jaenisch R, Raulet D. 1991. Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. *Nature* 349: 329-31

29. Karlhofer FM, Ribaudo RK, Yokoyama WM. 1992. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. *Nature* 358: 66-70

30. van Bergen J, Thompson A, Retière C, Trowsdale J, Koning F. 2009. Cutting edge: killer Ig-like receptors mediate “missing self” recognition in vivo. *J Immunol* 182: 2569-72

31. Valiante NM, Uhrberg M, Shilling HG, Lienert-Weidenbach K, Arnett KL, D’Andrea A, Phillips JH, et al. 1997. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity* 7: 739-51

32. Höglund P, Ohlén C, Carbone E, Franksson L, Ljunggren HG, Latour A, Koller B, et al. 1991. Recognition of beta 2-microglobulin-negative (beta 2m-) T-cell blasts by natural killer cells from normal but not from beta 2m- mice: nonresponsiveness controlled by beta 2m- bone marrow in chimeric mice. *Proc Natl Acad Sci USA* 88: 10332-6

33. Ljunggren HG, Van Kaer L, Ploegh HL, Tonegawa S. 1994. Altered natural killer cell repertoire in Tap-1 mutant mice. *Proc Natl Acad Sci USA* 91: 6520-4

34. Liao NS, Bix M, Zijlstra M, Jaenisch R, Raulet D. 1991. MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. *Science* 253: 199-202

35. Zimmer J, Donato L, Hanau D, Cazenave JP, Tongio MM, Moretta A, de la Salle H. 1998. Activity and phenotype of natural killer cells in peptide transporter (TAP)-deficient patients (type I bare lymphocyte syndrome). *J Exp Med* 187: 117-22

36. Dubey DP, Yunis I, Leslie CA, Mehta C, Yunis EJ. 1987. Homozygosity in the major histocompatibility complex region influences natural killer cell activity in man. *Eur J Immunol* 17: 61-6

37. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song Y-J, Yang L, French AR, et al. 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* 436: 709-13

38. Fernandez NC, Treiner E, Vance RE, Jamieson AM, Lemieux S, Raulet DH. 2005. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* 105: 4416-23

39. Anfossi N, André P, Guia S, Falk CS, Roetynck S, Stewart CA, Breso V, et al. 2006. Human NK cell education by inhibitory receptors for MHC class I. *Immunity* 25: 331-42

40. Fauriat C, Ivarsson MA, Ljunggren H-G, Malmberg K-J, Michaëlsson J. 2010. Education of human natural killer cells by activating killer cell immunoglobulin-like receptors. *Blood* 115: 1166-74

41. Joncker NT, Fernandez NC, Treiner E, Vivier E, Raulet DH. 2009. NK cell responsiveness is tuned commensurate with the number of inhibitory receptors for self-MHC class I: the rheostat model. *J Immunol* 182: 4572-80

42. Joncker NT, Shifrin N, Delebecque F, Raulet DH. 2010. Mature natural killer cells reset their responsiveness when exposed to an altered MHC environment. *J Exp Med* 207: 2065-72

43. Elliott JM, Wahle JA, Yokoyama WM. 2010. MHC class I-deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment. *J Exp Med* 207: 2073-9

44. Sola C, André P, Lemmers C, Fuseri N, Bonnafous C, Bléry M, Wagtmann NR, et al. 2009. Genetic and antibody-mediated reprogramming of natural killer cell missing-self recognition in vivo. *Proc Natl Acad Sci USA* 106: 12879-84

45. Sun JC, Beilke JN, Lanier LL. 2009. Adaptive immune features of natural killer cells. *Nature* 457: 557-61

46. O'Leary JG, Goodarzi M, Drayton DL, von Andrian UH. 2006. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat Immunol* 7: 507-16

47. Paust S, Gill HS, Wang B-Z, Flynn MP, Moseman EA, Senman B, Szczepanik M, et al. 2010. Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. *Nat Immunol* 11: 1127-35

48. Sun JC, Beilke JN, Bezman NA, Lanier LL. 2011. Homeostatic proliferation generates long-lived natural killer cells that respond against viral infection. *J Exp Med* 208: 357-68

49. Biron CA, Byron KS, Sullivan JL. 1989. Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med* 320: 1731-5

50. Bustamante J, Boisson-Dupuis S, Jouanguy E, Picard C, Puel A, Abel L, Casanova J-L. 2008. Novel primary immunodeficiencies revealed by the investigation of paediatric infectious diseases. *Curr Opin Immunol* 20: 39-48

51. Raulet DH, Guerra N. 2009. Oncogenic stress sensed by the immune system: role of natural killer cell receptors. *Nat Rev Immunol* 9: 568-80

52. Smyth MJ, Cretney E, Kelly JM, Westwood JA, Street SEA, Yagita H, Takeda K, et al. 2005. Activation of NK cell cytotoxicity. *Mol Immunol* 42: 501-10

53. Caligiuri MA. 2008. Human natural killer cells. *Blood* 112: 461-9

54. Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E. 2005. Natural-killer cells and dendritic cells: "l'union fait la force". *Blood* 106: 2252-8

55. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. 2008. Functions of natural killer cells. *Nat Immunol* 9: 503-10

56. Degli-Esposti MA, Smyth MJ. 2005. Close encounters of different kinds: dendritic cells and NK cells take centre stage. *Nat Rev Immunol* 5: 112-24

57. Robbins SH, Bessou G, Cornillon A, Zucchini N, Rupp B, Ruzsics Z, Sacher T, et al. 2007. Natural killer cells promote early CD8 T cell responses against cytomegalovirus. *PLoS Pathog* 3: e123

58. Krebs P, Barnes MJ, Lampe K, Whitley K, Bahjat KS, Beutler B, Janssen E, et al. 2009. NK-cell-mediated killing of target cells triggers robust antigen-specific T-cell-mediated and humoral responses. *Blood* 113: 6593-602

59. Lu L, Ikizawa K, Hu D, Werneck MBF, Wucherpfennig KW, Cantor H. 2007. Regulation of activated CD4+ T cells by NK cells via the Qa-1-NKG2A inhibitory pathway. *Immunity* 26: 593-604

60. Andrews DM, Estcourt MJ, Andoniou CE, Wikstrom ME, Khong A, Voigt V, Fleming P, et al. 2010. Innate immunity defines the capacity of antiviral T cells to limit persistent infection. *J Exp Med* 207: 1333-43

61. Cooper MA, Colonna M, Yokoyama WM. 2009. Hidden talents of natural killers: NK cells in innate and adaptive immunity. *EMBO Rep* 10: 1103-10

62. Koopman LA, Kopcow HD, Rybalov B, Boyson JE, Orange JS, Schatz F, Masch R, et al. 2003. Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J Exp Med* 198: 1201-12

63. Moffett-King A. 2002. Natural killer cells and pregnancy. *Nat Rev Immunol* 2: 656-63

64. King A, Burrows T, Loke YW. 1996. Human uterine natural killer cells. *Nat Immun* 15: 41-52

65. Long EO. 1999. Regulation of immune responses through inhibitory receptors. *Annu Rev Immunol* 17: 875-904

66. Lanier LL. 2009. DAP10- and DAP12-associated receptors in innate immunity. *Immunol Rev* 227: 150-60

67. Long EO, Colonna M, Lanier LL. 1996. Inhibitory MHC class I receptors on NK and T cells: a standard nomenclature. *Immunol Today* 17: 100

68. André P, Biassoni R, Colonna M, Cosman D, Lanier LL, Long EO, Lopez-Botet M, et al. 2001. New nomenclature for MHC receptors. *Nat Immunol* 2: 661

69. Marsh SGE, Parham P, Dupont B, Geraghty DE, Trowsdale J, Middleton D, Vilches C, et al. 2003. Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002. *Immunogenetics* 55: 220-6

70. Steffens U, Vyas Y, Dupont B, Selvakumar A. 1998. Nucleotide and amino acid sequence alignment for human killer cell inhibitory receptors (KIR), 1998. *Tissue Antigens* 51: 398-413

71. Dubey DP, Alper CA, Mirza NM, Awdeh Z, Yunis EJ. 1994. Polymorphic Hh genes in the HLA-B(C) region control natural killer cell frequency and activity. *J Exp Med* 179: 1193-203

72. Ciccone E, Pende D, Viale O, di Donato C, Tripodi G, Orengo AM, Guardiola J, et al. 1992. Evidence of a natural killer (NK) cell repertoire for (allo) antigen recognition: definition of five distinct NK-determined allospecificities in humans. *J Exp Med* 175: 709-18

73. Litwin V, Gumperz J, Parham P, Phillips JH, Lanier LL. 1993. Specificity of HLA class I antigen recognition by human NK clones: evidence for clonal heterogeneity, protection by self and non-self alleles, and influence of the target cell type. *J Exp Med* 178: 1321-36

74. Cella M, Longo A, Ferrara GB, Strominger JL, Colonna M. 1994. NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. *J Exp Med* 180: 1235-42

75. Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P. 1995. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J Exp Med* 181: 1133-44

76. Colonna M, Spies T, Strominger JL, Ciccone E, Moretta A, Moretta L, Pende D, et al. 1992. Alloantigen recognition by two human natural killer cell clones is associated with HLA-C or a closely linked gene. *Proc Natl Acad Sci USA* 89: 7983-5

77. Colonna M, Brooks EG, Falco M, Ferrara GB, Strominger JL. 1993. Generation of allospecific natural killer cells by stimulation across a polymorphism of HLA-C. *Science* 260: 1121-4

78. Biassoni R, Falco M, Cambiaggi A, Costa P, Verdiani S, Pende D, Conte R, et al. 1995. Amino acid substitutions can influence the natural killer (NK)-mediated recognition of HLA-C molecules. Role of serine-77 and lysine-80 in the target cell protection from lysis mediated by "group 2" or "group 1" NK clones. *J Exp Med* 182: 605-9

79. Moretta A, Sivori S, Vitale M, Pende D, Morelli L, Augugliaro R, Bottino C, et al. 1995. Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. *J Exp Med* 182: 875-84

80. Colonna M, Borsellino G, Falco M, Ferrara GB, Strominger JL. 1993. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc Natl Acad Sci USA* 90: 12000-4

81. Vitale M, Sivori S, Pende D, Moretta L, Moretta A. 1995. Coexpression of two functionally independent p58 inhibitory receptors in human natural killer cell clones results in the inability to kill all normal allogeneic target cells. *Proc Natl Acad Sci USA* 92: 3536-40

82. van Rood JJ, van Leeuwen A. 1963. Leukocyte grouping. A method and its application. *J Clin Invest* 42: 1382-90

83. Moretta A, Bottino C, Pende D, Tripodi G, Tambussi G, Viale O, Orengo A, et al. 1990. Identification of four subsets of human CD3-CD16+ natural killer (NK) cells by the expression of clonally distributed functional surface molecules: correlation between subset assignment of NK clones and ability to mediate specific alloantigen recognition. *J Exp Med* 172: 1589-98

84. Litwin V, Gumperz J, Parham P, Phillips JH, Lanier LL. 1994. NKB1: a natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. *J Exp Med* 180: 537-43

85. Moretta A, Vitale M, Bottino C, Orengo AM, Morelli L, Augugliaro R, Barbaresi M, et al. 1993. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *J Exp Med* 178: 597-604

86. Colonna M, Samaridis J. 1995. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science* 268: 405-8

87. Wagtmann N, Rajagopalan S, Winter CC, Peruzzi M, Long EO. 1995. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. *Immunity* 3: 801-9

88. Döhring C, Samaridis J, Colonna M. 1996. Alternatively spliced forms of human killer inhibitory receptors. *Immunogenetics* 44: 227-30

89. Selvakumar A, Steffens U, Dupont B. 1996. NK cell receptor gene of the KIR family with two IG domains but highest homology to KIR receptors with three IG domains. *Tissue Antigens* 48: 285-94

90. Biassoni R, Cantoni C, Falco M, Verdiani S, Bottino C, Vitale M, Conte R, et al. 1996. The human leukocyte antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. *J Exp Med* 183: 645-50

91. Rajagopalan S, Bryceson YT, Kuppusamy SP, Geraghty DE, van der Meer A, Joosten I, Long EO. 2006. Activation of NK cells by an endocytosed receptor for soluble HLA-G. *PLoS Biol* 4: e9

92. Vilches C, Rajalingam R, Uhrberg M, Gardiner CM, Young NT, Parham P. 2000. KIR2DL5, a novel killer-cell receptor with a D0-D2 configuration of Ig-like domains. *J Immunol* 164: 5797-804

93. Moesta AK, Graef T, Abi-Rached L, Older Aguilar AM, Guethlein LA, Parham P. 2010. Humans differ from other hominids in lacking an activating NK cell receptor that recognizes the C1 epitope of MHC class I. *J Immunol* 185: 4233-7

94. Katz G, Markel G, Mizrahi S, Arnon TI, Mandelboim O. 2001. Recognition of HLA-Cw4 but not HLA-Cw6 by the NK cell receptor killer cell Ig-like receptor two-domain short tail number 4. *J Immunol* 166: 7260-7

95. Graef T, Moesta AK, Norman PJ, Abi-Rached L, Vago L, Older Aguilar AM, Gleimer M, et al. 2009. KIR2DS4 is a product of gene conversion with KIR3DL2 that introduced specificity for HLA-A*11 while diminishing avidity for HLA-C. *J Exp Med* 206: 2557-72

96. D'Andrea A, Chang C, Franz-Bacon K, McClanahan T, Phillips JH, Lanier LL. 1995. Molecular cloning of NKB1. A natural killer cell receptor for HLA-B allotypes. *J Immunol* 155: 2306-10

97. Kollnberger S, Chan A, Sun M-Y, Chen LY, Wright C, di Gleria K, McMichael A, et al. 2007. Interaction of HLA-B27 homodimers with KIR3DL1 and KIR3DL2, unlike HLA-B27 heterotrimers, is independent of the sequence of bound peptide. *Eur J Immunol* 37: 1313-22

98. Hansasuta P, Dong T, Thananchai H, Weekes M, Willberg C, Aldemir H, Rowland-Jones S, et al. 2004. Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific. *Eur J Immunol* 34: 1673-9

99. Wilson MJ, Torkar M, Haude A, Milne S, Jones T, Sheer D, Beck S, et al. 2000. Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc Natl Acad Sci USA* 97: 4778-83

100. Döhring C, Scheidegger D, Samaridis J, Cella M, Colonna M. 1996. A human killer inhibitory receptor specific for HLA-A1,2. *J Immunol* 156: 3098-101

101. Pende D, Biassoni R, Cantoni C, Verdiani S, Falco M, di Donato C, Accame L, et al. 1996. The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/p70 family of inhibitory receptors that is characterized by three immunoglobulin-like domains and is expressed as a 140-kD disulphide-linked dimer. *J Exp Med* 184: 505-18

102. Gumperz JE, Barber LD, Valiante NM, Percival L, Phillips JH, Lanier LL, Parham P. 1997. Conserved and variable residues within the Bw4 motif of HLA-B make separable contributions to recognition by the NKB1 killer cell-inhibitory receptor. *J Immunol* 158: 5237-41

103. Winter C, Gumperz J, Parham P, Long E, Wagtmann N. 1998. Direct Binding and Functional Transfer of NK Cell Inhibitory Receptors Reveal Novel Patterns of HLA-C Allotype Recognition. *J Immunol* 161: 571

104. Khakoo SI, Carrington M. 2006. KIR and disease: a model system or system of models? *Immunol Rev* 214: 186-201

105. Parham P. 2005. MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol* 5: 201-14

106. Rajagopalan S, Long EO. 2005. Understanding how combinations of HLA and KIR genes influence disease. *J Exp Med* 201: 1025-9

107. Boyton RJ, Altmann DM. 2007. Natural killer cells, killer immunoglobulin-like receptors and human leucocyte antigen class I in disease. *Clin Exp Immunol* 149: 1-8

108. Martin MP, Gao X, Lee J-H, Nelson GW, Detels R, Goedert JJ, Buchbinder S, et al. 2002. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet* 31: 429-34

109. Qi Y, Martin MP, Gao X, Jacobson L, Goedert JJ, Buchbinder S, Kirk GD, et al. 2006. KIR/HLA pleiotropism: protection against both HIV and opportunistic infections. *PLoS Pathog* 2: e79

110. Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski J, Cheng J, et al. 2004. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* 305: 872-4

111. Artavanis-Tsakonas K, Eleme K, McQueen KL, Cheng NW, Parham P, Davis DM, Riley EM. 2003. Activation of a subset of human NK cells upon contact with Plasmodium falciparum-infected erythrocytes. *J Immunol* 171: 5396-405

112. Wauquier N, Padilla C, Becquart P, Leroy E, Vieillard V. 2010. Association of KIR2DS1 and KIR2DS3 with fatal outcome in Ebola virus infection. *Immunogenetics* 62: 767-71

113. Hollenbach JA, Ladner MB, Saeteurn K, Taylor KD, Mei L, Haritunians T, McGovern DPB, et al. 2009. Susceptibility to Crohn's disease is mediated by KIR2DL2/KIR2DL3 heterozygosity and the HLA-C ligand. *Immunogenetics* 61: 663-71

114. McGeough CM, Berrar D, Wright G, Mathews C, Gilmore P, Cunningham RT, Bjourson AJ. 2011. Killer immunoglobulin-like receptor and human leukocyte antigen-C genotypes in rheumatoid arthritis primary responders and non-responders to anti-TNF-αtherapy. *Rheumatology international*

115. van der Slik AR, Alizadeh BZ, Koeleman BPC, Roep BO, Giphart MJ. 2007. Modelling KIR-HLA genotype disparities in type 1 diabetes. *Tissue antigens* 69 Suppl 1: 101-5

116. Fusco C, Guerini FR, Nocera G, Ventrella G, Caputo D, Valentino MA, Agliardi C, et al. 2010. KIRs and their HLA ligands in remitting-relapsing multiple sclerosis. *J Neuroimmunol* 229: 232-7

117. Miller JS, Cooley S, Parham P, Farag SS, Verneris MR, McQueen KL, Guethlein LA, et al. 2007. Missing KIR ligands are associated with less relapse and increased graft-versus-host disease (GVHD) following unrelated donor allogeneic HCT. *Blood* 109: 5058-61

118. Cooley S, Weisdorf DJ, Guethlein LA, Klein JP, Wang T, Le CT, Marsh SGE, et al. 2010. Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood* 116: 2411-9

119. Naumova E, Mihaylova A, Stoitchkov K, Ivanova M, Quin L, Toneva M. 2005. Genetic polymorphism of NK receptors and their ligands in melanoma patients: prevalence of inhibitory over activating signals. *Cancer Immunol Immunother* 54: 172-8

120. Carrington M, Wang S, Martin MP, Gao X, Schiffman M, Cheng J, Herrero R, et al. 2005. Hierarchy of resistance to cervical neoplasia mediated by combinations of killer immunoglobulin-like receptor and human leukocyte antigen loci. *J Exp Med* 201: 1069-75

121. Hiby SE, Walker JJ, O'shaughnessy KM, Redman CWG, Carrington M, Trowsdale J, Moffett A. 2004. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J Exp Med* 200: 957-65

122. Hiby SE, Regan L, Lo W, Farrell L, Carrington M, Moffett A. 2008. Association of maternal killer-cell immunoglobulin-like receptors and parental HLA-C genotypes with recurrent miscarriage. *Hum Reprod* 23: 972-6

123. Hiby SE, Apps R, Sharkey AM, Farrell LE, Gardner L, Mulder A, Claas FH, et al. 2010. Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. *J Clin Invest* 120: 4102-10

124. Purdy AK, Campbell KS. 2009. Natural killer cells and cancer: regulation by the killer cell Ig-like receptors (KIR). *Cancer Biol Ther* 8: 2211-20

125. Billingham RE, Brent L, Medawar PB. 1953. Actively acquired tolerance of foreign cells. *Nature* 172: 603-6

126. Wende H, Colonna M, Ziegler A, Volz A. 1999. Organization of the leukocyte receptor cluster (LRC) on human chromosome 19q13.4. *Mamm Genome* 10: 154-60

127. Sambrook JG, Bashirova A, Andersen H, Piatak M, Vernikos GS, Coggill P, Lifson JD, et al. 2006. Identification of the ancestral killer immunoglobulin-like receptor gene in primates. *BMC Genomics* 7: 209

128. Trowsdale J, Barten R, Haude A, Stewart CA, Beck S, Wilson MJ. 2001. The genomic context of natural killer receptor extended gene families. *Immunol Rev* 181: 20-38

129. Carrington M, Cullen M. 2004. Justified chauvinism: advances in defining meiotic recombination through sperm typing. *Trends Genet* 20: 196-205

130. Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B, Tyan D, et al. 1997. Human diversity in killer cell inhibitory receptor genes. *Immunity* 7: 753-63

131. Uhrberg M, Parham P, Wernet P. 2002. Definition of gene content for nine common group B haplotypes of the Caucasoid population: KIR haplotypes contain between seven and eleven KIR genes. *Immunogenetics* 54: 221-9

132. Traherne JA, Martin M, Ward R, Ohashi M, Pellett F, Gladman D, Middleton D, et al. 2010. Mechanisms of copy number variation and hybrid gene formation in the KIR immune gene complex. *Hum Mol Genet* 19: 737-51

133. Pyo C-W, Guethlein LA, Vu Q, Wang R, Abi-Rached L, Norman PJ, Marsh SGE, et al. 2010. Different patterns of evolution in the centromeric and telomeric regions of group A and B haplotypes of the human killer cell Ig-like receptor locus. *PLoS ONE* 5: e15115

134. Witt CS, Dewing C, Sayer DC, Uhrberg M, Parham P, Christiansen FT. 1999. Population frequencies and putative haplotypes of the killer cell immunoglobulin-like receptor sequences and evidence for recombination. *Transplantation* 68: 1784-9

135. Single RM, Martin MP, Gao X, Meyer D, Yeager M, Kidd JR, Kidd KK, et al. 2007. Global diversity and evidence for coevolution of KIR and HLA. *Nat Genet* 39: 1114-9

136. Martin MP, Single RM, Wilson MJ, Trowsdale J, Carrington M. 2008. KIR haplotypes defined by segregation analysis in 59 Centre d'Etude Polymorphisme Humain (CEPH) families. *Immunogenetics* 60: 767-74

137. Parham P, Ohta T. 1996. Population biology of antigen presentation by MHC class I molecules. *Science* 272: 67-74

138. Rodgers JR, Cook RG. 2005. MHC class Ib molecules bridge innate and acquired immunity. *Nat Rev Immunol* 5: 459-71

139. de Groot NG, Otting N, Doxiadis GGM, Balla-Jhagjhoorsingh SS, Heeney JL, van Rood JJ, Gagneux P, et al. 2002. Evidence for an ancient selective sweep in the MHC class I gene repertoire of chimpanzees. *Proc Natl Acad Sci USA* 99: 11748-53

140. Adams EJ, Cooper S, Thomson G, Parham P. 2000. Common chimpanzees have greater diversity than humans at two of the three highly polymorphic MHC class I genes. *Immunogenetics* 51: 410-24

141. Cooper S, Adams EJ, Wells RS, Walker CM, Parham P. 1998. A major histocompatibility complex class I allele shared by two species of chimpanzee. *Immunogenetics* 47: 212-7

142. Gleimer M, Wahl AR, Hickman HD, Abi-Rached L, Norman PJ, Guethlein LA, Hammond JA, et al. 2011. Although divergent in residues of the peptide binding site, conserved chimpanzee Patr-AL and polymorphic human HLA-A*02 have overlapping peptide-binding repertoires. *J Immunol* 186: 1575-88

143. de Groot NG, Heijmans CMC, Zoet YM, de Ru AH, Verreck FA, van Veelen PA, Drijfhout JW, et al. 2010. AIDS-protective HLA-B*27/B*57 and chimpanzee MHC class I molecules target analogous conserved areas of HIV-1/SIVcpz. *Proc Natl Acad Sci USA* 107: 15175-80

144. Clark EA, Sturge JC. 1981. Phylogeny of NK cell reactivity against human and nonhuman primate lymphoblastoid cell lines: evolving and conserved target antigens. *J Immunol* 126: 969-74

145. Lawlor DA, Warren E, Taylor P, Parham P. 1991. Gorilla class I major histocompatibility complex alleles: comparison to human and chimpanzee class I. *J Exp Med* 174: 1491-509

146. Adams EJ, Thomson G, Parham P. 1999. Evidence for an HLA-C-like locus in the orangutan Pongo pygmaeus. *Immunogenetics* 49: 865-71

147. Adams EJ, Parham P. 2001. Species-specific evolution of MHC class I genes in the higher primates. *Immunol Rev* 183: 41-64

148. Chen ZW, McAdam SN, Hughes AL, Dogon AL, Letvin NL, Watkins DI. 1992. Molecular cloning of orangutan and gibbon MHC class I cDNA. The HLA-A and -B loci diverged over 30 million years ago. *J Immunol* 148: 2547-54

149. Abi-Rached L, Kuhl H, Roos C, ten Hallers B, Zhu B, Carbone L, de Jong PJ, et al. 2010. A small, variable, and irregular killer cell Ig-like receptor locus accompanies the absence of MHC-C and MHC-G in gibbons. *J Immunol* 184: 1379-91

150. Otting N, Heijmans CMC, Noort RC, de Groot NG, Doxiadis GGM, van Rood JJ, Watkins DI, et al. 2005. Unparalleled complexity of the MHC class I region in rhesus macaques. *Proc Natl Acad Sci USA* 102: 1626-31

151. Daza-Vamenta R, Glusman G, Rowen L, Guthrie B, Geraghty DE. 2004. Genetic divergence of the rhesus macaque major histocompatibility complex. *Genome Res* 14: 1501-15

152. Knapp LA, Cadavid LF, Watkins DI. 1998. The MHC-E locus is the most well conserved of all known primate class I histocompatibility genes. *J Immunol* 160: 189-96

153. Otting N, Bontrop RE. 1993. Characterization of the rhesus macaque (Macaca mulatta) equivalent of HLA-F. *Immunogenetics* 38: 141-5

154. Boyson JE, Iwanaga KK, Urvater JA, Hughes AL, Golos TG, Watkins DI. 1999. Evolution of a new nonclassical MHC class I locus in two Old World primate species. *Immunogenetics* 49: 86-98

155. Sambrook JG, Bashirova A, Palmer S, Sims S, Trowsdale J, Abi-Rached L, Parham P, et al. 2005. Single haplotype analysis demonstrates rapid evolution of the killer immunoglobulin-like receptor (KIR) loci in primates. *Genome Res* 15: 25-35

156. Guethlein LA, Older Aguilar AM, Abi-Rached L, Parham P. 2007. Evolution of killer cell Ig-like receptor (KIR) genes: definition of an orangutan KIR haplotype reveals expansion of lineage III KIR associated with the emergence of MHC-C. *J Immunol* 179: 491-504

157. Martin AM, Freitas EM, Witt CS, Christiansen FT. 2000. The genomic organization and evolution of the natural killer immunoglobulin-like receptor (KIR) gene cluster. *Immunogenetics* 51: 268-80

158. Valiante NM, Lienert K, Shilling HG, Smits BJ, Parham P. 1997. Killer cell receptors: keeping pace with MHC class I evolution. *Immunol Rev* 155: 155-64

159. Khakoo SI, Rajalingam R, Shum BP, Weidenbach K, Flodin L, Muir DG, Canavez F, et al. 2000. Rapid evolution of NK cell receptor systems demonstrated by comparison of chimpanzees and humans. *Immunity* 12: 687-98

160. Rajalingam R, Hong M, Adams EJ, Shum BP, Guethlein LA, Parham P. 2001. Short KIR haplotypes in pygmy chimpanzee (Bonobo) resemble the conserved framework of diverse human KIR haplotypes. *J Exp Med* 193: 135-46

161. Rajalingam R, Parham P, Abi-Rached L. 2004. Domain shuffling has been the main mechanism forming new hominoid killer cell Ig-like receptors. *J Immunol* 172: 356-69

162. Guethlein LA, Flodin LR, Adams EJ, Parham P. 2002. NK cell receptors of the orangutan (Pongo pygmaeus): a pivotal species for tracking the coevolution of killer cell Ig-like receptors with MHC-C. *J Immunol* 169: 220-9

163. Older Aguilar AM, Guethlein LA, Adams EJ, Abi-Rached L, Moesta AK, Parham P. 2010. Coevolution of killer cell Ig-like receptors with HLA-C to become the major variable regulators of human NK cells. *J Immunol* 185: 4238-51

164. Hershberger KL, Shyam R, Miura A, Letvin NL. 2001. Diversity of the killer cell Ig-like receptors of rhesus monkeys. *J Immunol* 166: 4380-90

165. Grendell RL, Hughes AL, Golos TG. 2001. Cloning of rhesus monkey killer-cell Ig-like receptors (KIRs) from early pregnancy decidua. *Tissue Antigens* 58: 329-34

166. Andersen H, Rossio JL, Coalter V, Poore B, Martin MP, Carrington M, Lifson JD. 2004. Characterization of rhesus macaque natural killer activity against a rhesus-derived target cell line at the single-cell level. *Cell Immunol* 231: 85-95

Chapter 2

Evidence for balancing selection acting on KIR2DL4 genotypes in rhesus macaques of Indian origin

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ABSTRACT

The interaction of killer-cell immunoglobulin-like receptors (KIR) and their respective major histocompatibility complex (MHC) ligands can alter the activation state of the natural killer (NK) cell. In both humans and rhesus macaques, particular types of non-classical MHC class I molecules are predominantly expressed on the trophoblast. In humans, human leukocyte antigen G has been demonstrated to act as a ligand for KIR2DL4, present on all NK cells, whereas Mamu-AG may execute a similar function in rhesus macaques. During primate evolution, orthologues of *KIR2DL4* appear to have been highly conserved, suggesting strong purifying selection. A cohort of 112 related and unrelated rhesus macaques of mostly Indian origin were selected to study their *KIR2DL4* genes for the occurrence of polymorphism. Comparison of the proximal region provided evidence for strong conservative selection acting on the exons encoding the Ig domains. As is found in humans, in the Indian rhesus macaque population, two different KIR2DL4 entities are encountered, which differ for their intra-cellular signalling motifs. One genotype contains a complex mutation in the distal region of exon 9, which negates a serine/threonine kinase site. Furthermore, both allelic entities are present in a distribution, which suggests that balancing selection is operating on these two distinct forms of *KIR2DL4*.

INTRODUCTION

Natural killer (NK) cells are important components in the first line of defence against pathogens and cancer. Moreover, they are thought to play a major regulatory role during pregnancy by maintaining a state of immunological tolerance. The interaction between major histocompatibility complex (MHC) class I molecules and their killer-cell immunoglobulin-like receptor (KIR) ligands may affect the balance between the inhibition or activation state of the NK cell. As such, these gene families must have had considerable impact on each other during their evolution (1). Moreover, these multigene families possess members that display varying levels of polymorphism (2, 3).

The *KIR2DL4* gene is reported to be highly conserved in humans. It is considered a framework gene, as it is present on most haplotypes, and only a few exceptions have been recorded (4). The promotor region is highly characteristic (5), and the protein itself is constitutively expressed on NK cells. *KIR2DL4* comprises multiple exons: exons 1 and 2 encode the signal peptide, exons 3 and 5 the immunoglobulin domains, exon 6 the stem, exon 7 the transmembrane section, and exons 8 and 9 the cytoplasmic domains, respectively. Most other KIR that comprise two Ig domains have a D1-D2 organisation encoded by exons 4 and 5 and contain a non-transcribed exon 3. *KIR2DL4* is different in that it has a D0-D2 organisation that lacks exon 4 and where exon 3 is not a pseudoexon (6).

From a functional point of view, KIR2DL4 has been attributed with both an

activating and an inhibitory role. Its transmembrane domain comprises a charged arginine residue, which can associate with an adapter molecule. This FcεRI-αchain contains immunoreceptor tyrosine- based activation motifs by which the NK cell can be activated (7). The cytoplasmic domain of KIR2DL4 contains an immunoreceptor tyrosine-based inhibition motif (ITIM) that can associate with Src homology 2-containing phosphatases 1 and 2 (8).

In humans, the natural ligand for KIR2DL4 is human leukocyte antigen G (HLA-G), a non-classical MHC class I molecule, which is selectively expressed on trophoblast cells (9). Through this interaction, NK cells are involved in maintaining a state of tolerance between mother and foetus. However, as has been observed for other complex systems, there are some levels of redundancy, since healthy individuals who lack either HLA-G or KIR2DL4 have been observed. Different human populations have been studied to under- stand the extent of polymorphism in KIR2DL4. The Ig domains in particular display polymorphism, and a number of alleles have been reported (10-12). In fact, there are two major forms of KIR2DL4, as exon 7 contains a polymorphism that may lead to the truncation of its cytoplasmic tail (13). Both forms seem to experience balancing selection, as they are found in approximately equal distribution among different human populations (14).

Rhesus macaques (*Macaca mulatta*) are used as animal models: for instance, to study infectious diseases, autoim- munity, and the induction of tolerance in the case of transplantation medicine. Different populations of Indian and Chinese rhesus macaques have been well characterised for their Mhc class I genes to facilitate biomedical research (15-18). It is equally important to have detailed knowledge of their natural ligands, such as the genes of the KIR cluster. On average, KIR genes seem to evolve rapidly, and a complex array of duplication and recombination (exon shuffling) processes seems to have generated most of the known diversity in primate species (19). *Mamu-KIR2DL4*, however, appears to have been subject to conservative evolution, although various subtle differences are documented. The detailed genomic organisation of the *Mamu-KIR2DL4* gene in rhesus macaques is known (20). The cytoplasmic region comprises two ITIMs, suggesting a more inhibitory role for this molecule. The *Mamu-KIR2DL4* gene seems to be present in all animals studied thus far (21, 22). Hershberger and colleagues observed two different forms of *Mamu-KIR2DL4* (4.1, 4.2), although no full-length cDNA sequences of *KIR2DL4.2* were reported. The ancestral *KIR2DL4* gene must have played an essential role in the generation of an array of activating KIRs in rhesus macaques, which were formed by recombination, subsequently followed by a splice site mutation (23). Such data underline the fact that *KIR2DL4* is an old entity, and that activating KIRs and KIR2DL4 in rhesus macaques potentially interact with the same adapter molecule. Recently, a cohort of Mauritian cynomolgous macaques (*Macaca fascicularis*) was analysed, and haplotypes that appear to lack the *Mafa-KIR2DL4* gene were documented (24). The *KIR2DL4* genes in both Old

World monkey species are, however, highly similar.

At this stage, it is not known whether *Mamu-KIR2DL4* displays any significant levels of polymorphism. To evaluate whether allelic variations do indeed exist, a large, outbred population of Indian rhesus macaques was examined.

MATERIALS AND METHODS

Animals and cells

In this study, 112 mostly Indian rhesus macaques belonging to the BPRC self-sustaining colony were analysed. Unrelated animals were selected to calculate gene frequency, and pedigreed animals were analysed to verify sequences and to monitor segregation. Whole blood was obtained during regular health checks, and peripheral blood mononuclear cells (PBMCs) were isolated as described previously (25). All animals were typed by different methods for their Mamu-A, -B, and -DR allelic repertoire (26-28). MtDNA analysis was performed to define or confirm the origin of the macaque population that was studied (29, 30).

RNA extraction, cDNA synthesis, and amplification

Total RNA was isolated from 5×10^6 PBMCs with Trizol reagent (Invitrogen, Paisley, Scotland), according to the manufacturer's instructions. First-strand cDNA synthesis was performed with an oligo$(dT)_{18}$ primer and RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany). PCR was performed with the primers prom2dl4fw and 2dl43utrrv (Table 1) synthesised by Invitrogen, using Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland), according to the manufacturer's conditions.

Table 1 Overview of primers used for amplification of full length cDNA, introns 3, 7, and cloning and sequencing of *Mamu-KIR2DL4*.

Primers Name	5′-sequence-3′
prom2dl4fw	CCACATCCTCTGCACCGGTCAGTC
2dl43utrrv	GCAGGGGTCAAGTGAAGGGGAGAA
2dl4_intr3fw	AGTTTCCTCATTAGCCCTGTGACTG
2dl4_intr3rv	CACTCGTAGGGAGAGTCACGGAG
2dl4_intr7fw	TTGGATTCCTATCTTCCTCCAGGTA
2dl4_intr7rv	TATATCCATGAKGCTGGGGAGAG
2dl4_intr7rv2	CATATCCATGAGGCTGGGCCGAG
340fwseq36	AGGGACCTACAGATGTCGAGTTTTTC
790fwseq44	GTGATTAGGTACTCGGTGGCCACCAT
520fwseq230	TTTGACATGTACCATCTATCC
780rvseq232	CACAATAGGCAGGTGTCTGG
440rvseq231	GGCTGGGCTGAGAGAGAAGG
2dl4fwatg	GCCATGTCGCCCACGGTCGTCATCCTGG
2dl4.1rv	TCAGATTCCAACTGCTGGTACATTGGAGC
2dl4.2rv	TCAGATTCCAGCTGCTGGCACTGGAGC

The amplification parameters were a 2-min initial denaturation step at 98°C, followed by 20 cycles of a 20-s 98°C denaturation step, a 45-s 65°C annealing step, and a 2-min 72°C extension step, then 20 cycles of a 29-s 98°C denaturation step, a 30-s 60°C annealing step, and a 2-min 72°C extension step. A final extension step was performed at 72°C for 10 min.

DNA extraction and amplification

Genomic DNA was extracted from heparinised blood samples or immortalised rhesus macaque B cell-lines according to standardised protocols (31).

PCR of *Mamu-KIR2DL4* of introns 3 and 7 was conducted based on newly designed primers (Table 1), using recombinant Taq Polymerase (Invitrogen), according to the manufacturer's instructions. Cycling parameters were a 1-min initial denaturation step, followed by 30 cycles of a 15-s 94°C denaturation step, a 30-s 63°C annealing step, and a 1-min 72°C extenstion step. A final extension step was performed at 72°C for 7 min. An annealing temperature of 60°C was used to amplify intron 7 with primers 2dl4_intr7fw and 2dl4intr7rv2.

Cloning and sequencing

PCR products were subjected to gel electrophoresis, and bands of appropriate size were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Products were blunt-end ligated into the cloning site of the PJET1.2/blunt vector (Fermentas) and were used to transform XL1Blue *Escherichia coli* cells with the TransformAid Bacterial Transformation Kit (Fermentas). A minimum of 16 clones were examined per PCR reaction. Plasmid was isolated and products were sequenced with pJET1.2 forward and reverse primers (Fermentas), as well as internal primers (Table 1), on a 3130XL ABI automatic sequencer (Applied Biosystems, Foster City, USA). Only sequences that were confirmed in multiple PCR products or animals are reported.

Mamu-KIR2DL4 transfectants and fluorescent microscopy

Full-length cDNA of two *Mamu-KIR2DL4* alleles (*001, *01501) from animal R05061 was cloned separately into mammalian expression vector pcDNA6.2/yellow fluorescent protein (YFP)-GW/TOPO (Invitrogen). TOP10 *E. coli* cells were transformed with these constructs and their insert sequenced to ensure correct orientation. Endotox free midiprep was performed (Qiagen) to obtain high-quality plasmid. HEK293 (ATCC, CRL-1573) cells were transfected with the plasmid using PolyFect transfection reagent (Qiagen). Cells were then cultured in complete Dulbecco's modified Eagle medium (Lonza, Basel, Switzerland), and transfectants were selected by adding a final concentration of 10 µg/ml blasticidin (Invivogen, San Diego, CA, USA). Microscopy was performed with a microphot FXA fluorescent microscope (Nikon, Tokyo, Japan) under fluorescein isothiocyanate filter settings.

Sequence alignments, in silico analysis, and nomenclature

The Seqman programme (Dnastar, Madison, WI, USA) was used to align the sequences and the Macvector programme, version 10.5 (MacVector, Cambridge, UK), was used to analyse and compare both nucleotide and protein sequences. This programme was also used to calculate pairwise distances using the Kimura-2

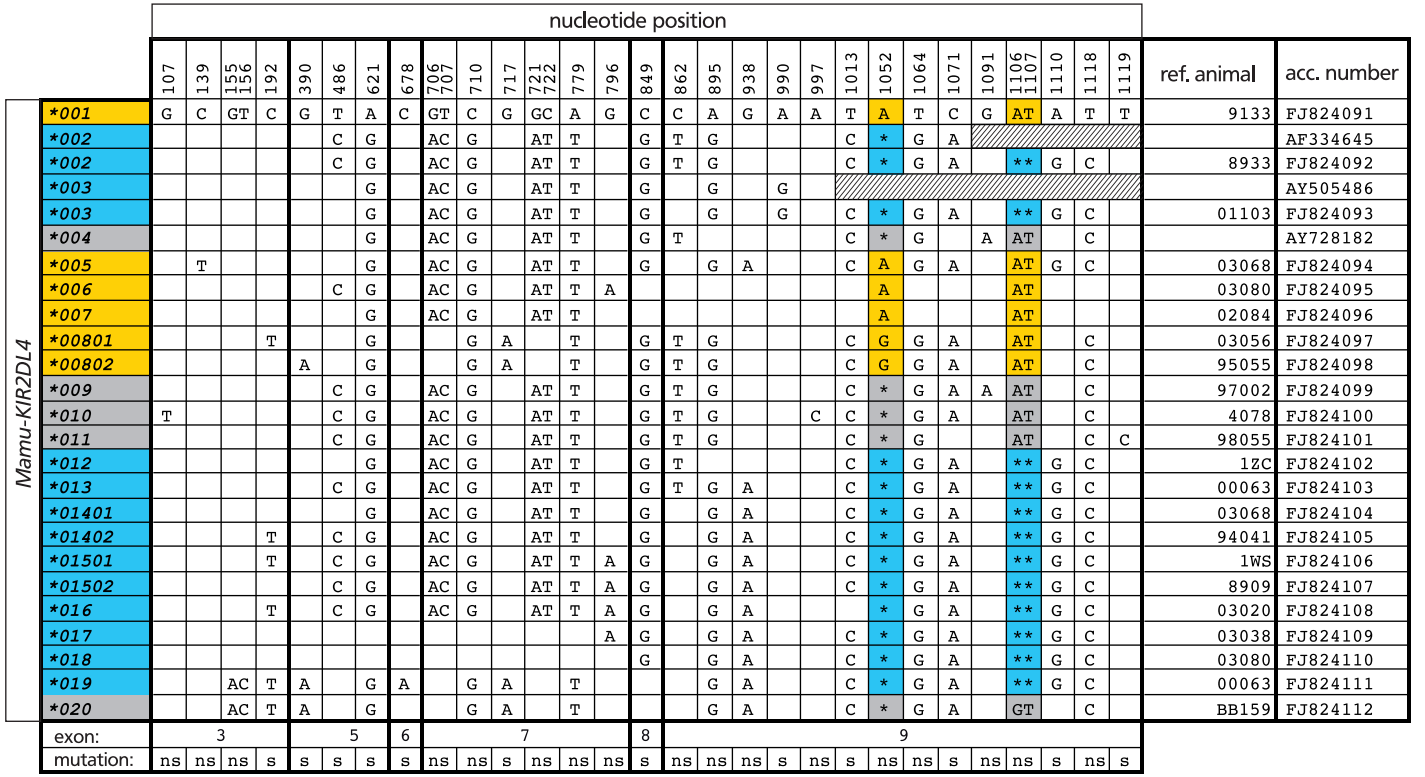

Figure 1 Overview of different *Mamu-KIR2DL4* alleles, polymorphic codons, reference animals, and accession numbers. The *Mamu-KIR2DL4*001* gene (GenBank accession number NM_001042661) was taken as a reference. Two previously reported sequences (AF334644 and AY505486) have been extended. Deletions have been depicted by an *asterisk*, and a cell was left blank if it matched the reference sequence. For each allele, a reference animal was added next to the accession number. Alleles are depicted, highlighted in *yellow*, with nucleotides at positions 1052, 1106, and 1107 that are characteristic for 2DL4.1. Highlighted in *blue*, alleles are shown with deletions at these positions characteristic for 2DL4.2. In *grey*, alleles with only a deletion at position 1052 are shown. The overview also provides information as to whether a mutation results in a non-synonymous (ns) or a synonymous (s) codon change.

parameters, and the neighbour-joining method was used to create a phylogram. Bootstrap values were calculated based on 1,000 interactions. These analyses were applied to compare sequences and to facilitate nomenclature. To calculate dN and dS values, the programme SNAP (http://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html) was used (32). Input sequences were all of the human *KIR2DL4* alleles listed by the Immunogenetics Polymorphism Database IPD-KIR (www.ebi.ac.uk/ipd/kir), release 2.1.0, and macaque *Mamu-KIR2DL4* alleles reported in this manuscript. Relevant GenBank accession numbers for the sequences described in this study have been summarised (Fig. 1). In collaboration with the IPD, an offical nomenclature designation was given to *Mamu-KIR2DL4* alleles according to the standardised rules described for KIR genes (33).

RESULTS AND DISCUSSION

Mamu-KIR2DL4 displays complex polymorphism profiles

Full-length cDNA screening of a population of mostly Indian rhesus macaques revealed the existence of 22 alleles (Fig. 1). This study significantly extends the repertoire of documented alleles. In rhesus macaques, an alternative form of the regular gene has been observed, which was named . That report, however, was based on the description of partial sequences (22). In our panel, both genotypes (KIR2DL4.1 and KIR2DL4.2) are present and the full-length structures are provided (Fig. 1).

Both forms are distinguishable by mutations in exon 8 at positions 1052, 1106, and 1107. The nucleotides at these positions have been deleted in KIR2DL4.2. The first deletion introduces a stretch of sixteen different codons before returning back in frame due to the subsequent deletion of two further nucleotides. Additionally, one *Mamu-KIR2DL4* allele (**003*) is identical to a partial cDNA sequence reported previously (AY505486), hereby confirming this sequence and extending it to full length. Finally, a polymorphism was observed in different alleles (**009*, **010*, **011*, **020*), resulting in a configuration that is similar to *KIR2DL4.2* but lacks the upstream deletion of two nucleotides. This configuration was reported previously (AY728182), and its translation results in an extended cytoplasmatic domain. However, these alleles are rare and observed in less than 5% of the analysed population. Further mitochondrial analysis revealed that these alleles were observed in animals that had been misrepresented as pure Indian macaques, and were in fact of either Burmese origin or were the offspring of Indian females with Burmese or Chinese males. All other alleles were observed in animals with confirmed Indian origin.

Many mutations observed among *Mamu-KIR2DL4* alleles represent synonymous mutations; nevertheless, these single nucleotide polymorphisms (SNP) might turn out to be useful for genomic typing. Conserved framework genes such as *KIR2DL5* and *2DL4* display abundant allelic variation that can be applied for haplotyping *KIR* region configurations (34), with an emphasis on activating family members. It is en-

visaged that, in the near future, much more information on different rhesus macaque *KIR* haplotypes will become available. The *KIR2DL4* alleles described in this paper could be used to serve a similar purpose.

Apart from allelic variability, different splice variants were observed, which are, however, present at much lower frequencies than the primary transcripts. Alternative splicing of 2DL4 resulting in different isoforms has also been reported in humans (12). One of the isoforms observed in rhesus macaques lacks exon 8, which may result in a truncated Mamu-KIR2DL4 protein highly similar to the family of *KIR2DL4* alleles *(*007, *008, *009,* and **011)* observed in humans (10). It is yet unknown whether these splice variants have any functional relevance; if so, this may add another level of complexity to the KIR system.

Differential selection acting on Mamu-KIR2DL4 gene segments

Comparison of the different alleles highlights a marked difference in the levels of polymorphism observed between the proximal exons 1–6, encoding the extracellular Ig-domains (D0 and D2), and the distal exons 7–9 that encode the transmembrane and intra-cellular section of the molecule. For instance, in exons 1 to 6, only a few nonsynonymous and synonymous mutations are observed among 700 nucleotide positions, while accumulation of nonsynomous mutations in the exons coding for the signal transduction domain is more abundant (Fig. 1). These data suggest that strong purifying selection is operative on the extracellular domains of Mamu-KIR2DL4.

A dN/dS comparison of different alleles in both rhesus macaque and humans (Fig. 2) revealed that the ligand-binding domains in rhesus macaques are highly conserved and are in contrast to the somewhat more polymorphic Ig domains of KIR2DL4

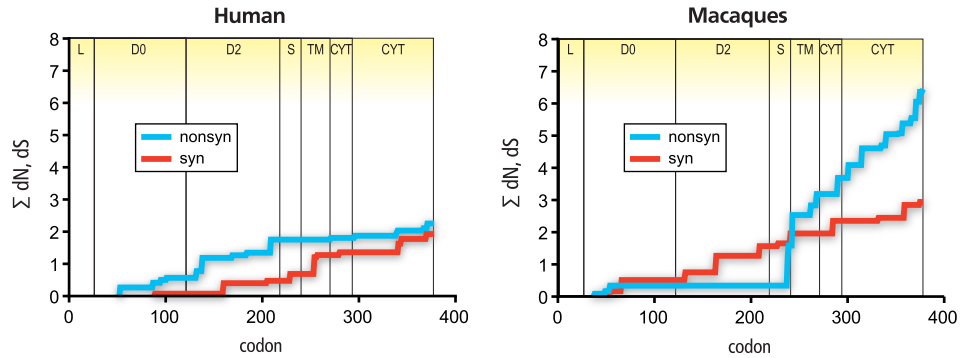

Figure 2 Analysis of dN (nonsynonymous mutations per synonymous site) and dS (synonymous mutations per nonsynonymous site) for human and rhesus macaque alleles of KIR2DL4. The y axis shows a cumulative value of either dN (nonsyn) and dS (syn) as calculated by the SNAP programme. The x axis shows the codon position of aligned human and macaque sequences. A schematic representation of the KIR2DL4 protein is shown, depicting the different domains: leader (L), immunoglobulin (D0–D2), stem (S), transmembrane (TM) and cytoplasmatic (CYT).

observed in humans. dN/dS analyses are most useful when analysing intra-species selection; however, one needs to be aware when performing inter-species comparisons that mutation rates may differ between species in a given gene system. Even though human KIR2DL4 is considered to be conserved, the proximal region of the rhesus macaque orthologue displays lower levels of polymorphism.

This suggests that, in rhesus macaques, an even stronger intensity of purifying selection may be operating on this region. In contrast, the distal section of *Mamu-KIR2DL4* is more polymorphic (Fig. 2), which suggests that either selection on the intra-cellular domain is less stringent, allowing it to evolve in a more neutral fashion, or selection on this region favoured the manifesting of more gradual differences in signal transduction. To investigate which evolutionary processes may be involved, the introns of various *Mamu-KIR2DL4* alleles were sequenced. Subsequent comparison shows that there are more SNP positions in the introns than in the adjacent exons. For example, intron 3 has an average of three SNP per 100 bp, whereas the adjacent proximal exons display only one SNP per 100 bp (Table 2).

Table 2 Comparison of intron and exon SNPs in *Mamu-KIR2DL4*.

Region	Nucleotides	SNPs	SNPs per 100 bp
intron 3	900	34	3.8
exon 1-6	700	8	1.1
intron 7	472	79	16.7
exon 7-9	428	21	4.9

In contrast, intron 7 has >16 SNP per 100 bp, while the distal exons have accumulated slightly less than five polymorphic sites per 100 bp. These SNP differences between introns 3 and 7 suggest that different rates of mutation may exist within one gene. This raises the question of whether all these introns evolve under neutral conditions. Differences may be due to the presence of regulatory sequences or they may be due to sequences that act as hot spots for the generation of mutations. Although polymorphism in the distal exons is more abundant, the ratio between SNP in the intron and exon is stable. These observations, together with the dN/dS analyses, suggest that strong purifying selection is operating on the proximal exons, and the distal exons of the gene seem to have been subject to divergence.

The ligand of KIR2DL4 in humans is the non-classical HLA-G gene product, which, although found in different isoforms, shows only limited levels of polymorphism (35). The high level of KIR2DL4 conservation in humans is thought to reflect its specialised function (9). The extracellular domains of the various Mamu-KIR2DL4 allotypes also show high levels of conservation. This is in contrast to the high levels of polymorphism observed for those domains in other *Mamu-KIR* genes (22, 23). This may be due to the fact that the inhibitory KIR gene products have the classical class I

MHC proteins as their ligand, and in rhesus macaques, the corresponding genes have undergone massive rounds of expansion (16, 36-39). This certainly must have had an impact on the KIR ligands, which co-evolved during these duplication processes. The expansion and contraction of the MHC region may result not only in the birth but also in the death of genes. Indeed, the rhesus macaque orthologue of *HLA-G* appears to be inactivated and is considered to be a pseudogene (40). Its specialised function, however, appears to have been taken over by *Mamu-AG*. Since no population-wide analysis of the allelic polymorphism of Mamu-AG has yet been performed, it is hypothesised that Mamu-AG has limited polymorphism in comparison with that of observed classical Mamu-A and -B molecules taking into account similar sample size. If Mamu-KIR2DL4 is indeed the ligand for Mamu-AG, then the limited polymorphism in the ligand-binding domain can be explained by the mutual restriction placed upon receptor and ligand during evolution. The different amounts of polymorphism may make sense, as they could reflect the time span during which KIR2DL4 and its ligand HLA-G, as compared to Mamu-KIR2DL*4* and Mamu-AG, had time to accumulate mutations. Indeed, there is evidence that the *HLA-G* gene is very old, as it is also present in New World monkeys (41, 42), whereas the *Mamu-AG* gene is considered to have a much more recent origin.

Inter-species KIR2DL4 comparisons

To determine similarities and dissimilarities, the deducted protein sequences obtained from humans, chimpanzees, and rhesus macaques were aligned (Fig. 3a). The alignment shows that the KIR2DL4 protein is indeed highly conserved between species. Moreover, the positions of the residues interacting with HLA-G were visualised by applying different colours. Since no crystal structure has been described of a DO–D2 configuration, a 2D-model was constructed based on the crystal structure of human KIR2DL2 (43) to visualise which regions of KIR2DL4 would interact with its ligand (Fig. 3b). Since, functionally, D0 of KIR2DL4 may have less of an enhancer function and be more directly involved with ligand binding, it is hypothesised that the Ig-like characteristics of KIR2DL4 D0 shared with those of KIR2DL2 D1 are similar enough to use the model and do comparative analysis.

If one compares the intra-species allelic variation, the polymorphic residues in humans do not correspond with those found in rhesus macaque and vice versa. Some of the amino acid sites that differ in rhesus macaques in comparison to humans and chimpanzees are located on residues most likely binding the ligand of 2DL4 (Fig. 3b). The D0 domain contains three loops, two of which have a polymorphic residue: for instance, a proline on position 89 in humans vs an alanine in macaques. Unchanged residues are located in the purple region that is considered to face the MHC molecule but does not have a direct interaction, and the red hinch-loop (LYE) that separates the D0 and D2 domains. The D2 domain contains three loops, all of which show intra-

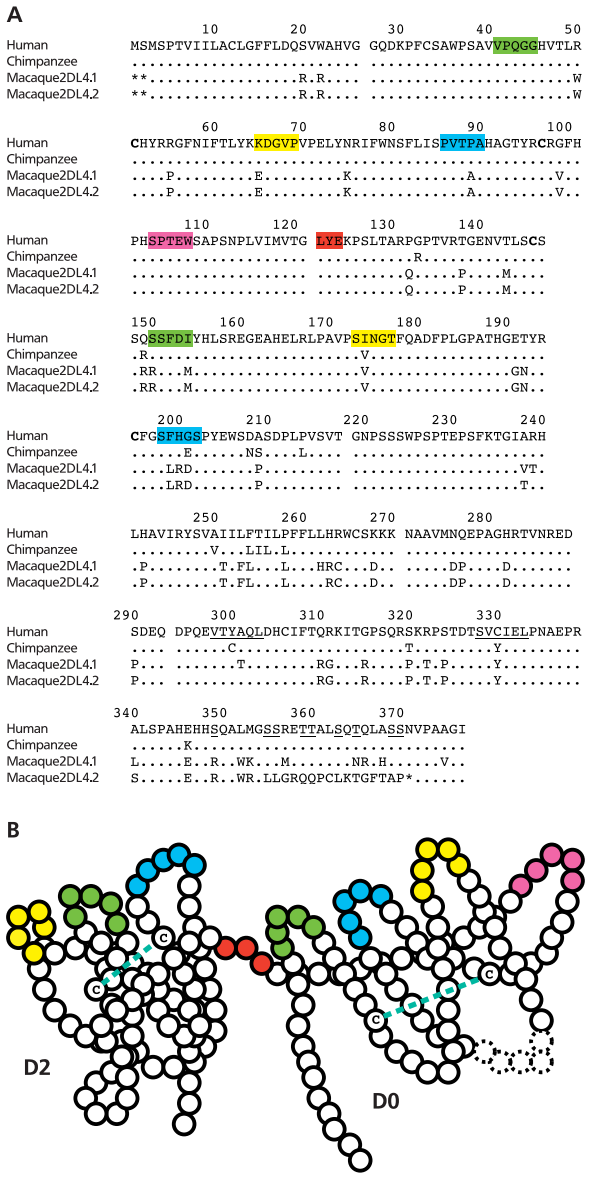

Figure 3 Inter-species comparison of KIR2DL4 proteins. **A** Amino acid comparison between human, chimpanzee and rhesus macaque KIR2DL4 proteins. Reference sequences of human KIR2DL4*0010101 (X99480), chimpanzee KIR2DL4 (X99480) and rhesus macaque Mamu-KIR2DL4.1 (*001) and Mamu-KIR2DL4.2 (*01501) have been aligned. Conserved cysteine residues in the Ig domains are shown in *bold*. Positions in the cytoplasmatic domains where ITIMs are present are *underlined*, as are serines and threonine residues in the C terminal cytoplasmic domain. *Highlighted* amino acids depict domains that may interact with the ligand. Polymorphic amino acid residues described in human 2DL4 are 53, 87, 95, 101, 132, 138, 169, 184, 209, 271, 296 and 371, whereas, for rhesus macaques, the polymorphic sites are 36, 47, 52, 238, 239, 243, 262, 268, 290, 301, 315, 340, 353, 357, 359 and 375. **B** 2D-Model of KIR2DL4 D0 and D2 domains based on the crystal structure of KIR2DL2. The hinch loop between the D0 and D2 domains has been depicted in *red*. The domains most likely to interact with a MHC class I ligand have been high lighted in *green*, *blue* and *yellow*, respectively. A loop that extends but might not interact with the MHC interface is shown in *purple*. Each *circle* represents an amino acid, and *dotted circles* represent amino acids present in KIR2DL2 but absent in KIR2DL4. Characteristic cysteines are represented.

-species differences, the most striking of which are the FHG residues starting at position 198 in humans, which are exchanged to the very different LRD residues in rhesus macaque. These differences appear to be a result of receptor-ligand co-evolution in macaques and humans.

It has been hypothesised that an ancient KIR2DL4 was initially formed by a deletion of part of intron 3, exon 4, and intron 4 from an ancestral KIR3DL structure (2).

The genetic footprint of this event is still visible in the presence of a break point at the place where intron 3 recombined to intron 4. This break point is present not only in humans and chimpanzees but is also observed in rhesus macaques (Fig. 4).

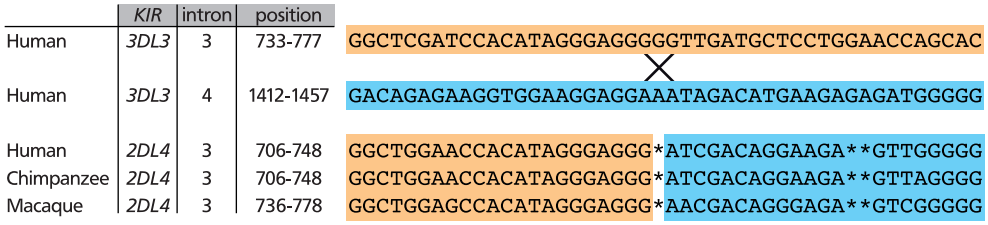

Figure 4 Schematic representation depicting the recombination event resulting in the birth of the 2DL4 gene. Intron 3 and 4 sequences of human *KIR3DL3*0010101* have been chosen because they best illustrate the ancestral sequences that have recombined. Intron 3 sequences of human (*KIR2DL4*0010101*), chimpanzee (BX842589) and macaque (*Mamu-KIR2DL4*001*) KIR2DL4 have been aligned, illustrating that this break point is shared by these three species, and as such, the recombination itself took place in a common ancestor.

This provides evidence that *KIR2DL4*, *PtKIR2DL4*, and *Mamu-KIR2DL4* are orthologues of each other, and that the genesis of the ancestral gene predates the speciation of humans, great apes, and Old World monkeys, which took place approximately 28 million years ago.

A comparison of the cytoplasmic tail section illustrates that humans and chimpanzees both have one ITIM (S/VxYxxL), which, however, maps to a different location (Fig. 3a). In rhesus macaques, both these ITIMs are present. The loss of one of these ITIMs in the human and chimpanzee lineages probably represents independent events, illustrating that selective forces continuously modify the activating/inhibitory capacity of KIR gene family members.

In rhesus macaques, two forms of Mamu-KIR2DL4 have been reported. Since the KIR2DL4.1 most resembles the human and chimpanzee form, it is likely that KIR2DL4.2 was formed independently in the macaque lineage. In this constitution, many original serine and threonine residues were lost, including a serine/threonine kinase site defined by SxR, which is thought to influence the intra-cellular signalling capacity or localisation of the protein. To determine whether there is a difference in the cellular localisation of both genotypes, HEK293 cells were transfected with constructs expressing recombinant Mamu-KIR2DL4 with a YFP attached to its C terminus. Both forms show similar expression and cellular distribution (Supplementary Fig. 1), although it is not yet clear whether they localise to endosomal compartments, as is observed in humans (44).

Population analyses of the *Mamu-KIR2DL4* forms

In human populations, alternative forms of KIR2DL4 have been described, which are differentiated by a truncated cytoplasmic tail resulting in a gene product that is not present on the cell surface. Thus, these forms may differ in their capacity to deliver intra-cellular signals. In rhesus macaques, two alternative forms of KIR2DL4 are also observed, which may differ in their strength to deliver signals. The genetic events modifying the signalling capacity of KIR2DL4 genes in humans and rhesus macaques probably happened independent of each other. Indeed, long and truncated alleles of 2DL4 are reported in chimpanzees, pygmy chimpanzees, and orangutans (19, 45, 46), whereas in gorillas, it was not observed (47), suggesting that this form was present in a common ancestor of humans and great apes.

These distinct types of genetic modification might have more or less the same effect and, as such, may point in the direction of convergent evolution. In the rhesus macaque, however, this effect would be much more subtle, since in humans, an entire cytoplasmic domain has been truncated, while in macaques, it has been altered.

The question is whether one of these alternative forms in rhesus macaques is present in more abundance than the other. To shed light on this issue, a large outbred population of Indian animals was examined. As can be seen, the population distribution of heterozygous or homozygous animals for a particular genotype has been determined (Fig. 5).

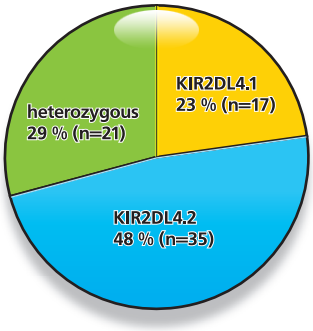

Figure 5 Frequency of *Mamu-KIR2DL4.1* and *-KIR2DL4.2* genotypes in the population of Indian rhesus macaques. The diagram shows the percentage of animals in which only the 2DL4.1 or 2DL4.2 form was detected or which have a heterozygous genotype.

Both alleles are not present in Hardy–Weinberg equilibrium, and the balance is shifted towards the *Mamu-KIR2DL4.2* form. This imbalance might be due to a linkage disequilibrium of this form with a neighbouring KIR gene or might have a technical nature, since one cannot rule out preferential amplification of certain forms, or it might be caused by a founder effect. However, both forms are still present in abundance, which suggests that the *Mamu-KIR2DL4* forms experience balancing selection and that the Indian rhesus macaques benefit from the existence of both genotypes.

A preliminary scan at the genomic level revealed that both forms are also present in animals of Burmese and Chinese origin, suggesting that the forms were present in an ancestral population. At this stage, we do not have enough independent material from outbred animals at hand to determine the gene frequencies in these populations. In conclusion, our data suggest that, due to convergent evolution, different primate species deployed distinct KIR2DL4 forms, which have differential capacities to deliver intra-cellular inhibitory signals and, as such, alter the activation state of the NK cell.

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REFERENCES

1. Valiante NM, Lienert K, Shilling HG, Smits BJ, Parham P. 1997. Killer cell receptors: keeping pace with MHC class I evolution. *Immunol Rev* 155: 155-64
2. Martin AM, Freitas EM, Witt CS, Christiansen FT. 2000. The genomic organization and evolution of the natural killer immunoglobulin-like receptor (KIR) gene cluster. *Immunogenetics* 51: 268-80
3. Single RM, Martin MP, Meyer D, Gao X, Carrington M. 2008. Methods for assessing gene content diversity of KIR with examples from a global set of populations. *Immunogenetics* 60: 711-25
4. Middleton D, Meenagh A, Gourraud PA. 2007. KIR haplotype content at the allele level in 77 Northern Irish families. *Immunogenetics* 59: 145-58
5. Stewart CA, Van Bergen J, Trowsdale J. 2003. Different and divergent regulation of the KIR2DL4 and KIR3DL1 promoters. *J Immunol* 170: 6073-81
6. Vilches C, Pando MJ, Parham P. 2000. Genes encoding human killer-cell Ig-like receptors with D1 and D2 extracellular domains all contain untranslated pseudoexons encoding a third Ig-like domain. *Immunogenetics* 51: 639-46
7. Kikuchi-Maki A, Catina TL, Campbell KS. 2005. Cutting edge: KIR2DL4 transduces signals into human NK cells through association with the Fc receptor gamma protein. *J Immunol* 174: 3859-63
8. Faure M, Long EO. 2002. KIR2DL4 (CD158d), an NK cell-activating receptor with inhibitory potential. *J Immunol* 168: 6208-14
9. Rajagopalan S, Long EO. 1999. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J Exp Med* 189: 1093-100
10. Gedil MA, Steiner NK, Hurley CK. 2005. Genomic characterization of KIR2DL4 in families and unrelated individuals reveals extensive diversity in exon and intron sequences including a common frameshift variation occurring in several alleles. *Tissue antigens* 65: 402-18
11. Goodridge JP, Lathbury LJ, Steiner NK, Shulse CN, Pullikotil P, Seidah NG, Hurley CK, et al. 2007. Three common alleles of KIR2DL4 (CD158d) encode constitutively expressed, inducible and secreted receptors in NK cells. *Eur J Immunol* 37: 199-211
12. Schellekens J, Tilanus MGJ, Rozemuller EH. 2008. The elucidation of KIR2DL4 gene polymorphism. *Mol Immunol* 45: 1900-6
13. Witt CS, Martin A, Christiansen FT. 2000. Detection of KIR2DL4 alleles by sequencing and SSCP reveals a common allele with a shortened cytoplasmic tail. *Tissue antigens* 56: 248-57
14. Witt CS, Whiteway JM, Warren HS, Barden A, Rogers M, Martin A, Beilin L, et al. 2002. Alleles of the KIR2DL4 receptor and their lack of association with pre-eclampsia. *Eur J Immunol* 32: 18-29
15. Kaizu M, Borchardt GJ, Glidden CE, Fisk DL, Loffredo JT, Watkins DI, Rehrauer WM. 2007. Molecular typing of major histocompatibility complex class I alleles in the Indian rhesus macaque which restrict SIV CD8+ T cell epitopes. *Immunogenetics* 59: 693-703
16. Otting N, de Vos-Rouweler AJM, Heijmans CMC, de Groot NG, Doxiadis GGM, Bontrop RE. 2007. MHC class I A region diversity and polymorphism in macaque species. *Immunogenetics* 59: 367-75
17. Otting N, Heijmans CMC, van der Wiel M, de Groot NG, Doxiadis GGM, Bontrop RE. 2008. A snapshot of the Mamu-B genes and their allelic repertoire in rhesus macaques of Chinese origin. *Immunogenetics* 60: 507-14

18. Karl JA, Wiseman RW, Campbell KJ, Blasky AJ, Hughes AL, Ferguson B, Read DS, et al. 2008. Identification of MHC class I sequences in Chinese-origin rhesus macaques. *Immunogenetics* 60: 37-46

19. Khakoo SI, Rajalingam R, Shum BP, Weidenbach K, Flodin L, Muir DG, Canavez F, et al. 2000. Rapid evolution of NK cell receptor systems demonstrated by comparison of chimpanzees and humans. *Immunity* 12: 687-98

20. Sambrook JG, Bashirova A, Palmer S, Sims S, Trowsdale J, Abi-Rached L, Parham P, et al. 2005. Single haplotype analysis demonstrates rapid evolution of the killer immunoglobulin-like receptor (KIR) loci in primates. *Genome Res* 15: 25-35

21. Grendell RL, Hughes AL, Golos TG. 2001. Cloning of rhesus monkey killer-cell Ig-like receptors (KIRs) from early pregnancy decidua. *Tissue Antigens* 58: 329-34

22. Hershberger KL, Shyam R, Miura A, Letvin NL. 2001. Diversity of the killer cell Ig-like receptors of rhesus monkeys. *J Immunol* 166: 4380-90

23. Blokhuis JH, Doxiadis GGM, Bontrop RE. 2009. A splice site mutation converts an inhibitory killer cell Ig-like receptor into an activating one. *Mol Immunol* 46: 640-8

24. Bimber BN, Moreland AJ, Wiseman RW, Hughes AL, O'Connor DH. 2008. Complete characterization of killer Ig-like receptor (KIR) haplotypes in Mauritian cynomolgus macaques: novel insights into nonhuman primate KIR gene content and organization. *J Immunol* 181: 6301-8

25. Slierendregt BL, van Noort JT, Bakas RM, Otting N, Jonker M, Bontrop RE. 1992. Evolutionary stability of transspecies major histocompatibility complex class II DRB lineages in humans and rhesus monkeys. *Hum Immunol* 35: 29-39

26. Bontrop RE, Otting N, de Groot NG, Doxiadis GG. 1999. Major histocompatibility complex class II polymorphisms in primates. *Immunol Rev* 167: 339-50

27. Penedo MCT, Bontrop RE, Heijmans CMC, Otting N, Noort R, Rouweler AJM, de Groot N, et al. 2005. Microsatellite typing of the rhesus macaque MHC region. *Immunogenetics* 57: 198-209

28. Doxiadis GGM, de Groot N, Claas FHJ, Doxiadis IIN, van Rood JJ, Bontrop RE. 2007. A highly divergent microsatellite facilitating fast and accurate DRB haplotyping in humans and rhesus macaques. *Proc Natl Acad Sci USA* 104: 8907-12

29. Doxiadis GGM, Otting N, de Groot NG, de Groot N, Rouweler AJM, Noort R, Verschoor EJ, et al. 2003. Evolutionary stability of MHC class II haplotypes in diverse rhesus macaque populations. *Immunogenetics* 55: 540-51

30. de Groot N, Doxiadis GGM, de Vos-Rouweler AJM, de Groot NG, Verschoor EJ, Bontrop RE. 2008. Comparative genetics of a highly divergent DRB microsatellite in different macaque species. *Immunogenetics* 60: 737-48

31. Doxiadis GGM, Rouweler AJM, de Groot NG, Louwerse A, Otting N, Verschoor EJ, Bontrop RE. 2006. Extensive sharing of MHC class II alleles between rhesus and cynomolgus macaques. *Immunogenetics* 58: 259-68

32. Korber B. 2000. HIV signature and sequence variation analysis. In *Computational analysis of HIV molecular sequeces*, ed. GL AG Rodrigo, pp. 55-72. Dordrecht: Kluwer

33. Marsh SGE, Parham P, Dupont B, Geraghty DE, Trowsdale J, Middleton D, Vilches C, et al. 2003. Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002. *Immunogenetics* 55: 220-6

34. Du Z, Sharma SK, Spellman S, Reed EF, Rajalingam R. 2008. KIR2DL5 alleles mark certain combination of activating KIR genes. *Genes Immun* 9: 470-80

35. Marsh SGE, Albert ED, Bodmer WF, Bontrop RE, Dupont B, Erlich HA, Geraghty DE, et al. 2005. Nomenclature for Factors of the HLA System, 2004. *Hum Immunol* 66: 571-636

36. Boyson JE, Shufflebotham C, Cadavid LF, Urvater JA, Knapp LA, Hughes AL, Watkins DI. 1996. The MHC class I genes of the rhesus monkey. Different evolutionary histories of MHC class I and II genes in primates. *J Immunol* 156: 4656-65

37. Daza-Vamenta R, Glusman G, Rowen L, Guthrie B, Geraghty DE. 2004. Genetic divergence of the rhesus macaque major histocompatibility complex. *Genome Res* 14: 1501-15

38. Otting N, Heijmans CMC, Noort RC, de Groot NG, Doxiadis GGM, van Rood JJ, Watkins DI, et al. 2005. Unparalleled complexity of the MHC class I region in rhesus macaques. *Proc Natl Acad Sci USA* 102: 1626-31

39. Doxiadis GGM, Heijmans CMC, Bonhomme M, Otting N, Crouau-Roy B, Bontrop RE. 2009. Compound evolutionary history of the rhesus macaque MHC class I B region revealed by microsatellite analysis and localization of retroviral sequences. *PLoS ONE* 4: e4287

40. Boyson JE, Iwanaga KK, Golos TG, Watkins DI. 1996. Identification of the rhesus monkey HLA-G ortholog. Mamu-G is a pseudogene. *J Immunol* 157: 5428-37

41. Watkins DI, Chen ZW, Hughes AL, Evans MG, Tedder TF, Letvin NL. 1990. Evolution of the MHC class I genes of a New World primate from ancestral homologues of human non-classical genes. *Nature* 346: 60-3

42. Bontrop RE. 2006. Comparative genetics of MHC polymorphisms in different primate species: duplications and deletions. *Hum Immunol* 67: 388-97

43. Boyington JC, Brooks AG, Sun PD. 2001. Structure of killer cell immunoglobulin-like receptors and their recognition of the class I MHC molecules. *Immunol Rev* 181: 66-78

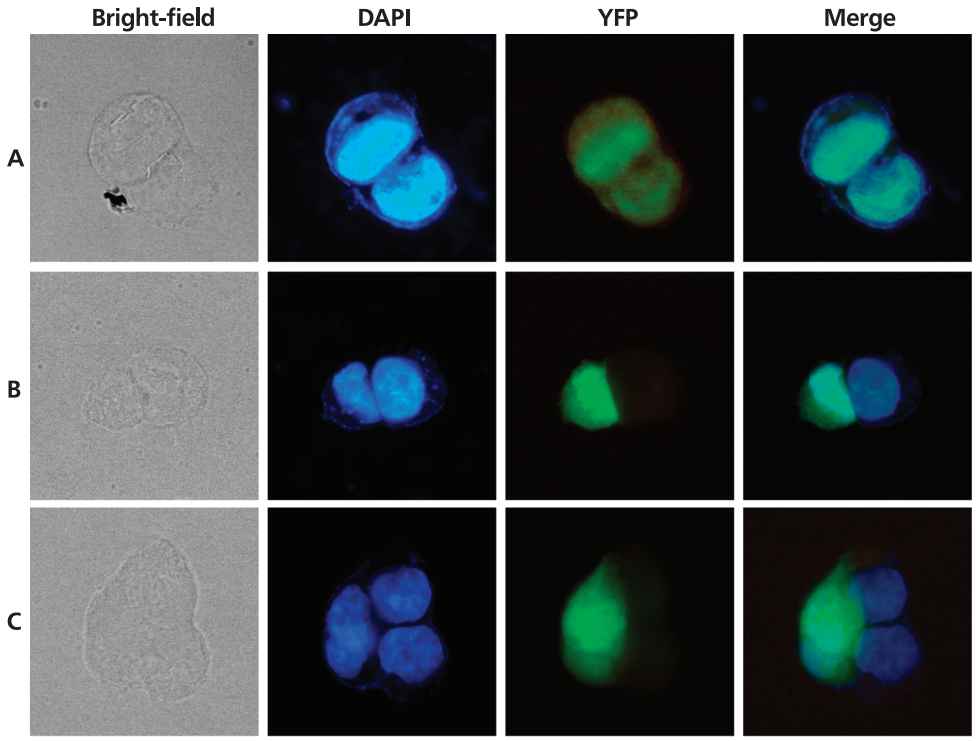
44. Rajagopalan S, Bryceson YT, Kuppusamy SP, Geraghty DE, van der Meer A, Joosten I, Long EO. 2006. Activation of NK cells by an endocytosed receptor for soluble HLA-G. *PLoS Biol* 4: e9

45. Rajalingam R, Hong M, Adams EJ, Shum BP, Guethlein LA, Parham P. 2001. Short KIR haplotypes in pygmy chimpanzee (Bonobo) resemble the conserved framework of diverse human KIR haplotypes. *J Exp Med* 193: 135-46

46. Guethlein LA, Flodin LR, Adams EJ, Parham P. 2002. NK cell receptors of the orangutan (Pongo pygmaeus): a pivotal species for tracking the coevolution of killer cell Ig-like receptors with MHC-C. *J Immunol* 169: 220-9

47. Rajalingam R, Parham P, Abi-Rached L. 2004. Domain shuffling has been the main mechanism forming new hominoid killer cell Ig-like receptors. *J Immunol* 172: 356-69

SUPPLEMENTARY INFORMATION



Supplementary Figure 1 Microscopy of HEK293 cells two days after transfection (magnification x400). From left to right: bright-field picture, DAPI stained fluorescent nucleus, YFP-fusion protein, merge of previous pictures. Cells were transfected with pcDNA6.2/C-YFP-GW/TOPO containing as an insert **A** *Mamu-KIR2DL4*001* (2DL4.1) **B** *Mamu-KIR2DL4*01501* (2DL4.2) **C** control chloramphenicol acetyl transferase (CAT). Since transfection efficiencies differ, some cells do not show YFP fluoresence because they were not transfected.

Chapter 3

A splice site mutation converts an inhibitory killer cell Ig-like receptor into an activating one

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ABSTRACT

The killer cell Ig-like receptor (KIR) 3DH protein in rhesus macaques (*Macaca mulatta*) is thought to be an activating one because it contains a charged arginine in its transmembrane domain and has a truncated cytoplasmic domain. *MmKIR3DH* has thus far been characterized by an analysis of cDNA. Its presence and polymorphism has been further investigated by examining mRNA transcripts and genomic sequences in families. Multiple copies of *MmKIR3DH* are present per animal, suggesting that the gene has been duplicated on some haplotypes. All transcripts are truncated and lack exon 8. Investigation of the gene itself shows that exon 8 is present, intact, and homologous to *MmKIR2DL4*. However, there is a mutation in the donor splice site of intron 8, which is absent in *MmKIR2DL4* genomic sequences. This mutation introduces a frameshift, subsequently resulting in a premature stopcodon. To further verify this mutation, a cohort of unrelated animals from different geographical locations was examined, and both exon 8 and the splice site mutation were seen to be present in their *MmKIR3DH* genes. The data suggest that the splice site mutation causes the truncation of the *MmKIR3DH* transcript and the subsequent loss of its inhibitory motifs further downstream. Loss of inhibitory potential through different mutations is observed in other primate species as well, suggesting convergent evolution; however, this is the first report to document that a mutation in an intron produces a similar effect.

INTRODUCTION

Killer cell Ig-like receptor (KIR) represent a family of proteins that interact with major histocompatibility complex (MHC) class I gene products as its ligand (1-3). In humans, KIR are expressed on natural killer (NK) cells and certain subpopulations of T cells (4, 5). KIR are type I transmembrane glycoproteins that consist of two or three Ig-like domains, a stem region, a transmembrane domain, and a cytoplasmic tail (6). KIR nomenclature is based on the number of Ig domains (2-3D) and on the presence of a long (L) or short (S) cytoplasmic tail (7). KIR can transduce an inhibitory signal into the cell through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) located on their cytoplasmic tail (8). If the protein has a polar membrane-embedded interaction site, it can activate the cell through an association with an adaptor molecule that has immunoreceptor tyrosine-based activating motifs (ITAMs) (9, 10). Inhibitory KIR control immunological tolerance by scanning for the presence of self-MHC class I molecules (2). Activating KIR play an important role in lysing transformed or infected cells or in producing pro-inflammatory cytokines. The balance in these signals determines the activation state of the NK cell.

Human KIR genes are situated in the leukocyte receptor cluster (LRC), which is located on chromosome 19q13.4 (11). The complex is highly plastic and harbors genes in different region configurations, and its gene content has been reshuffled by

recombination events and unequal crossovers (12, 13). In humans, a wide range of haplotypes has been described, and based on the number of activating genes a division has been made into A and B haplotypes (14). At the population level, some KIR genes display abundant levels of polymorphism (15, 16).

Allelic variation is governed by different genetic mechanisms like point mutations, domain exchange by recombination, and insertions or deletions, and more recently, a deletion and substitution event has been documented (17, 18). Amino acid polymorphisms may have an effect on ligand binding or prevent the protein from reaching the cell surface (19-21), and some KIR are being expressed as splice variants (22, 23), resulting in either membrane bound or secreted receptors. Specific KIR have been reported to have an association with the outcome of infectious diseases (24, 25), autoimmunity (26), and pre-eclampsia (27). In transplantation, certain MHC-KIR combinations are beneficial, while others give rise to less relapse and more graft versus host disease (28-30).

Rhesus macaques are used in preclinical models to study human infectious diseases, autoimmune disorders, and transplantation biology (31, 32), and a detailed description of the immune system is necessary to be able to interpret the research data.

Comparative genomic studies on different primate species illustrate that KIR are rapidly evolving receptors (33-41), presumably co-evolving differently in separate species alongside their respective ligands (42). In contrast, KIR2DL4 gene structure is highly conserved, most likely because it binds to the oligomorphic nonclassical HLA-G structure. Human KIR2DL4 can activate the NK cell (43) but it also has inhibitory potential (44). In rhesus macaques, the gene for 2DL4 encodes two ITIMs, possibly tipping the balance towards inhibition.

An ancestral KIR gene has recently been described that is located more centromeric in the LRC (45), and presumably a duplication event followed by rapid evolution has given rise to different KIR lineages for different species. In the orangutan, one KIR lineage has been suggested to have co-evolved along with *Popy-C*, the evolutionary equivalent of *HLA-C* (46). Since macaques have an extensive number of *Mamu-A* and *-B* genes displaying differential transcription levels (47), it is conceivable that *MmKIR* genes have evolved to cope with this diversity. KIR transcripts from macaques have been described for both inhibitory and activating genes, all of which showed some levels of polymorphism. One haplotype has been described at the genomic level, which lacks activating *MmKIR* genes (48).

MmKIR3DH appears to be a hybrid gene, probably created by a recombination event between ancestral *KIR3DL* and *KIR2DL4* structures (36). Its nucleotide sequence encodes a transmembrane domain that contains a charged arginine residue. However, due to the deletion of exon 8 and a subsequent frameshift mutation, the *MmKIR3DH* transcript does not encode ITIMs; it is thought that *MmKIR3DH* encodes for the only activating KIR. To gain more insight into its evolutionary history and

polymorphisms at the population level, this activating KIR gene was characterized at the transcription and genomic level.

MATERIALS AND METHODS

Animals and cells

In this study, 48 rhesus macaques belonging to the BPRC self- sustaining colony were analyzed, and animals were selected on the basis that they had different geographic origins. Whole blood was obtained during regular health checks, and peripheral blood mononuclear cells (PBMCs) were isolated as previously described (49). All animals were typed by different methods for their *Mamu-A, -B*, and *-DR* genes (50-53).

RNA extraction, cDNA synthesis, and amplification

Total RNA was isolated from 5×10^6 PBMC with Trizol reagent (Invitrogen, Paisley Scotland), according to the manufacturer's instructions. First-strand cDNA synthesis was performed with a oligo$(dT)_{18}$ primer and RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany). PCR was performed with newly designed primers synthesized by Invitrogen (Paisley, Scotland). The primer pairs are as follows: for *MmKIR2DL4*: 5′-CCACATCCTCTGCACCGGTCAGTC-3′ and 5′-GCAGGGGTCAAGTGAAGGGGAGAA-3′ ; for *MmKIR3DH*: 5′-TCCAGAKGGCCTGTCCACACAC-3′ and 5′-CTGTCTGTGGTGCTCCTGGGCT-3′. PCR was performed in a 20 µl reaction volume containing 1× Phusion HF Buffer, 3% DMSO, 5 µl first-strand cDNA, 0.5 µM Primers, 200 µM dNTPs, and 0.4 units Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland). The amplification parameters for both PCRs were a 45 s initial denaturation step at 98°C, followed by 30 cycles of a 15 s 98°C denaturation step, a 30 s 66°C annealing step, and a 90 s 72°C extenstion step. A final extension step was performed at 72°C for 7 min.

Genomic DNA extraction and amplification

Genomic DNA was extracted from heparinized blood samples or immortalized rhesus macaque B cells according to standard protocols (54). PCR of *MmKIR2DL4* and *KIR3DH* from exon 7 into the untranslated 3′-region was conducted with newly designed primers: 5′-GTGATTAGGTACTCGGTGGCCACCAT-3′ and 5′-GCAGGGGTCAAGTGAAGGGGAGAA-3′. The reaction was performed in a 20 µl volume containing 1× Phusion HF Buffer, 3% DMSO, 100ng genomic DNA, 0.5 µM primers, 200 µM dNTPs, and 0.4 units Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland). The cycling parameters were a 45 s initial denaturation step, followed by 30 cycles of a 15 s 98°C denaturation step, a 30 s 68°C annealing step, and a 3 min 72°C extenstion step. A final extension step was performed at 72°C for 7 min.

Accession numbers

The following sequences from the GenBank database were used for comparative analyses: *MmKIR3DHlike1-5* (AY505479-AY505483), *MmKIR3DH1-5* (AF334648-AF334651, NM001105173), *MmKIR3DL1-21* (AF334616-AF334626, AF408150 AF361086, NM001105174, AY728184, AY728188), *MmKIR3DH11* (AY505484), *MmKIR3DH17* (AY505485), *MmKIR3DM1* (AY505487), and *MmKIR2DL4* (AY728182). GenBank accession numbers for the sequences described in this study are provided in Table 1.

Table 1 Overview of alleles, animals and accession numbers.

Gene	Animals	Accession number
MmKIR3DH6	01087, 02039, 9133	EU702452
MmKIR3DH7	98016	EU702453
MmKIR3DH8	4065	EU702454
MmKIR3DH9	98016	EU702455
MmKIR3DH10	03100, 95020	EU702456
MmKIR3DH11	94056, 03100, KM	EU702457
MmKIR3DH12	01087, 9133	EU702458
MmKIR3DH13	01087, 8909	EU702459
MmKIR3DH14	94032, 05053, 03080	EU702460
MmKIR3DH15	4065	EU702461
MmKIR3DH16	9133	EU702462
MmKIR3DH17	00007, 4065, D55, 9133	EU702463
MmKIR3DH18	94056, 03100	EU702464
MmKIR3DH19	03080, KM, 05053, 94032	EU702465
MmKIR3DH20	98016, 05105	EU702466
MmKIR3DH21	03100, 05105, 95020	EU702467
MmKIR3DM1	KM, 94032, 4065	EU702468
MmKIR3DM2	94056, 03100	EU702469
MmKIR3DM3	D55	EU702470
MmKIR3DM4	4065	EU702471
MmKIR3DM5	01087, 9133	EU702472
MmKIR3DM6	8909, 00007	EU702473
Gene fragments	**Animals**	**Accession number**
MmKIR2DL4	94056, C122	EU702474
fragment1	BB109	EU702475
fragment2	1MP	EU702476
fragment3	9133, 8909	EU702477
MmKIR3DM2	94056, 4049	EU702478
fragment4	1087	EU702479
MmKIR3DH11	94056, C128	EU702480
MmKIR3DH18	94056, C128	EU702481
fragment5	BB109	EU702482
fragment6	1MP	EU702483
fragment7	C122	EU702484
fragment8	9133	EU702485

Cloning and sequencing

PCR products were subjected to gel electrophoresis, and bands of appropriate size

were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, USA). Products were blunt-end ligated into the cloning site of the PJET1.2/blunt vector (Fermentas, St. Leon-Rot, Germany) and were used to transform competent XL1Blue *E. coli* cells with the TransformAid Bacterial Transformation Kit (Fermentas, St. Leon-Rot, Germany). A minimum of 32 clones were examined per PCR reaction. Plasmid was isolated and products were sequenced with pJET1.2 forward and reverse primers (Fermentas, St. Leon-Rot, Germany) on a 3130XL ABI automatic sequencer (Applied Biosystems, Foster City, USA). Although many clones were selected and analyzed, only sequences that were present in multiple PCR products or animals are reported.

Sequence alignments and phylogenetic analysis

The Seqman program (Dnastar, Inc., Madison, USA) was used to align the sequences; the Macvector version 8.1.1. program (MacVector, Inc., Cambridge, UK) was used to analyze and compare sequences. A phylogenetic analysis was performed with the program PAUP*4.0b10 (55) (http://paup.csit.fsu.edu). Pairwise distances were calculated using the Kimura-2 parameters, and the Neighbor-Joining method was used to create a phylogram. Bootstrap values were calculated based on 1000 iterations. The program Genesplicer (56) was used to compute the effect of mutations on splicing.

RESULTS

MmKIR3DH segregates in families and displays copy number variation

A family of Indian rhesus macaques that had been pedigreed based on the segregation of several MHC markers was chosen for analysis. Segregation profiles could be constructed based on the manner in which combinations of *MmKIR3DH* genes were passed on to the offspring (Fig. 1).

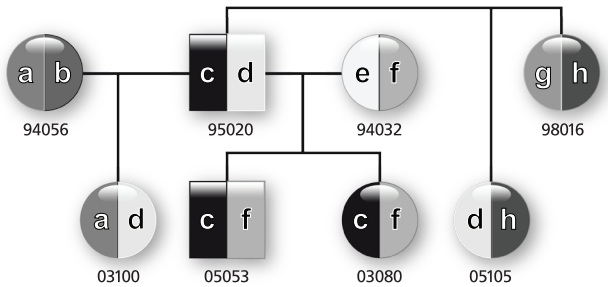

Figure 1 KIR genes segregate in a family of Indian rhesus macaques. In the top row are depicted the parental animals, the bottom row are offspring. Numbers correspond with the animal, and letters with the KIR haplotype. The "a" haplotype is passed on from 94056 to 03100. Haplotypes "c" and "f" are inherited by animals 05053 and 03080, haplotype "d" by 05105, and haplotype 'h" by 05105. Haplotypes "b", "e", and "g" are not inherited in this panel.

Multiple copies of *MmKIR3DH* genes were detected per individual and an overview of the haplotype content is provided (Table 2). There are haplotypes (b-d) that lack *MmKIR3DH*, in accordance with the genomic data on a published haplotype (48);

Table 2 *MmKIR3DH* and *MmKIR3DM* alleles observed on each haplotype.

Haplotype	*MmKIR3DH*	*MmKIR3DM*
a	*11, 18*	2
b	-	-
c	-	-
d	*10, 21*	-
e	-	-
f	*14, 19*	1
g	*7, 9*	-
h	*20*	-

however, haplotypes with 1 (h) or 2 (a, d, f, g) loci of *MmKIR3DH* are also observed. Additionally, two haplotypes (a, f) contain a KIR gene structure that is highly similar to a previously described sequence (accession number AY505487) (57), which has been designated *MmKIR3DM* (Medium) because of the intermediate length of its cytoplasmic domain encoding region compared to *MmKIR3DH* and *MmKIR3DL*.

MmKIR3DH displays extensive polymorphism

Together with the above family of eight individuals, cDNAs of five related (father 8909, mother 9133, and children 00007, 01087, 02039) and three unrelated animals (4065, KM, D55) were analyzed. In these animals, 14 unreported *MmKIR3DH* sequences were observed as well as 2 previously reported ones (*MmKIR3DH11*, *MmKIR3DH17*). The newly reported sequences were, provisionally, named *MmKIR3DH6-21*. All these *MmKIR3DH* transcripts share a 53bp deletion, indicating that exon 8 is lacking. Some animals (01087, 4065, 9133) expressed more than two *MmKIR3DH* transcripts, indicating that they too contain haplotypes with duplicated loci. The general protein structure encoded by *MmKIR3DH* transcripts was compared with those of *MmKIR2DL4* and *MmKIR3DM* (Fig. 2).

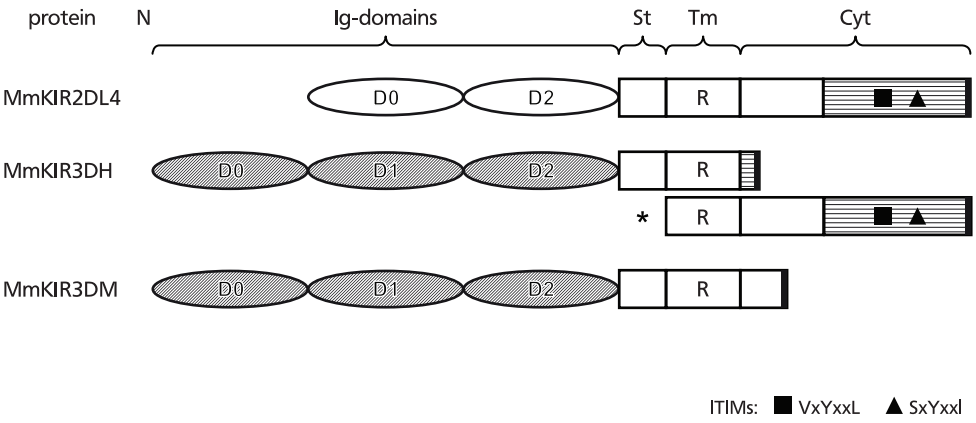

Figure 2 Schematic representation of KIR proteins that are encoded by observed cDNAs. Immunoglobulin (Ig) domains are depicted along with stem (St), transmembrane (Tm), and cytoplasmic (Cyt) domains. In all proteins a charged arginine (R) is located central in the transmembrane domain. Additionally, MmKIR2DL4 contains 2 ITIMs in the C-terminal part of its cytoplasmic domain. Both MmKIR3DH and 3DM have a truncated tail thus lacking these ITIMs. "*" Shows a prediction of the protein tail of MmKIR3DH that is based on translation of its intact exon sequences.

MmKIR2DL4 protein has a characteristic D0-D2 configuration along with a long cytoplasmic tail that contains two ITIMs. All molecules are highly similar from stem to transmembrane domain, and contain a central arginine in the transmembrane domain. Both MmKIR3DH and MmKIR3DM have three Ig-domains which although similar are polymorphic, and they comprise a truncated cytoplasmic tail lacking ITIMs. When analysing the translated *MmKIR3DH* transcripts, it is observed that most amino acids are conserved between different alleles, and cysteine residues are located on both ends of an Ig-like domain.

An alignment of the polymorphic residues shows they are located in all three Ig-like domains (Fig. 3).

Some genes differ by synonymous nucleotide mutations, explaining why they are depicted with a single amino acid sequence. Two genes, *MmKIR3DH16* and *MmKIR3DHlike-4*, share characteristics of multiple clades, most likely due to recombination events. When comparing the D1 and D2 domains with human KIR genes (58), similar positions involved with ligand binding seem to be polymorphic. Finally, transcripts of *MmKIR3DM* genes are different from KIR3DH because they contain exon 8, which has a single nucleotide deletion, resulting in a cytoplasmic tail of intermediate length (Fig. 2). The cause of the truncation of MmKIR3DH protein was not observed in its exons, since they appear to encode an intact long cytoplasmic tail.

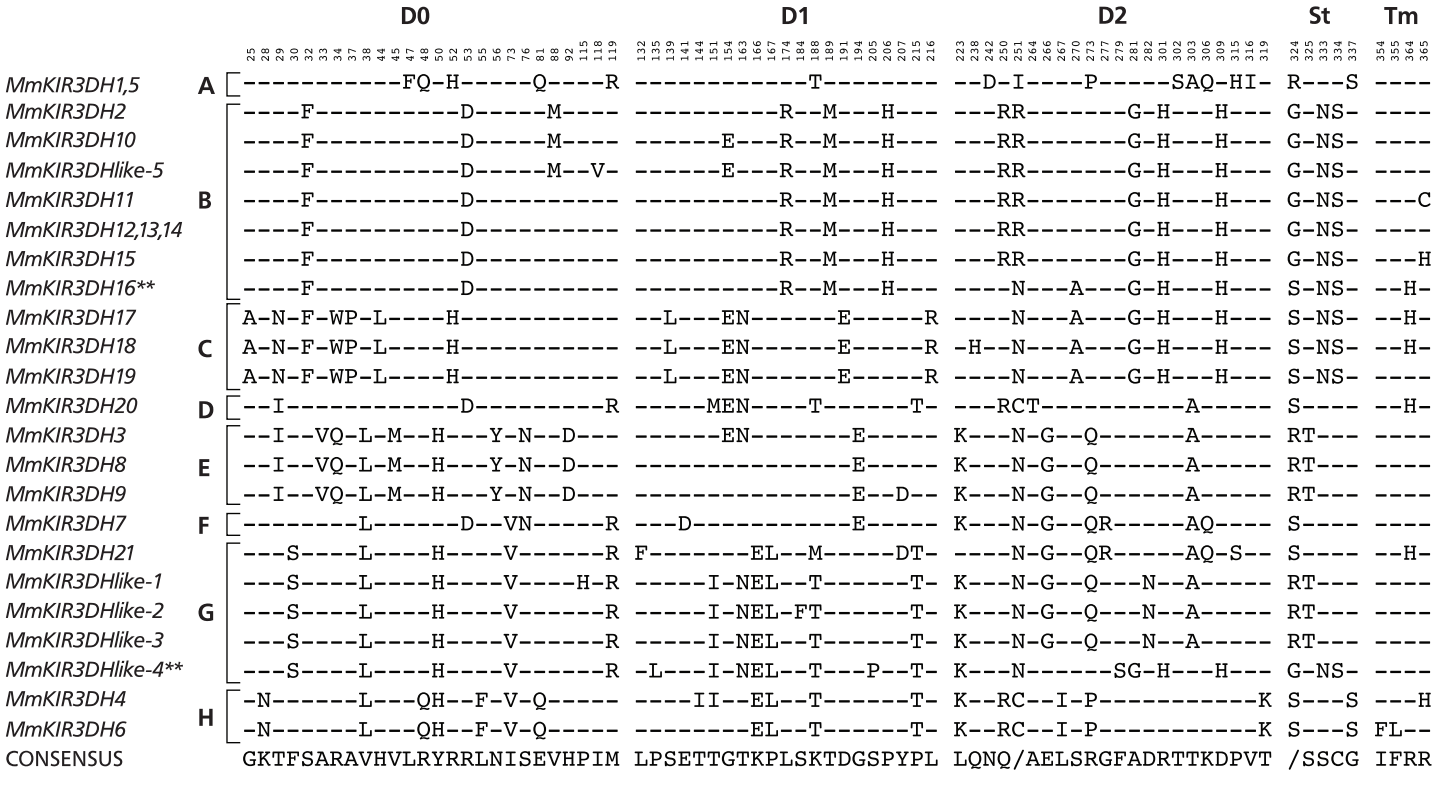

Figure 3 *MmKIR3DH* alleles are polymorphic in their ligand binding domains and show patterns of domain exchange. An alignment is shown of translated polymorphic amino acid residues of *MmKIR3DH* genes. Leader peptide sequences are not shown due to primer binding. The consensus sequence shows residues that are present >50% in the panel. "-" in the alignment indicates a residue that is identical to the consensus. "/" in the consensus means a position where no residue is represented >50%. Eight clades (A-H) are indicated based on phylogenetic analysis. In this panel, two alleles most likely formed by recombination are indicated by "**".

Phylogenetic analysis of *MmKIR3DH* genes defines paralogous relationships

A phylogenetic analysis was performed on nucleotide sequences that encode the Ig, stem, and transmembrane domains of the *MmKIR3DH* and *KIR3DM* transcripts. *MmKIR3DL* sequences were added as outgroups (Fig. 4).

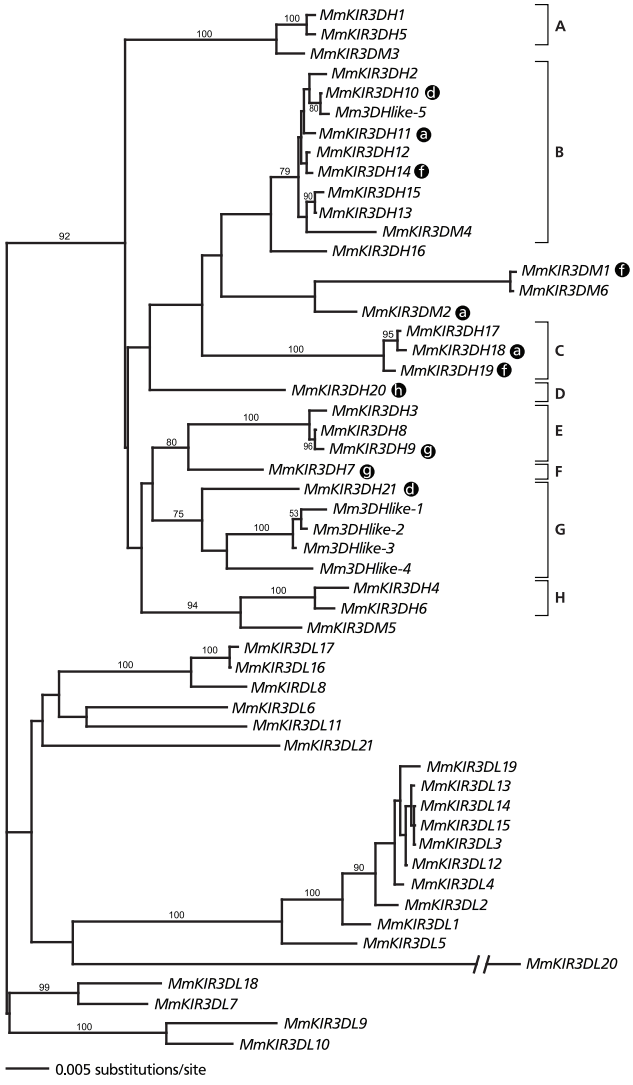

Figure 4 The *MmKIR3DH* gene family is polymorphic and comprises several lineages. A phylogram of *MmKIR3DH, MmKIR3DM,* and *MmKIR3DHlike* nucleotide sequences is depicted. *MmKIR3DL* sequences are shown as outgroup. The Neighbor-joining method was used for treebuilding and the Kimura-2 parameter to calculate distances. Bootstrap values were calculated based on 1000 iterations and values >75% are shown. Genes that are present on observed haplotypes are indicated with the corresponding haplotype in lowercase. Clades have been defined A to H.

Multiple clades were defined, indicating that *MmKIR3DH* is polymorphic and comprises separate lineages. The different *MmKIR3DH* loci found on one haplotype are situated in different sections of the tree. If for instance the genes on haplotype "a" are examined, *MmKIR3DH11* is located in clade B of the tree and *KIR3DH18* in clade C, the *KIR3DM2* gene clusters between these two clades. Haplotype "f" shows a similar pattern, as *MmKIR3DH14* is located in clade B, while *KIR3DH19* is located in clade C and *KIR3DM1* is positioned between these two clades. Finally, haplotype "d" shows *MmKIR3DH10* in clade B and *MmKIR3DH21* in clade G. These paralogs demonstrate nucleotide variation, indicating there was sufficient time after their duplication to acquire polymorphism. Some *MmKIR3DM* alleles cluster together but apart from *KIR3DH*, possibly indicating an old entity. Others like *MmKIR3DM3*, *-4*, and *-5* group between different *KIR3DH* clades, suggesting that a domain exchange has taken place here.

MmKIR3DH is present in all animals screened

A large panel of animals from different geographical regions such as India, Burma, and China were genomically typed for the presence of gene segments of *MmKIR2DL4* and *KIR3DH* regions encoding the transmembrane and cytoplasmic domain. Gel electrophoresis of this PCR product resulted in the detection of one single band of approximately 1250bp. Subsequent cloning and sequencing showed that multiple products were amplified. Comparison of the exons and untranslated region of these genomic sequences with *MmKIR2DL4* and *KIR3DH* transcripts showed that both entities were observed in all animals (Fig. 5). In some instances, a 3DM gene fragment was also detected, suggesting that this gene has a low gene frequency. In a comparison of exon 8 and intron 8 sequences, polymorphism was observed between animals from India, China, and Burma, but sequences were also shared between them.

A splice site mutation is discovered in intron 8 of *MmKIR3DH*

The *MmKIR2DL4*, *KIR3DH*, and *KIR3DM* genes share exon 8 sequences, which are highly similar. Two differences between these gene sections are that *MmKIR3DH* has a substitution positioned at the first nucleotide of intron 8, changing a guanine into an adenine residue (Fig. 5) and a deletion of the fifth nucleotide. When comparing the extended donor splice sites of these genes with the consensus GTRAGT sequence, it is observed that *MmKIR2DL4* and *KIR3DM* (GTAGGT) share five out of six nucleotides, whereas in the case of *MmKIR3DH* (ATAGTC) this drops to three out of six. All gene fragments comprise an acceptor splice site that matches the consensus Y_{7-10}NAG, and a branch site resembling the YNYURAY consensus. Exon 8 contains an alternative branch site sequence, while some fragments have acquired an alternative acceptor splice site sequence. Subsequent analysis of this region with the program Genesplicer shows that the complex mutation in the donor site disrupts the splicing

of *MmKIR3DH*, in concordance with the truncated transcript observed.

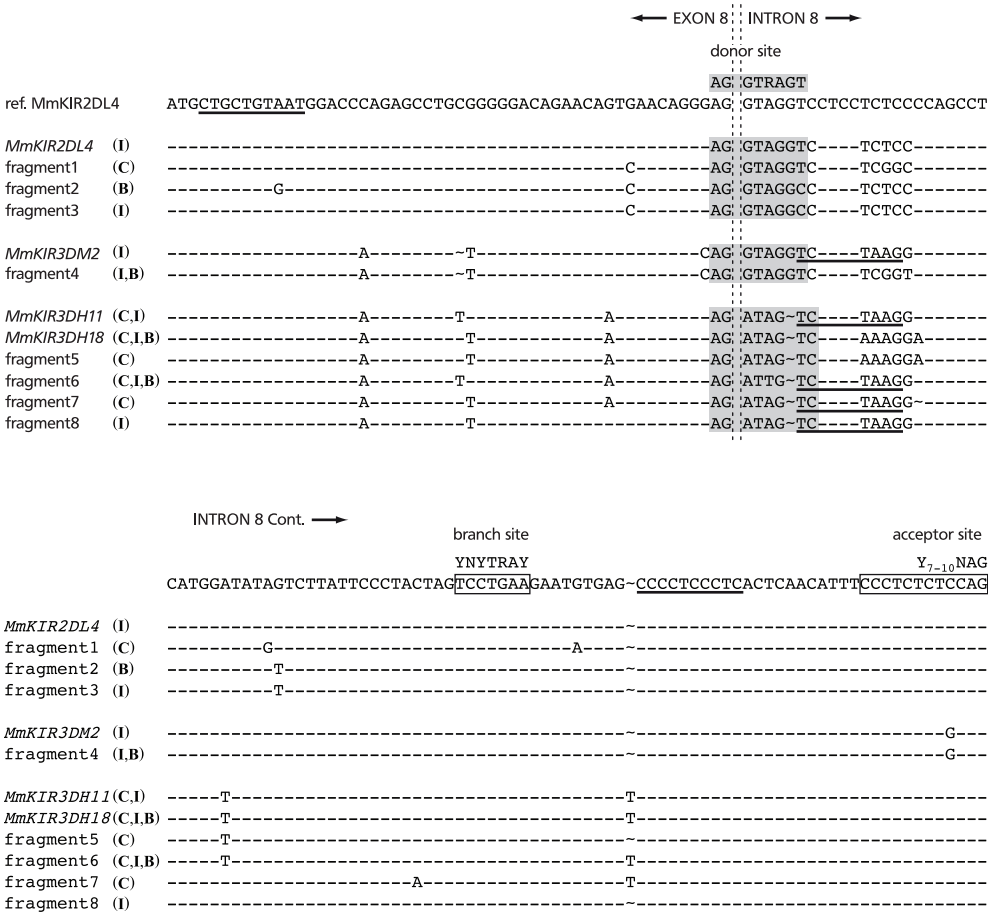

Figure 5 A comparison of (pseudo)exon 8 and intron 8 sequences of *MmKIR2DL4*, *KIR3DH*, and *KIR3DM* shows the presence of a complex splice site mutation. *MmKIR2DL4* (AY728182) is shown as reference sequence. The donor splice site is highlighted in gray and a consensus sequence AGGTRAGT is shown. The branch (YNYTRAY) as well as the donor acceptor site Y_{7-10}NAG is shown boxed in intron 8. An alternative branch site in exon 8 and alternative pyrimidine-rich domains and an acceptor site in intron 8 are underscored. "-" means a nucleotide similar to the reference sequence, "d" indicates a nucleotide deletion. Sequences of the haplotype "a" are presented along with gene fragments found in animals from different geographical regions. Fragments observed in Chinese, Indian and Burmese animals are indicated by "C", "I", and "B" respectively.

Alternatively, if this guanine to adenine substitution was replaced by the original guanine, a long transcript is predicted. This predicted transcript encodes 2 ITIMs, as is

the case for *MmKIR2DL4*. The *MmKIR3DM* gene looks similar to *MmKIR3DH*, but it lacks the splice site mutation and has a single nucleotide deletion in exon 8, in accordance with the cDNA data. Combining these data provides a model for the evolution of the *MmKIR3DH* gene family, which suggests a complex series of duplications and recombination (Fig. 6).

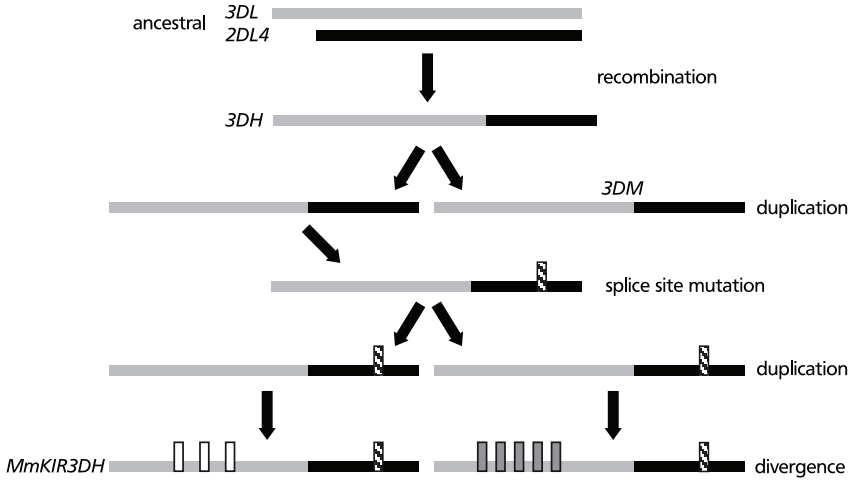

Figure 6 This model depicts the evolution and emergence of *MmKIR3DH*, followed by the introduction of duplications and polymorphism. An ancestral recombination event between *2DL4* and *3DL* created an hybrid ancestral gene. This gene subsequently duplicated and gave rise to the *KIR3DH* and *KIR3DM* lineage. A splice site mutation was introduced in *KIR3DH* and this gene became fixed in the population before the separation of Chinese, Burmese, and Indian macaques. Further duplications of this gene and the subsequent acquisition of polymorphism gave rise to different loci and *MmKIR3DH* alleles.

DISCUSSION

The present report illustrates that a single nucleotide substitution in a splice site has a major effect on the resulting gene product, as it transforms an inhibitory KIR protein into an activating one. It seems that through acquisition of this splice site mutation and possibly because an alternative branch site and multiple polypyrimidine tracks are available, splicing of *MmKIR3DH* is disrupted. As a consequence, the *MmKIR3DH* gene contains a pseudoexon, making it impossible to distinguish the *KIR2DL4* and *KIR3DH* genes based on the length of their cytoplasmatic domain-encoding regions. Attention should be drawn to the fact that on the basis of a genomic analysis of exon sequences, this gene would have been predicted to encode a product with a long cytoplasmic tail and two ITIMs. Only by an analysis of the corresponding transcripts was this truncation noticed. Based on the present results, by sequencing the exon-

intron boundaries it has become possible to genotype individuals for the presence or absence of these KIR genes. It cannot be ruled out, however, that other KIR in human and nonhuman primates have subtle mutations in their intron sequences, which affect splicing, skew alternative splicing, or influence transcription quantity. Due to the length of KIR genes, it is a challenge to correlate the genomic information of exons 1-5 with that of exons 6-9. Due to the recent description of the cloning and sequencing of the entire human *2DL4* gene, and with an increased knowledge of specific alleles, bead-based separation of these products has become possible (59, 60). This approach would be favorable for further analyses of KIR genes, since exon and intron data can be obtained within the same experiment.

MmKIR3DH is a hybrid gene, most likely formed by recombination of an ancestral 3DL and 2DL4 in the macaque lineage (36). The duplications observed in this communication confirm that the KIR gene cluster is highly plastic and rapidly evolving, as has been documented for other nonhuman primate species (38). Comparison of alleles and/or paralogs shows not only polymorphism by point mutation but also the presence of recombination events between various domains, a situation observed in hominoid species as well (41). The presence of a *MmKIR3DM* gene and multiple copies of *KIR3DH* suggest the existence of an ancestral *MmKIR3DH* gene that must have been duplicated at least once, followed by the introduction of a splice site mutation that has subsequently undergone an additional duplication event (Fig. 6). Duplication of genes may result in the generation of sequence variation; with selective pressure on one copy, its duplicate might have the freedom to accumulate mutations at a reduced cost. The differential clustering of the *MmKIR3DM* sequences suggests that further levels of heterogeneity must have been generated by recombination events.

All animals in our genomically screened panel possess *MmKIR3DH* sequences, with some macaques expressing as many as five activating KIR. Although single-cell-level expression profiling was not performed, it might well be that expression is limited on individual cells, as it is in humans (61). Additionally, macaques in which these transcripts were not found have been reported (36). In some of the animals that genomically show the presence of *MmKIR3DH*, the gene may not be expressed. In the family study, animals were detected that lacked *MmKIR3DH* on one of their haplotypes, although no animals were observed that were homozygous for this haplotype. This observation might indicate a selective advantage of having at least one gene without ITIMs present on the genotype. Loss of ITIMs combined with the presence of a charged amino acid in a transmembrane domain is thought to represent an activating function. However, a recent study indicated that there could be other motifs in the cytoplasmatic domain involved in intracellular signaling (62). With regard to the KIR gene cluster, it may be beneficial to have both long and short cytoplasmatic domains. In humans, there seems to be a balancing selection for A and B haplotypes (63). The presence of haplotypes lacking *MmKIR3DH* contrasted by haplotypes with

one or more copies of the *KIR3DH* gene indicates that this situation may also exist in rhesus macaques.

A search of the literature reveals that several genetic mechanisms are operative in humans and nonhuman primates, resulting in the generation of short KIR without ITIM. In humans, such receptors arose for example either by the introduction of a stopcodon (*3DS1*010*) (64) or by an insertion (*2DL4*007*) disrupting the reading frame (65). In the case of chimpanzees (*PtKIR3DS1*) and orangutan (PopyKIR3DS), a substitution has been documented resulting in a stopcodon (35, 48). Additionally, a truncated KIR transcript has also been observed in the African sabaeus monkey (*CsKIR3DH*) and the gorilla (*GgKIR2DSa*) (37, 41). In Old World monkeys like rhesus macaques, similar events may have taken place through the generation of a stopcodon in (*MmKIR1D*) (36). In this report, and for the first time, a recombination event followed by a splice site mutation is proposed to be responsible. As such, these data suggest that there is an evolutionary advantage to evolve KIR receptors with a truncated tail. And since this type of evolution is caused by different genetic mechanisms, it strongly suggests convergent evolution.

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REFERENCES

1. Litwin V, Gumperz J, Parham P, Phillips JH, Lanier LL. 1994. NKB1: a natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. *J Exp Med* 180: 537-43

2. Moretta A, Sivori S, Vitale M, Pende D, Morelli L, Augugliaro R, Bottino C, et al. 1995. Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. *J Exp Med* 182: 875-84

3. Pende D, Biassoni R, Cantoni C, Verdiani S, Falco M, di Donato C, Accame L, et al. 1996. The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/p70 family of inhibitory receptors that is characterized by three immunoglobulin-like domains and is expressed as a 140-kD disulphide-linked dimer. *J Exp Med* 184: 505-18

4. Ferrini S, Cambiaggi A, Meazza R, Sforzini S, Marciano S, Mingari MC, Moretta L. 1994. T cell clones expressing the natural killer cell-related p58 receptor molecule display heterogeneity in phenotypic properties and p58 function. *Eur J Immunol* 24: 2294-8

5. Uhrberg M, Valiante NM, Young NT, Lanier LL, Phillips JH, Parham P. 2001. The repertoire of killer cell Ig-like receptor and CD94:NKG2A receptors in T cells: clones sharing identical alpha beta TCR rearrangement express highly diverse killer cell Ig-like receptor patterns. *J Immunol* 166: 3923-32

6. Wagtmann N, Rajagopalan S, Winter CC, Peruzzi M, Long EO. 1995. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. *Immunity* 3: 801-9

7. Marsh SGE, Parham P, Dupont B, Geraghty DE, Trowsdale J, Middleton D, Vilches C, et al. 2003. Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002. *Immunogenetics* 55: 220-6

8. Lanier LL. 1998. NK cell receptors. *Annu Rev Immunol* 16: 359-93

9. Feng J, Call ME, Wucherpfennig KW. 2006. The assembly of diverse immune receptors is focused on a polar membrane-embedded interaction site. *PLoS Biol* 4: e142

10. Lanier LL, Corliss BC, Wu J, Leong C, Phillips JH. 1998. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature* 391: 703-7

11. Wende H, Volz A, Ziegler A. 2000. Extensive gene duplications and a large inversion characterize the human leukocyte receptor cluster. *Immunogenetics* 51: 703-13

12. Martin MP, Bashirova A, Traherne J, Trowsdale J, Carrington M. 2003. Cutting edge: expansion of the KIR locus by unequal crossing over. *J Immunol* 171: 2192-5

13. Wilson MJ, Torkar M, Haude A, Milne S, Jones T, Sheer D, Beck S, et al. 2000. Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc Natl Acad Sci USA* 97: 4778-83

14. Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B, Tyan D, et al. 1997. Human diversity in killer cell inhibitory receptor genes. *Immunity* 7: 753-63

15. Middleton D, Meenagh A, Gourraud PA. 2007. KIR haplotype content at the allele level in 77 Northern Irish families. *Immunogenetics* 59: 145-58

16. Middleton D, Meenagh A, Moscoso J, Arnaiz-Villena A. 2008. Killer immunoglobulin receptor gene and allele frequencies in Caucasoid, Oriental and Black populations from different continents. *Tissue antigens* 71: 105-13

17. Gardiner CM, Guethlein LA, Shilling HG, Pando M, Carr WH, Rajalingam R, Vilches C, et al. 2001. Different NK cell surface phenotypes defined by the DX9 antibody are due to KIR3DL1 gene polymorphism. *J Immunol* 166: 2992-3001

18. Valiante NM, Lienert K, Shilling HG, Smits BJ, Parham P. 1997. Killer cell receptors: keeping pace with MHC class I evolution. *Immunol Rev* 155: 155-64

19. Martin MP, Pascal V, Yeager M, Phair J, Kirk GD, Hoots K, O'Brien SJ, et al. 2007. A mutation in KIR3DS1 that results in truncation and lack of cell surface expression. *Immunogenetics* 59: 823-9

20. Thananchai H, Gillespie G, Martin MP, Bashirova A, Yawata N, Yawata M, Easterbrook P, et al. 2007. Cutting Edge: Allele-specific and peptide-dependent interactions between KIR3DL1 and HLA-A and HLA-B. *J Immunol* 178: 33-7

21. VandenBussche CJ, Dakshanamurthy S, Posch PE, Hurley CK. 2006. A single polymorphism disrupts the killer Ig-like receptor 2DL2/2DL3 D1 domain. *J Immunol* 177: 5347-57

22. Goodridge JP, Lathbury LJ, Steiner NK, Shulse CN, Pullikotil P, Seidah NG, Hurley CK, et al. 2007. Three common alleles of KIR2DL4 (CD158d) encode constitutively expressed, inducible and secreted receptors in NK cells. *Eur J Immunol* 37: 199-211

23. Schellekens J, Tilanus MGJ, Rozemuller EH. 2008. The elucidation of KIR2DL4 gene polymorphism. *Mol Immunol* 45: 1900-6

24. Alter G, Martin MP, Teigen N, Carr WH, Suscovich TJ, Schneidewind A, Streeck H, et al. 2007. Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. *J Exp Med* 204: 3027-36

25. Artavanis-Tsakonas K, Eleme K, McQueen KL, Cheng NW, Parham P, Davis DM, Riley EM. 2003. Activation of a subset of human NK cells upon contact with Plasmodium falciparum-infected erythrocytes. *J Immunol* 171: 5396-405

26. Nelson GW, Martin MP, Gladman D, Wade J, Trowsdale J, Carrington M. 2004. Cutting edge: heterozygote advantage in autoimmune disease: hierarchy of protection/susceptibility conferred by HLA and killer Ig-like receptor combinations in psoriatic arthritis. *J Immunol* 173: 4273-6

27. Hiby SE, Walker JJ, O'shaughnessy KM, Redman CWG, Carrington M, Trowsdale J, Moffett A. 2004. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J Exp Med* 200: 957-65

28. Hsu KC, Gooley T, Malkki M, Pinto-Agnello C, Dupont B, Bignon J-D, Bornhäuser M, et al. 2006. KIR ligands and prediction of relapse after unrelated donor hematopoietic cell transplantation for hematologic malignancy. *Biol Blood Marrow Transplant* 12: 828-36

29. Miller JS, Cooley S, Parham P, Farag SS, Verneris MR, McQueen KL, Guethlein LA, et al. 2007. Missing KIR ligands are associated with less relapse and increased graft-versus-host disease (GVHD) following unrelated donor allogeneic HCT. *Blood* 109: 5058-61

30. Schellekens J, Rozemuller EH, Petersen EJ, van den Tweel JG, Verdonck LF, Tilanus MGJ. 2008. Activating KIRs exert a crucial role on relapse and overall survival after HLA-identical sibling transplantation. *Mol Immunol* 45: 2255-61

31. Bontrop RE. 2001. Non-human primates: essential partners in biomedical research. *Immunol Rev* 183: 5-9

32. Vierboom MPM, Jonker M, Bontrop RE, 't Hart B. 2005. Modeling human arthritic diseases in nonhuman primates. *Arthritis Res Ther* 7: 145-54

33. Canavez F, Young NT, Guethlein LA, Rajalingam R, Khakoo SI, Shum BP, Parham P. 2001. Comparison of chimpanzee and human leukocyte Ig-like receptor genes reveals framework and rapidly evolving genes. *J Immunol* 167: 5786-94

34. Grendell RL, Hughes AL, Golos TG. 2001. Cloning of rhesus monkey killer-cell Ig-like receptors (KIRs) from early pregnancy decidua. *Tissue Antigens* 58: 329-34

35. Guethlein LA, Flodin LR, Adams EJ, Parham P. 2002. NK cell receptors of the orangutan (Pongo pygmaeus): a pivotal species for tracking the coevolution of killer cell Ig-like receptors with MHC-C. *J Immunol* 169: 220-9

36. Hershberger KL, Shyam R, Miura A, Letvin NL. 2001. Diversity of the killer cell Ig-like receptors of rhesus monkeys. *J Immunol* 166: 4380-90

37. Hershberger KL, Kurian J, Korber BT, Letvin NL. 2005. Killer cell immunoglobulin-like receptors (KIR) of the African-origin sabaeus monkey: evidence for recombination events in the evolution of KIR. *Eur J Immunol* 35: 922-35

38. Khakoo SI, Rajalingam R, Shum BP, Weidenbach K, Flodin L, Muir DG, Canavez F, et al. 2000. Rapid evolution of NK cell receptor systems demonstrated by comparison of chimpanzees and humans. *Immunity* 12: 687-98

39. LaBonte ML, Hershberger KL, Korber B, Letvin NL. 2001. The KIR and CD94/NKG2 families of molecules in the rhesus monkey. *Immunol Rev* 183: 25-40

40. Rajalingam R, Hong M, Adams EJ, Shum BP, Guethlein LA, Parham P. 2001. Short KIR haplotypes in pygmy chimpanzee (Bonobo) resemble the conserved framework of diverse human KIR haplotypes. *J Exp Med* 193: 135-46

41. Rajalingam R, Parham P, Abi-Rached L. 2004. Domain shuffling has been the main mechanism forming new hominoid killer cell Ig-like receptors. *J Immunol* 172: 356-69

42. Abi-Rached L, Parham P. 2005. Natural selection drives recurrent formation of activating killer cell immunoglobulin-like receptor and Ly49 from inhibitory homologues. *J Exp Med* 201: 1319-32

43. Rajagopalan S, Bryceson YT, Kuppusamy SP, Geraghty DE, van der Meer A, Joosten I, Long EO. 2006. Activation of NK cells by an endocytosed receptor for soluble HLA-G. *PLoS Biol* 4: e9

44. Faure M, Long EO. 2002. KIR2DL4 (CD158d), an NK cell-activating receptor with inhibitory potential. *J Immunol* 168: 6208-14

45. Sambrook JG, Bashirova A, Andersen H, Piatak M, Vernikos GS, Coggill P, Lifson JD, et al. 2006. Identification of the ancestral killer immunoglobulin-like receptor gene in primates. *BMC Genomics* 7: 209

46. Guethlein LA, Older Aguilar AM, Abi-Rached L, Parham P. 2007. Evolution of killer cell Ig-like receptor (KIR) genes: definition of an orangutan KIR haplotype reveals expansion of lineage III KIR associated with the emergence of MHC-C. *J Immunol* 179: 491-504

47. Otting N, Heijmans CMC, Noort RC, de Groot NG, Doxiadis GGM, van Rood JJ, Watkins DI, et al. 2005. Unparalleled complexity of the MHC class I region in rhesus macaques. *Proc Natl Acad Sci USA* 102: 1626-31

48. Sambrook JG, Bashirova A, Palmer S, Sims S, Trowsdale J, Abi-Rached L, Parham P, et al. 2005. Single haplotype analysis demonstrates rapid evolution of the killer immunoglobulin-like receptor (KIR) loci in primates. *Genome Res* 15: 25-35

49. Slierendregt BL, van Noort JT, Bakas RM, Otting N, Jonker M, Bontrop RE. 1992. Evolutionary stability of transspecies major histocompatibility complex class II DRB lineages in humans and rhesus monkeys. *Hum Immunol* 35: 29-39

50. Bontrop RE, Otting N, de Groot NG, Doxiadis GG. 1999. Major histocompatibility complex class II polymorphisms in primates. *Immunol Rev* 167: 339-50

51. Doxiadis GGM, de Groot N, Claas FHJ, Doxiadis IIN, van Rood JJ, Bontrop RE. 2007. A highly divergent microsatellite facilitating fast and accurate DRB haplotyping in humans and rhesus macaques. *Proc Natl Acad Sci USA* 104: 8907-12

52. Otting N, de Vos-Rouweler AJM, Heijmans CMC, de Groot NG, Doxiadis GGM, Bontrop RE. 2007. MHC class I A region diversity and polymorphism in macaque species. *Immunogenetics* 59: 367-75

53. Penedo MCT, Bontrop RE, Heijmans CMC, Otting N, Noort R, Rouweler AJM, de Groot N, et al. 2005. Microsatellite typing of the rhesus macaque MHC region. *Immunogenetics* 57: 198-209

54. Doxiadis GGM, Rouweler AJM, de Groot NG, Louwerse A, Otting N, Verschoor EJ, Bontrop RE. 2006. Extensive sharing of MHC class II alleles between rhesus and cynomolgus macaques. *Immunogenetics* 58: 259-68

55. Swofford DL. 2003. *PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods), Version 4*. Sunderland: Sinauer Associates

56. Pertea M, Lin X, Salzberg SL. 2001. GeneSplicer: a new computational method for splice site prediction. *Nucleic Acids Res* 29: 1185-90

57. Andersen H, Rossio JL, Coalter V, Poore B, Martin MP, Carrington M, Lifson JD. 2004. Characterization of rhesus macaque natural killer activity against a rhesus-derived target cell line at the single-cell level. *Cell Immunol* 231: 85-95

58. Vilches C, Parham P. 2002. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol* 20: 217-51

59. Roberts CH, Madrigal JA, Marsh SGE. 2007. Cloning and sequencing alleles of the KIR2DL4 gene from genomic DNA samples. *Tissue antigens* 69 Suppl 1: 88-91

60. Roberts CH, Turino C, Madrigal JA, Marsh SGE. 2007. Enrichment of individual KIR2DL4 sequences from genomic DNA using long-template PCR and allele-specific hybridization to magnetic bead-bound oligonucleotide probes. *Tissue antigens* 69: 597-601

61. Draghi M, Yawata N, Gleimer M, Yawata M, Valiante NM, Parham P. 2005. Single-cell analysis of the human NK cell response to missing self and its inhibition by HLA class I. *Blood* 105: 2028-35

62. Miah SMS, Hughes TL, Campbell KS. 2008. KIR2DL4 differentially signals downstream functions in human NK cells through distinct structural modules. *J Immunol* 180: 2922-32

63. Parham P. 2005. Immunogenetics of killer cell immunoglobulin-like receptors. *Mol Immunol* 42: 459-62

64. Colonna M, Samaridis J. 1995. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science* 268: 405-8

65. Gedil MA, Steiner NK, Hurley CK. 2005. Genomic characterization of KIR2DL4 in families and unrelated individuals reveals extensive diversity in exon and intron sequences including a common frameshift variation occurring in several alleles. *Tissue antigens* 65: 402-18

Chapter 4

The mosaic of KIR haplotypes in rhesus macaques

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ABSTRACT

To further refine and improve biomedical research in rhesus macaques, it is necessary to increase our knowledge concerning both the degree of allelic variation (polymorphism) and diversity (gene copy number variation) in the killer cell immunoglobulin-like receptor (KIR) gene cluster. Pedigreed animals in particular should be studied, as segregation data will provide clues to the linkage of particular KIR genes/alleles segregating on a haplotype and to its gene content as well. A dual strategy allowed us to screen the presence and absence of genes and the corresponding transcripts, as well as to track differences in transcription levels. On the basis of this approach, 14 diverse KIR haplotypes have been described. These haplotypes consist of multiple inhibitory and activating *Mamu-KIR* genes, and any gene present on one haplotype may be absent on another. This suggests that the cost of accelerated evolution by recombination may be the loss of certain framework genes on a haplotype.

INTRODUCTION

Rhesus macaques (*Macaca mulatta*) are often used as animal models for human disease or biology: for instance, with regard to infectious diseases, autoimmunity and transplantation (1-3). Therefore, it is necessary to refine the existing knowledge with regard to the immunogenetic factors that can influence either disease or pathology. Natural killer (NK) cells are sentinels of the immune system, influencing both innate and adaptive immune responses, and thus may play a key role in the susceptibility to and outcome of disease (4). A group of leucocyte receptors that can determine the activation state of NK cells is the killer cell immunoglobulin-like receptors (KIR) (5). KIR are cell surface molecules of the immunoglobulin superfamily, and their protein name is based on the number of extracellular domains, which can vary between one and three (1D–3D), and the length of their intracellular tail, which can be either long (L) or short (S) (6, 7). Generally, the interaction between KIR and their respective ligands can have an activating or inhibitory influence on the cell, depending on the balance of these signals (8).

The ligands for KIR are the cell surface proteins encoded by the major histocompatibility complex (MHC) class I genes. Since these latter molecules are important for the presentation of degraded pathogen particles (intracellular peptides) to the immune system, there is an ongoing struggle involving pathogens that are trying to escape recognition by the MHC class I presentation pathway (9). This persistent selective pressure on the part of pathogens may be one of the reasons why the MHC gene family is highly polymorphic. Since MHC and KIR interactions are entwined, these two gene systems must have had a considerable impact upon each other during evolution (10, 11). Similar levels of polymorphism for the KIR region mirror the degree of polymorphism observed at the human leucocyte antigen (*HLA*)*-A, -B* and *-C* genes. However, whereas extensive diversity (gene copy number variation) is a hallmark of

the KIR system in humans, the number of HLA class I genes is fixed in the population. In contrast, the classical MHC class I genes in rhesus macaques, *Mamu-A* and *-B*, have experienced extensive duplications (12-14). This aspect of gene copy number variation in concert with polymorphism gives rise to an unparalleled complexity of the MHC class I system in macaques. At present, it is not known whether this has any impact on the KIR gene complex in macaques. Moreover, some *Mamu-A* and *-B* genes are characterised by high (majors) or low (minors) expression levels, respectively (15). The extent to which this quantitative feature will influence their capacity as immune response genes is not yet understood.

Since a KIR haplotype can contain multiple genes encoding for activating or inhibitory proteins and these can be gained or lost by recombination events, there seems to be an enormous amount of plasticity in the overall KIR repertoire. Despite this plasticity, some loci are present on most haplotypes and are considered to be framework genes. Many studies have reported on the haplotype composition of KIR genes encountered within different human populations (16). This has increased substantially the number of known region configurations but has also shown that haplotype repertoires may vary between populations (17, 18). Since particular alleles may be linked to specific haplotypes, which are undoubtedly subject to different modes of natural selection, these alleles may be enriched in a certain population yet be absent in another.

In non-human primates, KIR genes have been described for great apes, as well as for Old and New World monkeys (19-23). Comparative genetic analysis has revealed that the mechanisms responsible for expansion and contraction of the KIR region are most likely recombination events. These events can lead to the birth of new genes with a hybrid character or to the shuffling of particular exons. Further allelic variation can be added by occurrence of point mutations. Point mutations can lead to single amino acid changes or may have more drastic effects if the polymorphism maps to a splice site, which can result in a change in the overall domain composition of a protein (24). A recent example of co-evolution of MHC and KIR genes was documented for orangutans, and it was speculated that KIR with two extracellular domains evolved together with MHC-C orthologues (25).

Although certain individual *Mamu-KIR* sequences have been described, to date, information is limited with regard to alleles in the context of neighbouring ones. Studies on rhesus macaque KIR have resulted in the availability of some genotypes, but only one complete haplotype has been published (26). One reason for the lack of reports on haplotypes may be that it is necessary to have access to a pedigreed breeding colony to perform segregation analyses. Moreover, the same tools that are applied for fast and accurate KIR genotyping in humans do not yet exist for use with rhesus macaques. Ideally, to refine the use of rhesus macaques in biomedical research, it is required to characterise breeding colonies and to track the segregation of KIR haplo-

types with speedy methods. In this manner, it will be possible to begin exploring the controlled effect of different KIR and MHC interactions in the context of, for instance, infectious diseases (27). To start this process, multiple families from a breeding colony of Indian rhesus macaques, which are genotyped for their MHC class I and II repertoire, were now analysed for KIR gene polymorphism and diversity.

MATERIALS AND METHODS

Animals and cells

For the purpose of this particular study, four families of pedigreed animals from the BPRC self-sustaining colony were analysed. Whole blood of 19 rhesus macaques was obtained during regular health checks, and peripheral blood mononuclear cells (PBMC) were isolated as described previously (28). All animals had been haplotyped by different methods for their *Mamu-A, -B* and *-DR* allelic repertoire (15, 29, 30).

DNA/RNA extraction, cDNA synthesis and amplification

Genomic DNA was extracted from heparinised blood samples or immortalised rhesus macaque B cell lines according to standardised protocols (31). Total RNA was isolated from 5×10^6 PBMC with Trizol reagent (Invitrogen, Paisley, Scotland), according to the manufacturer's instructions. First-strand cDNA synthesis was conducted based on an oligo$(dT)_{18}$ primer and RevertAid H Minus M-MulV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany). Polymerase chain reaction (PCR) was performed with different primers specific for genomic KIR intron 4 and exon 5 and cDNA of *Mamu-KIR2DL04*, *-KIR3DL* and *-KIR3DS* (Table 1) synthesised by Invitrogen (Paisley, Scotland), using Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland), according to the manufacturer's instructions.

Table 1 Overview of primers used for PCR amplification of full-length *Mamu-KIR* cDNA and exon 5 genomic DNA.

	target	orientation	5′-sequence-3′
cDNA	*Mamu-KIR2DL04*	forward	CCACATCCTCTGCACCGGTCAGTC
	Mamu-KIR2DL04	reverse	GCAGGGGTCAAGTGAAGGGGAGAA
	Mamu-KIR3DL/1D/S	forward	AACATCCTGTGYRCTGCKGAGCWGAG
	Mamu-KIR3DS	reverse	CTGTCTGTGGTGCTCCTGGGCT
	Mamu-KIR3DL/1D	reverse	TGGAKAATTGTGGGYTAAGCAARGGAG
gDNA	*Mamu-KIR* exon 5	forward	AGAACTAGAGAGAMYRWGAGGCAGAG
	Mamu-KIR exon 5	reverse	CRTGGGACAGRCATGGGSTTTCCTCAC

Cloning and sequencing

PCR products were subjected to gel electrophoresis, and bands of appropriate size were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, USA). Products

were ligated in the cloning site of the Pjet1.2/blunt vector (Fermentas, St. Leon-Rot, Germany) and were used to transform XL1Blue *Escherichia coli* cells. A minimum of 48 clones were examined per PCR reaction. Plasmid was isolated and sequenced with forward and reverse primers supplied by the manufacturer, on a 3130XL ABI automatic sequencer (Applied Biosystems, Foster City, USA). Because KIR paralogues may be highly similar, recombination can occur frequently, potentially resulting in the formation of in vitro artefacts. Although a high-fidelity enzyme was used, polymerase-induced errors, represented as single nucleotide polymorphisms, were indeed observed. The reported sequences went through a stringent process of quality control; only sequences that were found to segregate or were obtained from more than two independent PCR reactions are reported, which might have resulted in an underestimation of their actual number.

Sequence alignments, in silico analysis and nomenclature

Sequences were aligned with the Seqman program (Dnastar, Inc., Madison, USA). Phylogenetic trees were drawn using the MEGA4.0 program (32). The JTT algorithm (33) was used to construct a distance model for amino acid substitution. The neighbour-joining method (34) was used to build a phylogenetic tree, and a bootstrap reliability test was run with 1,000 iterations. Sequences were submitted to Genbank and given the unique accession numbers GU112255– GU12332 and GU134802. Formal names have been assigned, and annotated sequences will be available through the immunopolymorphism database (www.ebi.ac.uk/ipd) (35).

RESULTS AND DISCUSSION

Mamu-KIR genotypes: correlation between transcripts and genomic sequences

Based on the cloning and sequencing of full-length KIR transcripts, genotype profiles were constructed (Fig. 1).

As can be seen, genotypes are characterised by different combinations of activating and inhibitory *Mamu-KIR*. Moreover, transcript information correlates with the presence of partial genomic sequences as revealed by scanning for the presence of exon 5. However, exceptions emerged. In animal 1UE, five additional segregating exon 5 sequences were only observed by genomic analysis. These sequences match with an inhibitory *Mamu-KIR3DL20*, *-KIR3DL02* and *-KIR3DL07* gene, respectively. In animal 98016, two genomic exon 5 segments were observed that are homologous to an inhibitory *Mamu-KIR3DL07* and an activating *-KIR3DSW09* gene, respectively (Fig. 1). Nevertheless, the corresponding transcripts were not detected. Since KIR genes in humans are known to display quantitative transcription level differences, it could be that some transcripts in rhesus monkeys are present at a low frequency as well and fall under the threshold of detection. Alternatively, these genes could be pseudogenes, or epigenetic factors may have silenced their transcription.

animal	*Mamu-KIR3DL20*	*Mamu-KIR1D*	*Mamu-KIR2DL04*	*Mamu-KIR3DL01*	*Mamu-KIR3DL02*	*Mamu-KIR3DL04*	*Mamu-KIR3DL05*	*Mamu-KIR3DL07*	*Mamu-KIR3DL08*	*Mamu-KIR3DL10*	*Mamu-KIR3DL11*	*Mamu-KIR3DS01*	*Mamu-KIR3DS02*	*Mamu-KIR3DS03*	*Mamu-KIR3DS05*	*Mamu-KIR3DS06*	*Mamu-KIR3DSW07*	*Mamu-KIR3DSW08*	*Mamu-KIR3DSW09*	total
95055	*002	*002	*001 *00802	*025					*010						*00201sv			*009 *008		9
95041	*002 *013	*002sv	*001 *003 *01401	*024		*00103							*011 *009		*00201sv	*006		*009		13
R01103	*002	*002 *002sv	(*001) *001 *003	*024					*010				*011		*00201sv			*009		10
R03068	*013	*002	(*001) *001 *01401			*00103							*009		*00201sv	*006		*009		9
96077	*013 *014	*002sv	*01501	*003		*00102	*009		*002			*00102		*00101						10
98024	*012		*001	*01901								*00102			rec				*004	6
R02091	*013 *012			*003 *01901					*002			*00102							*004	7
R04035	*014 *012	*002sv	*01501	*01901		*00102	*009					*00102		*00101					*004	10
1UE	gen1 gen2			*023	gen1 gen2		*008 *00602	gen1	*009	*00502	*006		*010	*00102		*00202				14
8719	*011 *015		*001 *003	*012 *0902	*008		*00602	*00902	*008					*00102	*00201sv					12
R01021	gen1 *011		*001	*012 *023	gen1		*00602		*008 *009					*00102	*00201sv					11
R04020	gen2 *015		*003	*01902	gen2 *008		*00602 *008	*00902 gen1		*00502	*006		*010	*00102		*00202				15
R03004	gen2 *011		*001	*012	gen2		*00602 *008	gen1	*008	*00502	*006		*010	*00102	*00201sv	*00202				15
98016	*009 *008	*002	*020	*022				gen1	*007		*001			*002			*001		*003 gen1	12
95020	*010		*002 *01501		*007						*005		*008							6
94056	*007 *006	*002	*001	*016						*002			*009		*00201	*005				9
R05105	*010 *008	*002	*002	*022	*007				*007		*005		*008	*002						10
R07121	*010 *009		*002 *020		*007			gen1			*001 *005		*008				*001		*003 gen1	12
R03100	*010 *007	*002	*002 *001	*016	*007					*002	*005		*009 *008		*00201	*005				13

Figure 1 Genotypic composition of a panel of Indian rhesus macaques. On the *top*, *Mamu-KIR* genes are displayed. Note that the order, in which genes are depicted for *KIR3DL20*, *KIR1D* and *KIR2DL04*, is based on the physical map. However, the copies of KIR3DL and KIR3DS are simply positioned numerically, since the order in which loci are positioned on a haplotype, is still unknown. Inhibitory KIR have been depicted in *blue* whereas activating ones are shown in *red*. KIR genes, for which only genomic exon 5 sequences are detected, are marked as *gen* and highlighted in *red*. KIR alleles, of which the observed transcript could be a splice variant, are indicated as *sv*, whereas a transcript that might be a recombinant is marked as *rec*.

KIR genotypes: presence and absence of genes

Within the present panel of 19 animals, covering four families, a total of 70 different KIR sequences were observed, 49 of which have not been reported before in the scientific literature. The other 21 full-length KIR sequences have already been described, and thus, they verify the existence of a particular allele, although the relevant sequence information is often extended (Supplementary Information Table 1).

The first KIR genotypes for rhesus macaques were deduced by a research team that studied five unrelated animals. In this case, the maximum number of KIR on one genotype was estimated to be five (20). Our genotyping data revealed that the number of KIR genes detected might vary substantially (Fig. 1). Overall, between 6 and 13 transcribed KIR genes were observed in any one animal, suggesting the presence of copy number variation.

Phylogenetic analysis and allelic variation of KIR genes

Phylogenetic analysis of full-length sequences of both activating KIR3DS and inhibitory 3DL alleles reveals extensive variation (Fig. 2).

Based on the depth of branches and bootstrap values, the phylogenetic trees allow the definition of lineages for the KIR3DL and 3DS genes, and these clusters have been indicated. It is realised that the robustness of such clustering will become more definitive when more alleles become available. At this stage, the distinction between a locus/lineage cannot be made, and it was decided to introduce a "W" designation to define its workshop status, a tradition that is also used in the HLA field (36). For instance, the *Mamu-KIR3DLW09* lineage, which comprises **003* and **004* (bold, Fig. 2), for which at this stage it is unclear whether these represent alleles of one locus or define separate loci. Alternatively, the lineage that comprises GU112327 and GU112317 has been designated *Mamu-KIR3DL20* because there is strong phylogenetic evidence and genomic data supporting the existence of a separate locus. Sequence analysis revealed that the sequence GU112302 (rec, Fig. 2a) is a recombinant between exons 1–7 of *Mamu-KIR3DS05* and exons 8–9 of *Mamu-KIR2DL04*. It is for this reason that this sequence stands out within the tree of inhibitory KIR. A combined phylogenetic tree of all the Ig-like domain sequences revealed no new lineages. However, it did show that activating and inhibitory lineages cluster together instead of separate, which suggests that some of these lineages are closely related, for instance, *Mamu-KIR3DLW03* and *Mamu-KIR3DS05*.

The number of transcribed KIR3DL and 3DS copies can vary substantially in one animal; however, some lineages appear to be more common. For instance, *Mamu-KIR3DL01* alleles were observed in 16 out of 19 animals, while members of the lineages *Mamu-KIR3DL08* and *-3DS02* were reported in 10 and 11 animals, respectively. The current sample size might well be too small to give these numbers any significance, but the observed trend may be a result of recent selective pressures, ensuring

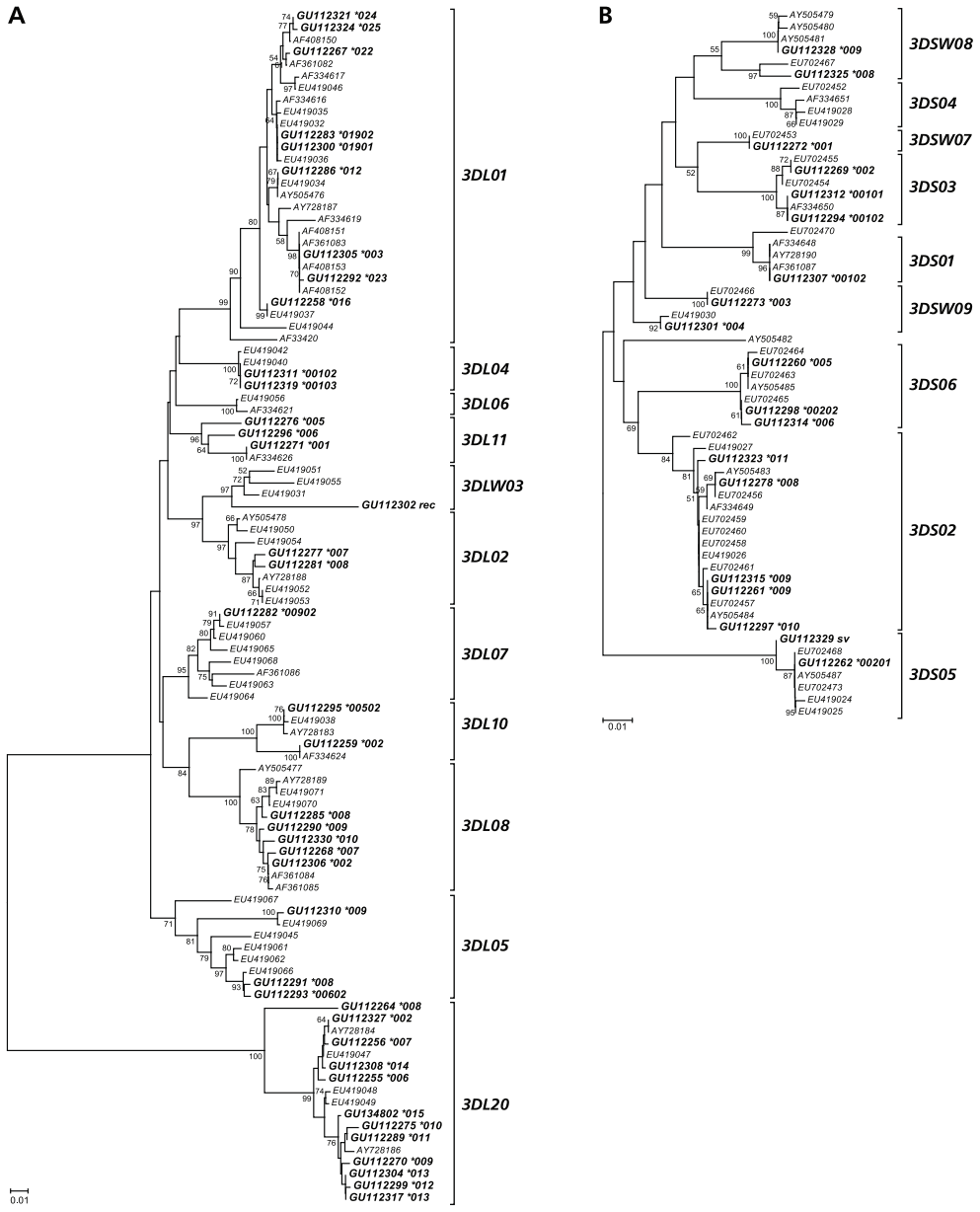

Figure 2 Phylogenetic trees of KIR3DL (**A**) and -3DS (**B**) genes depicting evolutionary distances. The *scale bar* represents amino acid substitutions per site. The JTT matrix method was used to calculate evolutionary distances. The neighbour-joining method was used to construct the phylogenetic tree. Bootstraps were calculated with 1,000 iterations, and confidence values above 50% are depicted. Genbank accession numbers are depicted of most reported macaque KIR sequences. The sequences in *bold* with an allele designation represent those observed in this study. Based on the clustering of sequences, KIR lineages have been indicated next to the alleles they comprise.

the presence of these lineages in at least part of the population. In combination with allelic polymorphism, this will result in a degree of genotypic complexity that may rival the situation observed in humans (37).

In the four macaque families, 12 alleles were observed that cluster together within the *KIR3DL20* lineage (Fig. 2). On the one haplotype that has been published (26), this locus is positioned most centromeric. This locus appears to be an orthologue of human *KIR3DL3*, which is considered a framework gene of the KIR gene cluster (38). The gene transcript was observed in all animals except for 1UE, which would suggest that haplotypes exist where *Mamu-KIR3DL20* is not expressed or that lack this locus. No genotypes were observed with more than two copies of this gene (Fig. 1), although copy number variations of the locus cannot be excluded. However, this locus displays allelic variation (Fig. 2). This report brings the total number of *3DL20* alleles to 15. This amount represents "the tip of the iceberg" as only a limited number of samples has been analysed.

A locus that was also observed on the genomic haplotype that has been sequenced (26) is that of Mamu-KIR1D. In ten genotypes, transcripts of this gene were observed, and a total of four alleles are reported, three of which are novel, thus doubling the number of reported alleles (Fig. 1).

One KIR gene that is observed in many primates is named *KIR2DL4* in humans, of which the orthologue in macaques has been named *Mamu-KIR2DL04*. A previous study reported extensive *Mamu-KIR2DL04* polymorphism in a large cohort of Indian rhesus macaques (22), and in the present set of animals, no unreported *2DL04* alleles were detected. However, genotyping revealed two animals (1UE, R02091) that lack the KIR2DL04 gene, the absence of which was established by combined genomic and transcription analysis. Although *KIR2DL04* was thought to be a framework gene in rhesus macaques, the recent analysis of a cohort of Mauritian cynomolgus macaques has also demonstrated the absence of the orthologue in animals of this species (39). Moreover, retrospective analysis demonstrated that 1UE has given successful birth to at least three offspring, suggesting that the presence of *KIR2DL04* is not essential for a successful pregnancy. This is similar to humans, with respect to women who have been reported to lack a functional *KIR2DL4* gene but have given birth successfully (40). One animal (95041) was found to possess three *KIR2DL04* sequences, suggesting that the locus has been duplicated on one haplotype. This is a novel observation in macaques but has been described for humans as well (41). It was also noted that identical *2DL04* alleles are shared by different genotypes, and as such, most likely are present on different haplotypes. This phenomenon was not observed, in the present panel, for any other gene besides *KIR2DL04* and *-1D*.

Mamu-KIR genes are subject to considerable splicing

Complex splicing events were observed frequently for all KIR gene transcripts except

for *Mamu-KIR2DL04*, where frequency was much lower. Alternatively spliced transcripts usually comprise a complete or partial deletion of one of exons 3, 4, 5, 6 and 7. These splice variants were observed to segregate, for instance, if a parental animal had a deletion of exon 5 in one transcript; a similar transcript would also be observed in the offspring, if the gene were inherited. It is yet unclear what determines the induction of splice variants. One possible explanation is the prevalence of GU-AG donor–acceptor splice motifs: These are highly frequent in the exons of many KIR genes, which could lead to a general proneness to alternative splicing in KIR transcripts. Because these products are shorter than the corresponding full-length transcript, they can be amplified more efficiently, which may result in an increase in the frequency of observed splice products in final cloned products.

Members of the *Mamu-KIR3DL20* gene produce splice variants in which exon 4 is missing (20). The consequence is an expressed protein that would have the characteristic D0-D2 domain structure reminiscent of human *KIR2DL4* and *-2DL5*. Indeed, *Mamu-KIR3DL20* genes contain an exon 3 that seems to be orthologous to *KIR2DL5*, so it is possible that by alternative splicing, a similar gene product is formed in macaques. This would be an example of convergent evolution, as KIR2DL5-like structures are observed in different primate species (19). At this stage, no ligand or function is known; however, one might speculate from its conservation that the ligand, too, has maintained certain structural characteristics that would enable co-evolution.

The *Mamu-KIR1D* gene product is also generated by alternative splicing. On a genomic level, *KIR1D* has three exons that potentially can encode for Ig-like domains. However, much as in human *KIR2DL1*, *2* and *3*, exon 3 is a pseudo exon, so the 1D transcript comprises exons 1–2 and 4–9. Since this gene contains a deletion in exon 5, resulting in a premature stop codon, the corresponding gene product might be soluble. In human, a common allele of *KIR2DS4*, which has a 22-bp deletion in exon 5, is thought to also encode a soluble product (42). In animals 95041 and 96077, only an alternatively spliced transcript of the *KIR1D* gene is observed, resulting in a 1DL conformation which has retained its intracellular tail. No function or ligand is known for these short KIR.

The mosaic of KIR haplotypes: extensive gene copy number variation in rhesus macaques

Genotyping in concert with segregation analyses revealed that different combinations of KIR genes segregate in rhesus monkey families according to Mendelian rules (Fig. 3). This approach allowed the definition in detail of 14 of 18 haplotypes that are present in the parental animals. For four haplotypes, marked H-02, -07, -13 and -18, informative segregation profiles were absent (Fig. 3).

The minimal gene content could be deduced, since genes that are not present on the haplotype, for which segregation data is available, should be present on the opposite one. However, the gene content of the haplotype could represent an under

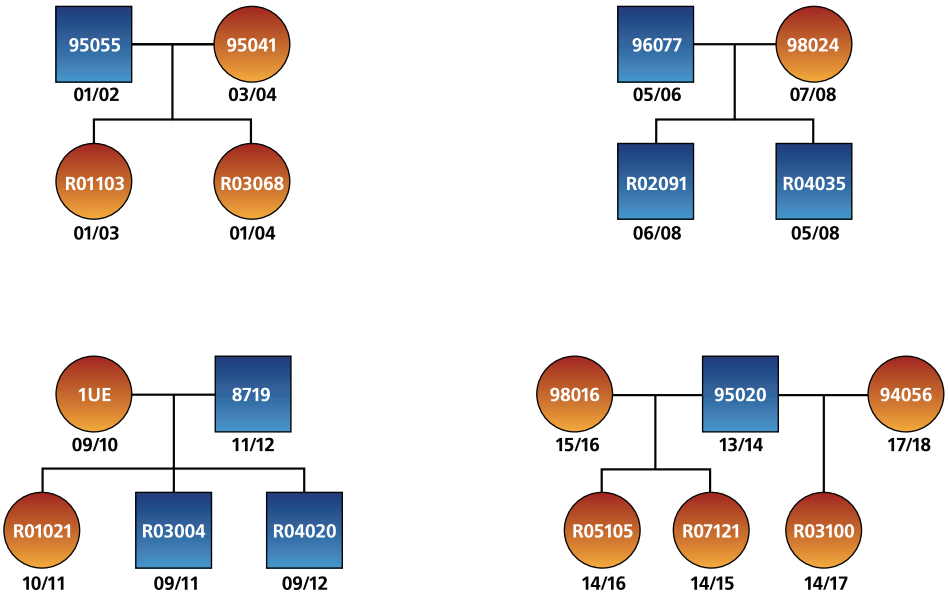

Figure 3 Segregation of KIR haplotypes in a pedigree of four families of Indian rhesus macaques. Depicted are the sires and dames with their offspring, and H-01 to H-18 represent haplotypes. Four haplotypes, H-02, -07, -13 and -18, were not inherited by the offspring.

estimation because homozygosity issues are not resolved by segregation. One should also keep in mind that low-frequency transcripts observed in these animals, which could not be confirmed by segregation analysis, were not reported.

For the other haplotypes, the discrimination power is high, as segregation of genes that differ by single nucleotide polymorphisms could be observed. As such, highly detailed haplotypes could be defined in 14 cases (Fig. 4). On these given haplotypes, the highest number of transcribed genes appears to be eight (H-17) while the average seems to be approximately six for most haplotypes (Fig. 4).

For instance, it was possible to distinguish highly similar *Mamu-KIR2DL04* alleles. Nevertheless, interpretation of segregation profiles may be hampered if both parents share the same genes/alleles. For example, this was observed for *2DL04* in animal 95041; since both offspring inherited an identical allele from the father, it was impossible to determine which haplotype comprises that of the mother.

To complicate matters further, all family members were genotyped positively for the same alleles of the lineages *Mamu-KIR3DSW08* and *-3DS05*, which means that these genes are present on at least one haplotype in both father and mother. A similar situation occurred in the family of 96077 and 98024, where the same allele of

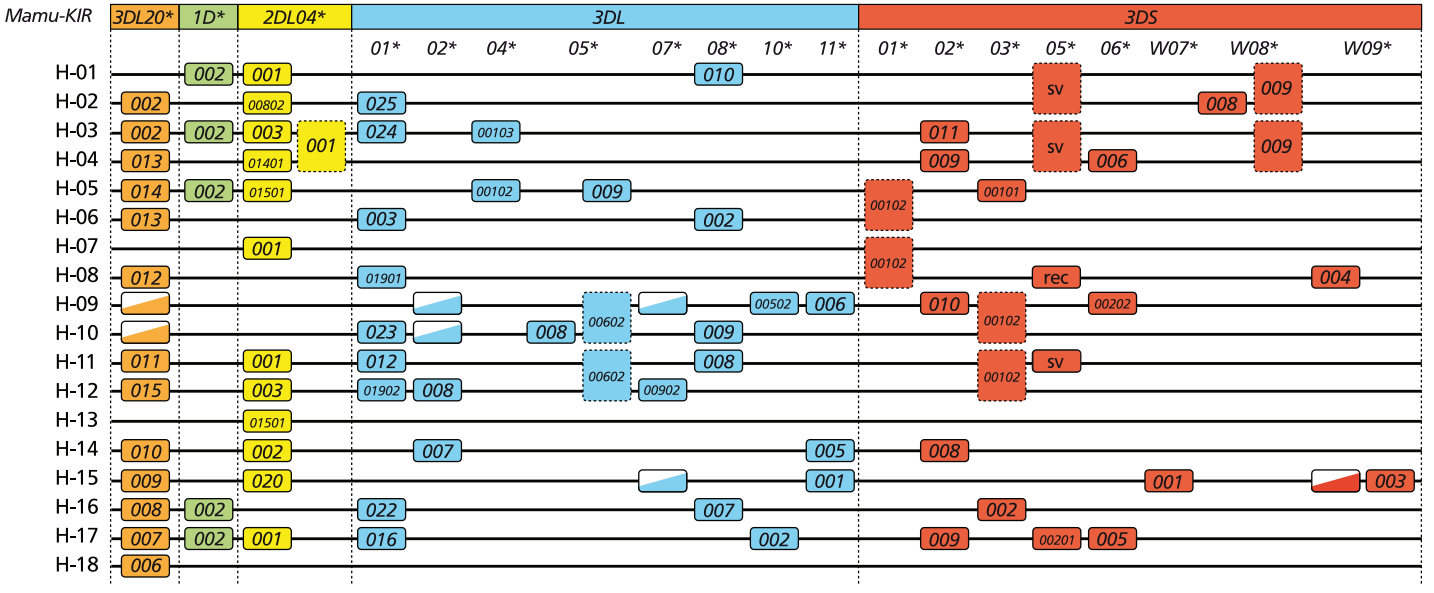

Figure 4 Definition of *Mamu-KIR* haplotypes H-01 to H-18 based on segregation analysis. On the *top axis*, the different Mamu-KIR genes are shown, and allele designation are provided in the *boxes*. Although haplotype gene content is reported, the order of the loci on the chromosome, however, is not known. For haplotypes H-02, -07, -13 and -18 the gene content could not be determined by segregation analysis, and a minimal haplotype is represented. If for one animal it is undeterminable whether an allele is present on both haplotypes or a single one, the allele is represented covering both haplotypes with an *interrupted outline*. On haplotype H-09, H-10 and H-15, genes are depicted *half full* because only an exon 5 sequence has been detected on a genomic level. This exon 5 sequence is similar to that of the particular lineage to which it has been appointed.

KIR3DS01 was observed in all animals. Finally, an allele of *KIR3DS03* was observed in all members of the family with parents 1UE and 8719. Although it is a coincidence that these alleles are shared between parents, many alleles are not shared.

The earlier observation that *KIR2DL04* may be absent on a genotype was confirmed at the haplotype level for animals 1UE and R02091. Segregation analyses confirmed that there are also parental animals that have *KIR2DL04* present on only one of their haplotypes (96077, 98024, 98016, 94056).

The exact mechanisms are still unclear as to how the expansion and contraction of haplotypes is generated, but it is thought that meiotic recombination events are involved (43). Unequal crossing-over in particular is a prominent mechanism that may influence gene copy number variation. This would account for the plasticity observed in the region, and as a consequence, it could very well be that activating and inhibitory KIR loci may be located juxtaposed on a haplotype.

One should realise that the haplotypes described here reflect gene content and not the localisation on the chromosome. Therefore, the terms downstream and upstream are based on the manner in which genes have been represented on the reported haplotypes. If, for instance, the region upstream of *KIR2DL04* would recombine with a similar more telomeric one, this could account for a haplotype without *KIR2DL04*, but with the genes telomeric of *2DL04*, like haplotype H-16. As a consequence, another recombinant haplotype would be formed that would contain a duplicated *2DL04* locus as one of haplotypes H-03 or H-04 might possibly posses. In a similar manner, if a recombination occurs upstream of *3DL20* with a region downstream of *2DL04*, this would result in the formation of a haplotype that lacks its most telomeric genes: for instance, as observed in haplotypes H-09 and H-10. As can be seen (Fig. 4), some haplotypes share particular segments, for instance, in H-06, H-10, H-11 and H-16, the *3DL01* and *3DL08* genes segregate together. Likewise, *3DS02* and *3DS06* are both present on haplotypes H-04, H-09 and H-17.

In conclusion, it seems likely that unequal crossing-over promotes the generation of mosaic haplotypes and rearrangement of gene content on a regular basis. The reported haplotypes display extensive gene copy number variation and gene polymorphism (Fig. 4). The present approach to obtain detailed alleles and discriminative haplotypes is highly accurate but also time consuming and could be complemented by an approach to track segregation of identified alleles. In the current issue of Immunogenetics, Kruse and co-workers report a rapid typing method to identify the presence or absence of KIR lineage specific markers (44). In this manner, informative genotypes and haplotypes could be detected. Based on expansion of detailed knowledge of allelic variation, additional refinement can be added to sequence-specific typing to make distinctions between highly similar sequences, for instance, alleles **009* and **010* of *Mamu-KIR3DS02*.

Activating and inhibitory haplotypes in rhesus macaques

In human, a distinction can be made between group A and B haplotypes, the former comprising on average six KIR genes and being more inhibitory than the latter (45). In human, nine common B haplotypes contain between seven and eleven KIR genes (46). In rhesus macaque, such clear distinction cannot be made. However, some haplotypes seem to comprise at least one inhibitory KIR, except for haplotype H-04 but appears to lack activating KIR (e.g. see haplotypes H-11, H-12 and possibly H-06 (Fig. 4)). In this panel, possibly only H-06 and one or two of H-10, -11 and -12 comprises solely inhibitory KIR genes. Overall, it appears that rhesus macaque haplotypes comprise a combination of activating and inhibitory KIR genes, and these can originate from several lineages. This mosaic pattern is also observed by another research group (44). Possibly because recombination events have taken place, there is no definite trend for a group A or B haplotype. Although it is true that haplotypes without activating KIR are reported, in humans the frequency of group A and B haplotypes is about 50%. It would take a much larger population study to estimate the ratio of A- and B-like haplotypes in rhesus macaques.

KIR3D expansion, restricted haplotype distribution and their impact on MHC repertoire

The present data suggest that the KIR3DS and 3DL families have experienced several rounds of duplications (Fig. 2). Based on segregation studies, the gene content of some KIR haplotypes has been established. As can be seen, these haplotypes comprise different numbers of KIR3DL and 3DS genes, ranging in the order of 0–3 and 0–4, respectively. The observed limit to the amount of genes on one haplotype implicates that there is a restricted haplotype distribution of KIR3DS and 3DL genes with regard to copy number. How can one explain the expansion of KIR3D lineages and the observed mosaic of KIR haplotypes from an evolutionary perspective? At this stage, it is understood that products of the KIR locus may exert different types of functions. On one hand, KIR on NK cells scan changes in the MHC class I expression status of cells. On the other hand, it is thought that some KIR recognise epitopes of pathogens or set apart pathogen derived peptides in the context of MHC class I. Such a complex task is difficult to achieve with one gene. Moreover, copy number variation of KIR3D minimises the chance that particular pathogens may escape immune recognition. However, if all individuals in a population share the same KIR repertoire, they could again become vulnerable to the adaptation of pathogens. As such, haplotypes with differential gene content would provide a selective advantage. The next issue is how haplotypes with differential gene content are maintained in the population. Selection by pathogens may enrich or even deplete particular haplotypes, altering gene frequencies in a population. NK cells play also a pivotal role during reproductive biology. Pregnancy can be regarded as a complex model for transplantation as the foetus consists from an immunological point of view 50% of nonself (paternal) material. NK

cell activation plays an important role in successful gestation (47). If this is true, then incompatibility between the KIR gene repertoire of the mother and inherited MHC repertoire of the father is favoured. As a consequence, an overrepresentation of MHC heterozygous individuals in the population can be expected. One can envision that this would in turn affect co-evolution of the KIR gene cluster.

In conclusion, to cope with a highly polymorphic gene system that is variable in both quality and quantity - such as the rhesus macaque MHC class I - a complex class of receptors is required that can detect such differences. This prerequisite is fulfilled by the highly plastic KIR gene system in rhesus macaques which displays copy number variation as well as extensive levels of polymorphism.

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References

1. Knechtle SJ, Burlingham WJ. 2004. Metastable tolerance in nonhuman primates and humans. *Transplantation* 77: 936-9
2. Bontrop RE, Watkins DI. 2005. MHC polymorphism: AIDS susceptibility in non-human primates. *Trends Immunol* 26: 227-33
3. Vierboom MPM, Jonker M, Bontrop RE, 't Hart B. 2005. Modeling human arthritic diseases in nonhuman primates. *Arthritis Res Ther* 7: 145-54
4. Moretta A, Bottino C, Mingari MC, Biassoni R, Moretta L. 2002. What is a natural killer cell? *Nat Immunol* 3: 6-8
5. Vilches C, Parham P. 2002. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol* 20: 217-51
6. Marsh SGE, Parham P, Dupont B, Geraghty DE, Trowsdale J, Middleton D, Vilches C, et al. 2003. Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002. *Immunogenetics* 55: 220-6
7. Kelley J, Walter L, Trowsdale J. 2005. Comparative genomics of natural killer cell receptor gene clusters. *PLoS Genet* 1: 129-39
8. Lanier LL. 1998. NK cell receptors. *Annu Rev Immunol* 16: 359-93
9. Vossen MTM, Westerhout EM, Söderberg-Nauclér C, Wiertz EJHJ. 2002. Viral immune evasion: a masterpiece of evolution. *Immunogenetics* 54: 527-42
10. Parham P. 2005. MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol* 5: 201-14
11. Single RM, Martin MP, Gao X, Meyer D, Yeager M, Kidd JR, Kidd KK, et al. 2007. Global diversity and evidence for coevolution of KIR and HLA. *Nat Genet* 39: 1114-9
12. Otting N, de Vos-Rouweler AJM, Heijmans CMC, de Groot NG, Doxiadis GGM, Bontrop RE. 2007. MHC class I A region diversity and polymorphism in macaque species. *Immunogenetics* 59: 367-75
13. Karl JA, Wiseman RW, Campbell KJ, Blasky AJ, Hughes AL, Ferguson B, Read DS, et al. 2008. Identification of MHC class I sequences in Chinese-origin rhesus macaques. *Immunogenetics* 60: 37-46
14. Otting N, Heijmans CMC, van der Wiel M, de Groot NG, Doxiadis GGM, Bontrop RE. 2008. A snapshot of the Mamu-B genes and their allelic repertoire in rhesus macaques of Chinese origin. *Immunogenetics* 60: 507-14
15. Otting N, Heijmans CMC, Noort RC, de Groot NG, Doxiadis GGM, van Rood JJ, Watkins DI, et al. 2005. Unparalleled complexity of the MHC class I region in rhesus macaques. *Proc Natl Acad Sci USA* 102: 1626-31
16. Martin MP, Single RM, Wilson MJ, Trowsdale J, Carrington M. 2008. KIR haplotypes defined by segregation analysis in 59 Centre d'Etude Polymorphisme Humain (CEPH) families. *Immunogenetics* 60: 767-74
17. Witt CS, Dewing C, Sayer DC, Uhrberg M, Parham P, Christiansen FT. 1999. Population frequencies and putative haplotypes of the killer cell immunoglobulin-like receptor sequences and evidence for recombination. *Transplantation* 68: 1784-9

18. Rajalingam R, Du Z, Meenagh A, Luo L, Kavitha VJ, Pavithra-Arulvani R, Vidhyalakshmi A, et al. 2008. Distinct diversity of KIR genes in three southern Indian populations: comparison with world populations revealed a link between KIR gene content and pre-historic human migrations. *Immunogenetics* 60: 207-17

19. Khakoo SI, Rajalingam R, Shum BP, Weidenbach K, Flodin L, Muir DG, Canavez F, et al. 2000. Rapid evolution of NK cell receptor systems demonstrated by comparison of chimpanzees and humans. *Immunity* 12: 687-98

20. Hershberger KL, Shyam R, Miura A, Letvin NL. 2001. Diversity of the killer cell Ig-like receptors of rhesus monkeys. *J Immunol* 166: 4380-90

21. Rajalingam R, Parham P, Abi-Rached L. 2004. Domain shuffling has been the main mechanism forming new hominoid killer cell Ig-like receptors. *J Immunol* 172: 356-69

22. Blokhuis JH, van der Wiel MK, Doxiadis GGM, Bontrop RE. 2009. Evidence for balancing selection acting on KIR2DL4 genotypes in rhesus macaques of Indian origin. *Immunogenetics* 61: 503-12

23. Cadavid LF, Lun C-M. 2009. Lineage-specific diversification of killer cell Ig-like receptors in the owl monkey, a New World primate. *Immunogenetics* 61: 27-41

24. Blokhuis JH, Doxiadis GGM, Bontrop RE. 2009. A splice site mutation converts an inhibitory killer cell Ig-like receptor into an activating one. *Mol Immunol* 46: 640-8

25. Guethlein LA, Older Aguilar AM, Abi-Rached L, Parham P. 2007. Evolution of killer cell Ig-like receptor (KIR) genes: definition of an orangutan KIR haplotype reveals expansion of lineage III KIR associated with the emergence of MHC-C. *J Immunol* 179: 491-504

26. Sambrook JG, Bashirova A, Palmer S, Sims S, Trowsdale J, Abi-Rached L, Parham P, et al. 2005. Single haplotype analysis demonstrates rapid evolution of the killer immunoglobulin-like receptor (KIR) loci in primates. *Genome Res* 15: 25-35

27. Khakoo SI, Carrington M. 2006. KIR and disease: a model system or system of models? *Immunol Rev* 214: 186-201

28. Otting N, Bontrop RE. 1993. Characterization of the rhesus macaque (Macaca mulatta) equivalent of HLA-F. *Immunogenetics* 38: 141-5

29. Bontrop RE, Otting N, Slierendregt BL, Lanchbury JS. 1995. Evolution of major histocompatibility complex polymorphisms and T-cell receptor diversity in primates. *Immunol Rev* 143: 33-62

30. Doxiadis GGM, Rouweler AJM, de Groot NG, Louwerse A, Otting N, Verschoor EJ, Bontrop RE. 2006. Extensive sharing of MHC class II alleles between rhesus and cynomolgus macaques. *Immunogenetics* 58: 259-68

31. Slierendregt BL, van Noort JT, Bakas RM, Otting N, Jonker M, Bontrop RE. 1992. Evolutionary stability of transspecies major histocompatibility complex class II DRB lineages in humans and rhesus monkeys. *Hum Immunol* 35: 29-39

32. Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 24: 1596-9

33. Jones DT, Taylor WR, Thornton JM. 1992. The rapid generation of mutation data matrices from protein sequences. *Comput Appl Biosci* 8: 275-82

34. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406-25

35. Robinson J, Mistry K, McWilliam H, Lopez R, Marsh SGE. 2010. IPD--the Immuno Polymorphism Database. *Nucleic Acids Res* 38: D863-9

36. Marsh SGE, Albert ED, Bodmer WF, Bontrop RE, Dupont B, Erlich HA, Geraghty DE, et al. 2005. Nomenclature for Factors of the HLA System, 2004. *Hum Immunol* 66: 571-636

37. Yawata M, Yawata N, Abi-Rached L, Parham P. 2002. Variation within the human killer cell immunoglobulin-like receptor (KIR) gene family. *Crit Rev Immunol* 22: 463-82

38. Wilson MJ, Torkar M, Haude A, Milne S, Jones T, Sheer D, Beck S, et al. 2000. Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc Natl Acad Sci USA* 97: 4778-83

39. Bimber BN, Moreland AJ, Wiseman RW, Hughes AL, O'Connor DH. 2008. Complete characterization of killer Ig-like receptor (KIR) haplotypes in Mauritian cynomolgus macaques: novel insights into nonhuman primate KIR gene content and organization. *J Immunol* 181: 6301-8

40. Gómez-Lozano N, de Pablo R, Puente S, Vilches C. 2003. Recognition of HLA-G by the NK cell receptor KIR2DL4 is not essential for human reproduction. *Eur J Immunol* 33: 639-44

41. Storset AK, Slettedal IO, Williams JL, Law A, Dissen E. 2003. Natural killer cell receptors in cattle: a bovine killer cell immunoglobulin-like receptor multigene family contains members with divergent signaling motifs. *Eur J Immunol* 33: 980-90

42. Maxwell LD, Wallace A, Middleton D, Curran MD. 2002. A common KIR2DS4 deletion variant in the human that predicts a soluble KIR molecule analogous to the KIR1D molecule observed in the rhesus monkey. *Tissue antigens* 60: 254-8

43. Norman PJ, Abi-Rached L, Gendzekhadze K, Hammond JA, Moesta AK, Sharma D, Graef T, et al. 2009. Meiotic recombination generates rich diversity in NK cell receptor genes, alleles, and haplotypes. *Genome Res* 19: 757-69

44. Kruse PH, Rosner C, Walter L. 2010. Characterization of rhesus macaque KIR genotypes and haplotypes. *Immunogenetics* 62: 281-93

45. Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B, Tyan D, et al. 1997. Human diversity in killer cell inhibitory receptor genes. *Immunity* 7: 753-63

46. Uhrberg M, Parham P, Wernet P. 2002. Definition of gene content for nine common group B haplotypes of the Caucasoid population: KIR haplotypes contain between seven and eleven KIR genes. *Immunogenetics* 54: 221-9

47. Trowsdale J, Moffett A. 2008. NK receptor interactions with MHC class I molecules in pregnancy. *Semin Immunol* 20: 317-20

SUPPLEMENTARY INFORMATION

Supplementary Table 1 Overview of *Mamu-KIR* sequences observed in reference animals. Lineage and allele designations as well as Genbank accession numbers are provided.

Lineage	Allele	Haplotype	Reference animal	Genbank accession number
Mamu-KIR3DL20	*002	H-02/-3	95055	GU112327
Mamu-KIR3DL20	*006	H-18	94056	GU112255
Mamu-KIR3DL20	*007	H-17	94056	GU112256
Mamu-KIR3DL20	*008	H-16	98016	GU112264
Mamu-KIR3DL20	*009	H-15	98016	GU112270
Mamu-KIR3DL20	*010	H-14	95020	GU112275
Mamu-KIR3DL20	*011	H-11	8719	GU112289
Mamu-KIR3DL20	*012	H-08	98024	GU112299
Mamu-KIR3DL20	*013	H-06	96077	GU112304
Mamu-KIR3DL20	*013	H-04	95041	GU112317
Mamu-KIR3DL20	*014	H-05	96077	GU112308
Mamu-KIR3DL20	*015	H-12	8719	GU134802
Mamu-KIR1D	*002	H-01	95055	GU112332
Mamu-KIR1D	*002sv	H-03	95041	GU112320
Mamu-KIR1D	*002sv	H-05	96077	GU112309
Mamu-KIR1D	*002	H-16	98016	GU112266
Mamu-KIR1D	*002	H-17	94056	GU112257
Mamu-KIR2DL04	*001	H-01	95055	GU112331
Mamu-KIR2DL04	*001	H-03/-04	95041	GU112318
Mamu-KIR2DL04	*001	H-17	94056	GU112263
Mamu-KIR2DL04	*001	H-07	98024	GU112303
Mamu-KIR2DL04	*001	H-11	8719	GU112287
Mamu-KIR2DL04	*002	H-14	95020	GU112279
Mamu-KIR2DL04	*003	H-03	95041	GU112322
Mamu-KIR2DL04	*003	H-12	8719	GU112284
Mamu-KIR2DL04	*00802	H-02	95055	GU112326
Mamu-KIR2DL04	*01401	H-04	95041	GU112316
Mamu-KIR2DL04	*01501	H-05	96077	GU112313
Mamu-KIR2DL04	*01501	H-13	95020	GU112280
Mamu-KIR2DL04	*020	H-15	98016	GU112274
Mamu-KIR3DL01	*003	H-06	96077	GU112305
Mamu-KIR3DL01	*012	H-11	8719	GU112286
Mamu-KIR3DL01	*016	H-17	94056	GU112258
Mamu-KIR3DL01	*01901	H-08	98024	GU112300
Mamu-KIR3DL01	*01902	H-12	8719	GU112283
Mamu-KIR3DL01	*022	H-16	98016	GU112267
Mamu-KIR3DL01	*023	H-10	1UE	GU112292
Mamu-KIR3DL01	*024	H-03	95041	GU112321
Mamu-KIR3DL01	*025	H-02	95055	GU112324
Mamu-KIR3DL02	*007	H-14	95020	GU112277
Mamu-KIR3DL02	*008	H-12	8719	GU112281
Mamu-KIR3DL04	*00102	H-05	96077	GU112311
Mamu-KIR3DL04	*00103	H-03	95041	GU112319
Mamu-KIR3DL05	*00602	H-09/-10	1UE 8719	GU112293
Mamu-KIR3DL05	*008	H-10	1UE	GU112291
Mamu-KIR3DL05	*009	H-05	96077	GU112310
Mamu-KIR3DL07	*00902	H-12	8719	GU112282
Mamu-KIR3DL08	*002	H-06	96077	GU112306
Mamu-KIR3DL08	*007	H-16	98016	GU112268
Mamu-KIR3DL08	*008	H-11	8719	GU112285
Mamu-KIR3DL08	*009	H-10	1UE	GU112290
Mamu-KIR3DL08	*010	H-01	95055	GU112330
Mamu-KIR3DL10	*002	H-17	94056	GU112259
Mamu-KIR3DL10	*00502	H-09	1UE	GU112295
Mamu-KIR3DL11	*001	H-15	98016	GU112271
Mamu-KIR3DL11	*005	H-14	95020	GU112276
Mamu-KIR3DL11	*006	H-09	1UE	GU112296
Mamu-KIR3DS01	*00102	H-05/-06 H-07/-08	96077 98024	GU112307
Mamu-KIR3DS02	*008	H-14	95020	GU112278
Mamu-KIR3DS02	*009	H-04	95041	GU112315
Mamu-KIR3DS02	*009	H-17	94056	GU112261
Mamu-KIR3DS02	*010	H-09	1UE	GU112297
Mamu-KIR3DS02	*011	H-03	95041	GU112323
Mamu-KIR3DS03	*00101	H-05	96077	GU112312
Mamu-KIR3DS03	*00102	H-09/-10 H-11/-12	1UE 8719	GU112294
Mamu-KIR3DS03	*002	H-16	98016	GU112269
Mamu-KIR3DS05	*00201	H-17	94056	GU112262
Mamu-KIR3DS05	*00201sv	H-01/-02 H-03/-04	95055 95041	GU112329
Mamu-KIR3DS06	*00202	H-09	1UE	GU112298
Mamu-KIR3DS06	*005	H-17	94056	GU112260
Mamu-KIR3DS06	*006	H-04	95041	GU112314
Mamu-KIR3DSW07	*001	H-15	98016	GU112272
Mamu-KIR3DSW08	*008	H-02 H-01/-02	95055 95055	GU112325
Mamu-KIR3DSW08	*009	H-03/-04	95041	GU112328
Mamu-KIR3DSW09	*003	H-15	98016	GU112273
Mamu-KIR3DSW09	*004	H-08	98024	GU112301
Mamu-KIR3DS05/2DL04	*rec*	H-07	98024	GU112302

CHAPTER 5

The extreme plasticity of killer cell Ig-like receptor (KIR) haplotypes differentiates rhesus macaques from humans

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ABSTRACT

Natural killer (NK) cells are essential in shaping immune responses, and play an important role during pregnancy and in controlling infections. Killer cell immunoglobulin-like receptors (KIR) educate the NK cell and determine its state of activation. Our goal was to determine how the KIR repertoire of the rhesus macaque (*Macaca mulatta*) has been shaped during evolution. The presence or absence of 22 KIR gene groups was determined in 378 animals. Some unexpected observations were made in an outbred colony comprising animals of different origins. For instance, the KIR region appears to be highly plastic, and an unprecedented number of genotypes and haplotypes was observed. In contrast to humans, there is no distinction between group A and B haplotypes, suggesting that different selective forces may be operative. Moreover, specific genes appear to be either present or absent in animals of different geographic origins. This extreme plasticity may have been propelled by co-evolution with the rhesus macaque MHC class I region, which shows signatures of expansion. The mosaic-like complexity of KIR genotypes as observed at the population level may represent an effective strategy for surviving epidemic infections.

INTRODUCTION

Natural killer (NK) cells are regarded as sentinels of the immune system. They bridge the gaps between innate and adaptive immunity (1). NK cells not only provide defence against infection (2) and cancer (3) but they are also involved with the vascularisation process during placentation, and thus contribute to reproductive success (4). The education and activation state of the NK cell is determined by the interactions of its receptors with their cognate ligands (5). It is the shift in equilibrium of inhibitory and activating receptor signaling that ultimately leads to NK cell activation in the form of cytokine production, cytotoxicity, or priming of the adaptive immune system (6).

Killer cell immunoglobulin-like receptors (KIR) may influence this balance through interactions with their ligands, the major histocompatibility complex (MHC) molecules, which are called human leukocyte antigens (HLA) in humans. Since both the HLA system and the KIR gene complex are characterized by variation in locus content, and segregate independent of each other, the potential array of interactions can vary considerably between individuals. Understanding the evolution and complexity of these receptor systems has broad medical relevance, since particular combinations of KIR and HLA alleles are associated with the outcome of viral infection, relapse of leukemia after transplantation, susceptibility to autoimmune disease, and successful pregnancy (7-10). Because of its close evolutionary relationship to humans, and evidenced by similar immunological responses, the rhesus macaque (*Macaca mulatta*) is an important animal model to study the onset, progression, and outcome of infectious diseases, experimentally induced autoimmunity, and transplantation (11-13).

Moreover, certain human pathogens or their simian-trophic family members have adapted to primates as their natural host, and may show a host-specific pathology. Since in an experimental setting the onset of disease or the actual challenge with a pathogen can be controlled, it is possible to study the first line of defence of innate immune systems. To improve the predictive value of rhesus macaques as a model system to study human biology and disease, it is essential to determine the degree of similarity between human and macaque KIR receptor repertoire.

The MHC class I genes of human and rhesus macaque have been investigated thoroughly by various methods. In humans, the classical *HLA-A, -B* and *-C* genes are restricted to a single locus, and are characterized by extensive polymorphism (14). In the rhesus macaque, the homologs of *HLA-A* and *-B*, designated *Mamu-A* and *-B*, respectively, are present, while the ortholog of *HLA-C* is absent (15).

Contrary to *HLA*, the *Mamu-A* and *-B* genes show a lesser degree of polymorphism but are expanded in their copy number, leading to many haplotypes with variable gene content, referred to as region configurations (16, 17). In both species, the MHC has evolved in its own manner; functionally, however, these molecules behave in the same way. For instance, one haplotype can comprise multiple *Mamu-A* and *-B* genes, which can be subdivided, however, into high-level (majors) and low-level (minors) transcribed genes (18). As a consequence, the end result may resemble the situation in humans. Apart from a shared ancestry, evidence for convergent evolution has been illustrated by the fact that unrelated alleles in both species may share highly similar peptide binding motifs; in essence, they may functionally behave the same (19). Whether the KIR gene system of both species may show similar convergent evolutionary traits has not yet been reported. Humans and rhesus macaques also share the MHC class I-like *E* and *F* genes (20, 21). The ortholog of *HLA-G* has been inactivated, and its function has been taken over by *Mamu-AG* (22). Moreover, rhesus macaques possess at least one extra nonclassical gene, *Mamu-I*, which is absent in humans (23).

The KIR gene complex is characterized by allelic polymorphism as well as by variation in locus content (24). It appears to have evolved rapidly in a species-specific manner, most likely because of co-evolution with the MHC (25). In humans, this has resulted in different KIR molecules that can recognize specific motifs present on either *HLA-A, -B*, and *-C* gene products (26, 27). The gene distributions of many geographically diverse human populations have been catalogued (28). Although three more or less conserved framework genes – *KIR2DL4, KIR3DL3*, and *KIR3DL2*, respectively – could be defined, the plasticity of the system is apparent. Not only can the gene content within haplotypes be expanded or contracted by recombination events but gene conversion, and the shuffling of functional domains may expand the repertoire even further (29). Nevertheless, two haplotype groups, A and B, can be distilled from this complexity (30, 31). Where the group B haplotypes are characterized

by the presence of one or more of the following genes – *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5*, and *KIR3DS1* – the group A haplotypes are defined by the absence of these genes. Moreover, much of the observed haplotype diversity can be explained by dividing these haplotype groups into centromeric and telomeric parts, whereby new haplotypes may be formed by equal and unequal crossover events between these regions (32).

With regard to the rhesus macaque, only a few individual animals have been analyzed for their KIR content, since the full-length KIR transcripts of some unrelated animals have been sequenced (33, 34). The subsequent analyses showed that the KIR system in this macaque species is polygenic and polymorphic, and can encode inhibitory and activating molecules with three extracellular domains. A genomic analysis of two BAC clones covering one haplotype comprising five KIR genes revealed that members of KIR lineage I, II (sublineage IV), and V are present, which are homologous to human *KIR2DL4*, *KIR3D*, and *KIR3DL3*, respectively (35). On this haplotype, a truncated *Mamu-KIR1D* was discovered, which clusters in lineage III and most likely is a distant homolog of human KIR2D. More recently, we have analyzed many more transcripts in the context of four families of pedigreed animals, resulting in a phylogenetic classification of lineage II/IV gene groups (36). Genotypes can comprise three particular loci (*Mamu-KIR3DL20*, *-KIR1D*, and *-KIR2DL04*) with a centromeric location, and ten inhibitory KIR3DL and nine activating KIR3DS genes that map to the telomeric region, respectively. Segregation analysis of these KIR genotypes revealed at least 18 unique haplotypes (36). The existing knowledge on haplotype data is based on a relatively small number of animals (n = 9 (36), n=11 (37)), which displays, however, a complexity similar to that found in humans. This begs the question as to how much of the KIR system variability is actually present in rhesus macaques. Furthermore, a trend is apparent that no similar distinction of group A and B haplotypes, as observed in humans, can be made (36, 37). Therefore, to further investigate these questions, an extensive KIR gene content analysis has been performed on an outbred colony of rhesus macaques. Additionally, samples of animals of different origins were included in this study, since it is known that MHC haplotype variation can be linked to geographical origin (15, 38). Whether this may also impact KIR gene content is not yet known.

MATERIALS AND METHODS

Animals and cells

The composition of a cohort of 378 rhesus macaques is depicted (Supplementary Fig. 1). The cohort contains animals that are of either Indian, Burmese, Chinese, or mixed origin, as well as their offspring. For the purpose of this particular study, whole blood was obtained during regular health checks. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples according to

standard protocol (36). Immortalized B cells were subsequently generated by culturing PBMCs after infection with herpes papiovirus, as previously described (21).

Isolation of genomic DNA and cDNA

Genomic DNA was obtained from either EDTA whole blood or immortalized B cells using a standard salting-out procedure. RNA was isolated from PBMCs with Trizol reagent (Invitrogen, Paisley, Scotland), and first-strand cDNA synthesis was performed with a poly-(18)dT primer according to the standard protocol (Fermentas, St. Leon-Rot, Germany).

KIR plasmid library preparation

PCR was performed on cDNA with KIR-specific primers, as was reported (36). Products were ligated in a Pjet1.2 vector (Fermentas) and transformation of XL1 Blue E. coli was performed. Single colonies were selected and grown, and mini-prep (alkaline lysis method) was performed. Subsequently, isolated vector was sequenced on an ABI 3130*XL* (Applied Biosystems, Foster City, CA, USA) with Pjet1.2 forward, Pjet1.2 reverse and one internal primer specific for Mamu-KIR: 5'-CGCAGGGACCTACAGATGTCG-3'. Sequences were obtained that phylogenetically cluster in previously described gene groups (36), with the exception of *Mamu-KIR3DLW03*, for which no sequences were retrieved.

KIR nomenclature and Genbank accession numbers

Sequences were submitted to Genbank and for each particular allele corresponding accession numbers are provided (Supplementary Table 1). Genes that are designated with *Mamu-KIR* have been assigned official nomenclature in accordance with the Immuno-Polymorphism Database (www.ebi.ac.uk/ipd/kir/). Genes that are designated mmKIR have not been assigned official nomenclature yet.

In silico analysis and primer design

A nucleotide alignment was performed on all rhesus macaque KIR sequences available in GenBank. Based on phylogenetic analyses, and in accordance with official rhesus macaque KIR gene nomenclature, 22 different gene groups of alleles could be designated. Sequence-specific primer pairs have been constructed to cover every one of these groups without ambiguity. Additionally, a group of previously described primers (37) was evaluated on their application for quantitative PCR (qPCR), however, because many of these primers could amplify only specific alleles, only the primer pair specific for *Mamu-KIR3DS03* was used. Although all currently described alleles of *Mamu-KIR3DL02* are covered by a reported primer pair (37), some false positives were observed in our qPCR system. In this instance, a novel primer pair for *-KIR3DL02* was designed, which however does not cover all alleles. For *Mamu-*

3DL07 and *-3DL08* several alleles could not be covered with one primer pair, whereas *Mamu-KIR3DSW08* and *-3DSW09* were each divided in two groups of alleles (1), and (2), respectively. This analysis yielded a total of 24 primer pairs (Supplementary Table 2).

Quantitative PCR and melt curve analysis

To perform quantitative PCR, a 20 µl reaction was prepared containing: 1x PCR buffer, 1x EvaGreen dye (Biotium, Hayward, CA, USA), 0.5 unit platinum Taq DNA polymerase, 5 pmol forward and reverse primer, 1.5 mM $MgCl_2$, 200 µM dNTPs and 25 ng genomic DNA. PCR was performed on the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Reaction conditions for each primer pair were identical: a 2 min hot-start at 94°C followed by 50 cycles of 15 s 94°C denaturing, 20 s 60°C annealing, and 20 s 72°C extension with measurement of the SYBR channel after each cycle. Melt curves were obtained by increasing the temperature from 75°C to 95°C in 0.2°C intervals with measurement of fluorescence after each interval. Analyses were performed using the CFX96 manager software (Bio-Rad). In this manner, genomic DNA samples were screened once with each primer set. Although one cannot rule out false negatives, in the studied cohort the segregation of particular loci could be witnessed. False negatives may lead to a situation where both parental animals test negative for a certain locus, while any offspring may test positive. This situation was not observed in the present study.

Validation of primer set

To validate the specificity of each primer set, qPCR was performed on a dilution series of plasmid that contains the corresponding KIR. This yielded a specific melt curve for each amplicon. Additionally, qPCR was performed on a mix of all KIR containing plasmids that are expected to give a negative results. A selection of PCR reactions of DNA samples that tested positive for a specific primer set, was analyzed on a 1% agarose gel, and the corresponding product was excised and directly sequenced. In this manner, the correct sequences could be correlated with the qPCR results and melt curve profiles.

Statistical analyses

To check for significance in differences between populations in number of loci, number of inhibitory and activating KIR, standard errors were calculated and a Welch t-test was performed. The p-values were calculated and any value under 0.05 was deemed significant. For calculating statistical significance between the frequencies of specific KIR in different populations, a Yates' chi-square test with one degree of freedom was performed and p-values under 0.05 were deemed significant.

RESULTS

A myriad of genotypes is present in rhesus macaques

Genotypes have been determined by screening for the presence or absence of 22 particular Mamu-KIR genes. For all respective animals, information on their KIR genotypes, their origin, and their parental animals has been provided (Supplementary Table 3). In the case of families, the segregation of KIR genes can be studied. In the cohort of 378 animals, a total of 272 different genotypes were observed. In total, 60 genotypes were shared between animals, but out of these, only 7 genotypes were shared by siblings. This indicated that 218 out of 378 animals possess a unique KIR genotype, which was not observed twice in this cohort.

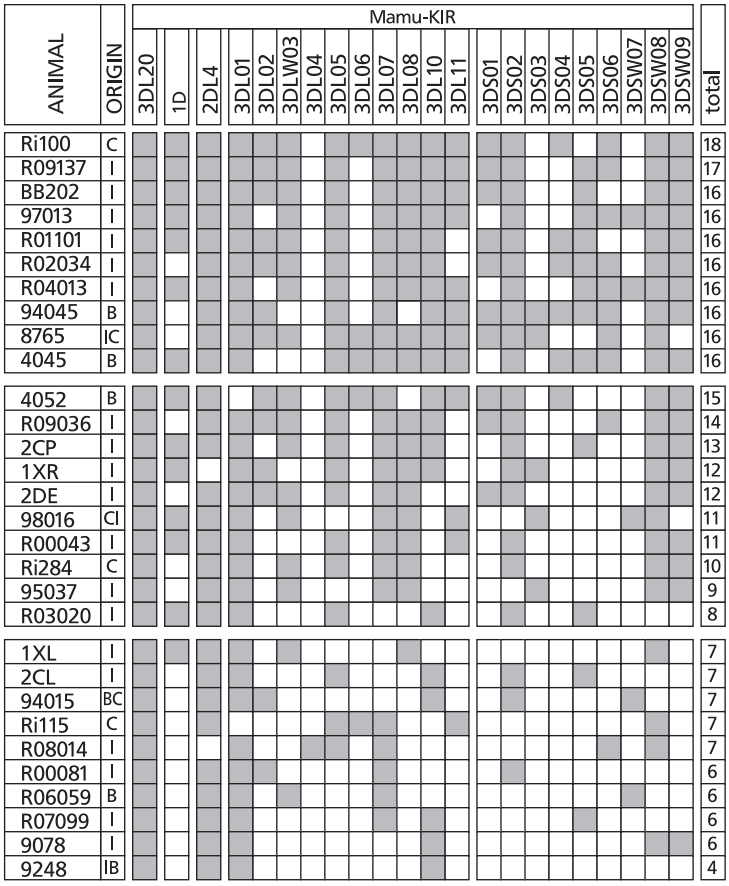

Figure 1 KIR genotypes in a panel of rhesus macaques. Depicted for each animal is its origin, the presence (*filled*) or absence (*empty*) of different Mamu-KIR loci, and the total number of loci present on its genotype. The panel is divided into three groups; at the top are the ten animals with the most number of loci per genotype, in the middle are ten animals ranging from 8 to 15, at the bottom are ten animals with the smallest number of loci per genotype.

Individual genotypes differ dramatically in their gene content

Since 22 loci were screened, an individual could theoretically comprise between 0 and 22 genes. As a reflection of the cohort, a selection of 30 animals and their gene content is highlighted (Fig. 1). This panel comprises 10 animals with the lowest number of genes, 10 animals with the highest number, and 10 subjects with intermediate numbers. This panel illustrates the variation in locus content that is present in the cohort. Genotypes are observed, for instance, comprising from 4 (animal 9248) up to 18 (animal Ri100) genes. As an overview, for each genotype as defined by the number of KIR loci, the corresponding number of animals present in the cohort is represented (Table 1). The average number of loci per individual is about 11 or 12.

Table 1 Division of number of loci and I:A score.

# Loci	# Animals	Average I : A
4	1	no activ.
6	4	2.50
7	8	2.86
8	25	1.75
9	31	1.58
10	43	1.51
11	69	1.64
12	69	1.40
13	70	1.46
14	33	1.36
15	14	1.30
16	9	1.22
17	1	1.33
18	1	1.50

Activating and inhibitory genotypes are present in the cohort

For each genotype with a particular number of loci, the average I:A ratio between inhibitory and activating KIR genes can be calculated (Table 1). For most animals, this is in the order of 1.5, which implies that for every three inhibitory genes, two activating genes are present. However, this ratio increases dramatically for several animals that have genotypes with seven or fewer loci (Table 1). For instance, no activating KIR was detected in animal 9248 (Fig. 1), while animals 1XL, R00081, R06059, and R07099 display an I:A ratio of 3, all comprising three inhibitory versus one activating gene. To illustrate that this phenomenon is also observed for animals with more numbers of loci, two groups of animals and their genotypes are depicted, in which both extremes of I:A ratio are present (Fig. 2). Here, for example, animal R05068 shows an I:A ratio of 0.4, with three KIR3DL and seven KIR3DS present on its genotype. Similarly, animals C56 and R05083 possess two inhibitory and four activating genes. On the other end of the spectum, however, animal Ri134 displays eight inhibitory and one activating

gene on its genotype, and animal 1GX shows four inhibitory and one activating gene.

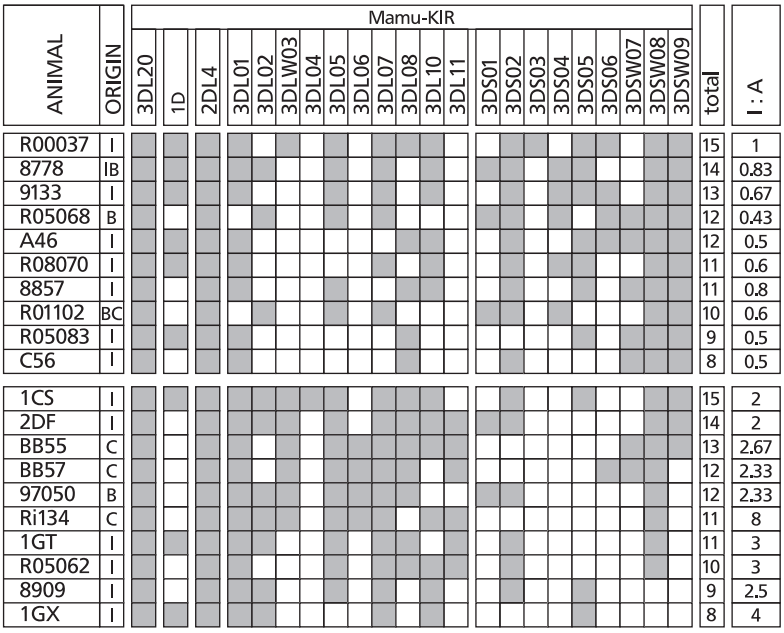

Figure 2 Extremes in inhibitory and activating genotypes. Depicted for each animal is its origin, the presence (*filled*) or absence (*empty*) of different *Mamu-KIR* loci, the total number of loci present on its genotype, and the ratio (I:A) between inhibitory and activating KIR. The panel is divided into two groups of animals with number of loci ranging from 8 tot 15; at the top are ten animals with the lowest I:A score (activating), at the bottom are ten animals with the highest I:A score (inhibitory).

Haplotypes display extreme plastic compositions

Based on segregation analysis, haplotypes can be deduced from some of the documented genotypes. A snapshot of 8 animals with different genotypes and 16 corresponding haplotypes is depicted (Fig. 3).

In a previous study, 18 haplotypes had been reported based on transcriptional analyses, and designated H-01 to H-18 (36). In line with this, these novel haplotypes are designated H-19 to H-34. Although, in theory, unrelated individuals from the cohort may share haplotypes, this was not observed in this small subpanel of animals. Furthermore, chances to observe homozygous animals for a particular KIR haplotype in the cohort appear to be very slim, and to our knowledge have not been recorded.

In this panel, two haplotypes were detected for each animal, which are not shared with other panel members (Fig. 3). Moreover, haplotypes can vary dramatically, both in their content as well as the number of loci present. Notably, two copies of *Mamu-*

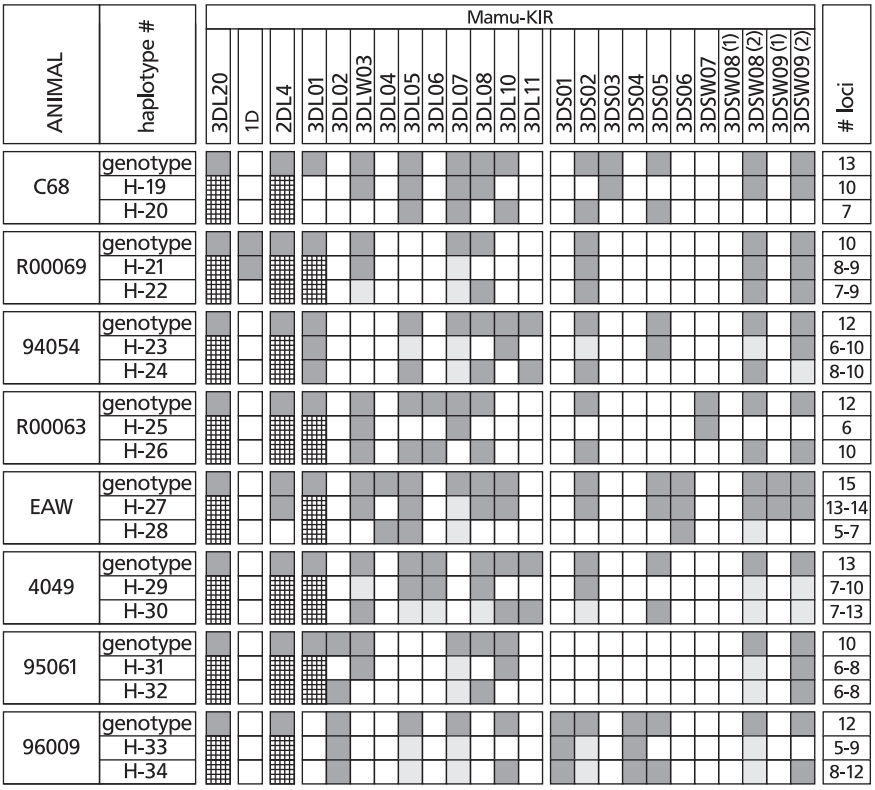

Figure 3 Plasticity of haplotypes. Depicted for each animal is the KIR content of its genotype as well as haplotypes. The presence (*dark-grey*) or absence (*empty*) of different *Mamu-KIR* loci is shown. A cell is filled *light-grey* when segregation analysis was inconclusive for this particular locus, and depicted as a *mesh* when it is highly likely a particular locus is situated on both haplotypes. The *Mamu-KIR3DSW08* and *-3DSW09* loci are each divided in (1) and (2) to indicate different allele groups of that particular locus that have been screened for. The final column depicts the number of loci that may be present on a genotype or on a haplotype.

KIR3DSW09 are reported on haplotype H-27, suggesting that other loci may also have been duplicated. This plasticity is further illustrated in the panel by the observation that every gene that may be present on one haplotype may be absent on another. The one exception may be recorded for *Mamu-KIR3DL20*, which is present on all analyzed genotypes, and therefore cannot be assigned to haplotypes by segregation analysis. In this panel, no *KIR2DL04* has been detected on haplotype H-28, which is in accordance with observations that this loci may be absent on certain haplotypes (39).

Animals of Indian, Chinese, and Burmese origin possess differential KIR gene contents

The gene content of animals from different populations was compared (Table 2), and significant differences became apparent, especially between Indian- and Chinese-origin animals.

Table 2 Differences in KIR gene content and composition between populations.

Origin	# Animals	Average # loci	Average # inhibitory	Average # inhibitory	I : A
Indian	89	11.1 (± 2.1) I-C*	5.0 (±1.3) I-C*	3.7 (±1.3)	1.3 I-C*
Burmese	14	11.5 (±2.2)	5.3 (±1.3) B-C*	3.9 (±1.3)	1.4 B-C*
Chinese	18	12.3 (±2.7)	6.6 (±1.5)	3.5 (±1.4)	1.9

*$p < 0.05$ between population

Chinese animals, for instance, have on average one additional inhibitory *KIR* gene present at the genotype level. Moreover, the balance between inhibitory and activating KIR is shifted more towards inhibition in Chinese animals as compared to Burmese and Indian animals. In this respect, the KIR gene profile of Burmese animals resembles the situation observed in Indian subjects.

To provide a more comprehensive overview of population differences, a comparison of the KIR gene frequencies is depicted (Fig. 4). As can be seen, the presence of particular genes can differ dramatically between animals originating from different geographic areas.

For the centromeric genes *KIR3DL20*, *-KIR1D*, and *-KIR2DL04*, none of the observed differences are significant, although there seems to exist a trend showing an increase in frequency of *KIR1D* from Chinese to Burmese and Indian animals, respectively. For the inhibitory KIR genes, two differences in population distribution are highly significant. First, the *KIR3DL04* gene is observed exclusively in Indian animals, albeit at very low frequency. Second, the *KIR3DL06* gene appears to be absent in Indian animals. For this locus, the difference in frequency between Burmese (43%) and Chinese (94%) animals is also significant. For other inhibitory genes, differences between Chinese and Indian animals are significant for *KIR3DL02* and *KIR3DLW03*, while Burmese and Chinese animals differ significantly for *KIR3DL07* and *KIR3DL10*. Additionally, for *KIR3DL11* there is a significant increase in frequency from Indian to Birmese and Chinese animals, and three activating KIR genes have highly significant population distributions. First, *KIR3DS05* was not observed in Chinese animals. Second, *KIR3DSW07* is more frequent in Chinese and Burmese animals as compared to Indian animals. Finally, *KIR3DS02* is present more than twice in Burmese and Indian animals as compared to Chinese animals. Although *KIR3DS01* appears to be enriched in Indian animals, this is below the threshold value for significance.

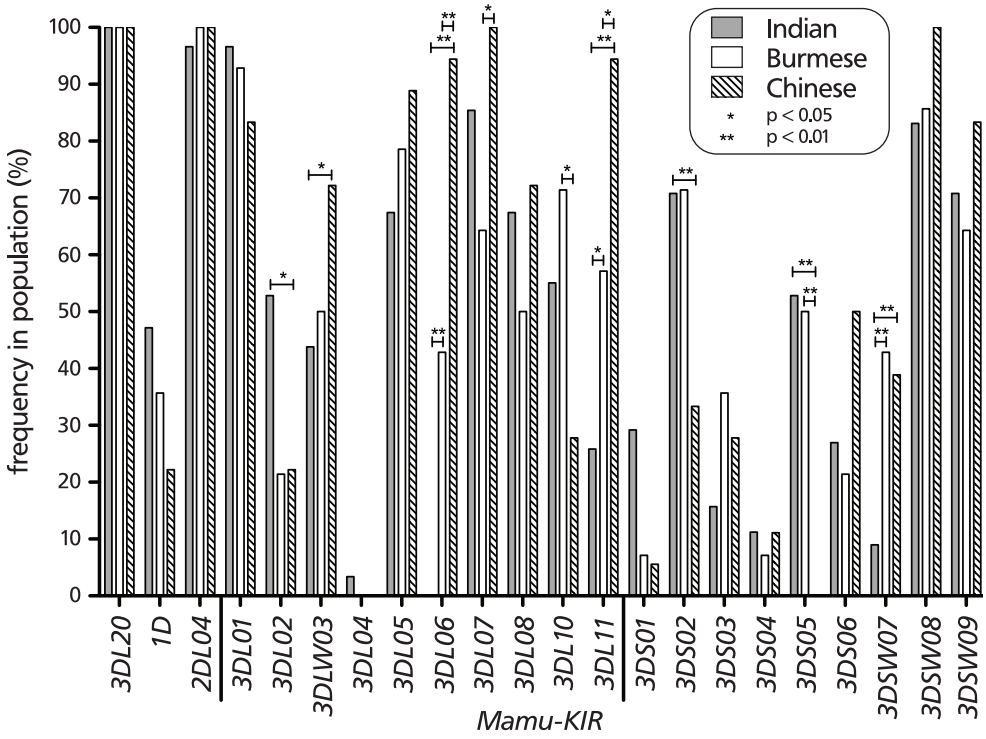

Figure 4 Frequencies of different *Mamu-KIR* genes observed in parental animals that originate in India, Burma, and China. On the x-axis, the three centromeric loci (*Mamu-KIR3DL20*, *-1D*, and *-2DL04*) are depicted, followed by ten inhibitory loci (*Mamu-KIR3DL*) and nine activating loci (*Mamu-KIR3DS*). The y-axis depicts the frequencies of these loci as observed in different populations. Statistical significant differences between populations have been shown.

DISCUSSION

In humans, much is known about the diversity of the KIR gene cluster (30). Moreover, due to the availability of KIR sequence-specific primer typing assays, many laboratories have examined the gene content in different human ethnic populations (28, 40). Furthermore, large numbers of transplantation donors and recipients are currently routinely screened for their KIR genotype, as are cohorts of patients and control individuals in disease association studies. One could claim that – with regard to KIR genotype diversity – humans are the most well-defined species. Because of its broad medical relevance, we wished to compare KIR diversity in humans with that of an important animal model, such as the rhesus macaque. Contrary to rodent models, outbred non-human primate colonies show similar levels of natural diversity in many of their immune-related genes, as is encountered in the human population. There are

hints that this may also apply to the KIR gene system (33, 36).

The present large-scale cohort study revealed an unprecedented diversity of KIR genotypes in rhesus macaques (Fig. 1). To put this into a proper context, one has to compare the numbers. To date, KIR population data have been reported for 108 human populations, of which 12.741 individuals were genotyped (41). In this study 378 rhesus macaques from three populations (India, Burma, China) have been genotyped. One should take into account that animals from Burma and China were under represented in our cohort. In humans 396 different KIR genotypes are reported (41), whereas in the present relatively small cohort of rhesus macaques 272 genotypes were observed (Table 3).

Table 3 KIR genotype statistics of humans and rhesus macaques.

	Human*	Rhesus macaque
Populations	108	3
Individuals	12,741	378
KIR genotypes	396	272

* information derived from www.allelefrequencies.net

It is likely that if more samples are analyzed, it will become evident that the rhesus macaque KIR genotypes display significantly more variation than humans. It is not only a question of numbers. Rhesus macaques are a successful species and they live in a large geographic area. Sampling animals from other areas will undoubtely reveal more KIR genotype variation. Indeed, this high number of genotypes may only represent the tip of the iceberg, since a comparison with 25 previously reported genotypes (37) showed only a very limited level of overlap.

We have recorded a snapshot of haplotypes based on segregation analysis, and already 34 unique haplotypes have been identified. Moreover, sharing of haplotypes seems to be a rare event between unrelated animals (Fig. 3). Furthermore, of these 272 genotypes, many lack the presence of specific loci. Therefore, the underlying haplotype structure may be similarly complex, and we anticipate that many haplotypes will be described in the near future. In humans, haplotypes can more or less be divided into inhibitory group A and activating group B haplotypes. This observation led to the hypothesis that this division originated due to different modes of selection (42). Whereas genes on the group A haplotype may have a protective effect against infectious diseases, genes on the group B haplotype may be more beneficial for reproductive success (43). In the rhesus macaque, some extreme examples of genotype variation have become evident, which have their balance shifted in one or another direction (Fig. 2). However, based on our observations, no clearcut division between inhibitory or activating haplotypes, as observed in humans, exists in rhesus macaques, which is a confirmation of previous findings (36, 37). Indeed, if placentation

is a strong selective force in humans (44), in rhesus macaques this influence may be less because the trophoblast-maternal interface is much smaller (45).

Recently, human KIR gene organization was compared with that of chimpanzee (46). Together with the present data, this sheds light on the evolution of KIR. Originally, a common simian ancestor comprised loci of four KIR lineages. In rhesus macaques, after their divergence, lineage II genes expanded telomerically (35). In the common ancestor of humans and chimpanzees, certain lineage III genes expanded centromerically. After divergence of chimpanzees and humans, chimpanzee lineage III genes continued colonization of the centromeric region, while telomeric lineage II genes collapsed, leaving no room for telomeric variability (46). In humans allocation of lineage II and III continued both centromerically and telomerically, and atone moment the group B haplotype was formed by introduction of specific activating KIR. Further diversity in humans is generated by recombination events, and a model has been proposed whereby centromeric and telomeric blocks of A or B haplotypes can be recombined to explain the birth of new ones (32). Therefore, certain KIR combinations are in strong linkage-disequilibrium on these haplotype blocks (47). This is in stark contrast to the present data on Mamu-KIR, which suggests an expansion only of the lineage II genes (35). Moreover, this expanded region appears to be even more plastic than that of humans, since haplotypes and genotypes are observed that can contain or lack any of the inhibitory or activating KIR genes, and many types of combinations are observed (Fig. 1 and 3).

For the first time, a comparative analysis was performed on KIR in different populations of rhesus macaques. With regard to their KIR repertoire, some highly significant differences were observed. Specifically, animals of Chinese origin seem to have on average one additional inhibitory KIR present on their genotype. In humans, the more inhibitory their genotype, the better an individual might be equipped to detect "missing-self", and, by extension, one can hypothesize that the more their NK cells can potentially be activated. Although functional evidence is lacking, the same might hold true for rhesus macaques. Numerous studies have, for instance, shown that Chinese-origin animals are more resistant to infection with SIV than are Indian-origin animals (48). Genetic differences are generally considered to account for these observations.

With regard to gene frequencies, we observed specific differences. For instance, the absence of *Mamu-KIR3DL06* and a very low presence of *KIR3DSW07* in Indian animals as well as the absence of *KIR3DS05* in Chinese animals (Fig. 4). Moreover, the *KIR3DL04* gene was observed exclusively in a very small number of Indian animals. It is not possible to tell whether Indian animals have gained or Chinese and Burmese animals have lost this gene, and whether its presence at such a low frequency is biologically significant. Phylogenetically, its D0 domain clusters with *Mamu-KIR3DL01*, whereas its D1 domain clusters separately and its D2 domain branches with *Mamu-*

KIR3DS01. Although this gene therefore has a hybrid nature, it is not a recently duplicated gene (data not shown). Based on comparative studies of primate species, a rapid evolution of the KIR gene system was proposed (25). In the light of our intraspecies data, this proposal can be expanded to extremely rapid evolution.

Since the repertoire is so diverse, it can be stated with confidence that in most biomedical studies the chance is very slim that rhesus macaques share a KIR genotype, in addition, this statement does not yet take into account the reported allelic variation (49). However, by knowing the repertoire, one may consider the effects of individual KIR genes, since animals can be selected for the presence or absence of particular KIR. The functional implications of candidate KIR genes can already be investigated. Recently, it was reported that Mamu-KIR3DLW03, KIR3DL05, and possibly KIR3DS05 gene products can interact with Mamu-A1*001:01 and -A3*13:11 molecules (50). Notably, these first two inhibitory genes are enriched in our animals of Chinese origin while the latter activating one is absent (Fig 4). Moreover, markers for *Mamu-KIR3DL05* alleles were more prevalant in SIV-infected animals with high viral loads (51). Finally, Mamu-KIR3DL05 allotypes can differentially bind Mamu-A1*002 tetramers, based on the type of SIV-peptide with which they were loaded (52).

Although it is clear that MHC class I genes are expanded in rhesus macaques, not all functional implications of this are yet understood. Because of the division in major and minor transcripts, the overall expression on peripheral blood lymphocytes in rhesus macaques appears to be quite similar to humans. Certainly, from a T-cell receptor point of view it appears that only the majors are restricting immune responses (53). Perhaps with this expansion a divison of labors has also occurred, while some loci, like *Mamu-A1*, (*-A7)*, and certain *Mamu-B*, may encode molecules whose functions have remained relatively conserved. Other duplicated loci, like *Mamu-A2 to -A6*, certain *Mamu-B*, and the newly generated *Mamu-I*, may encode products that have gained more specialized expression patterns and functions. We hypothesize that through co-evolution this division may be reflected in the KIR gene system.

Whereas conserved loci like *Mamu-KIR3DL20*, and *-2DL04* encode receptors that may perhaps target conserved ligands (like nonclassicals), the inhibitory and activating loci observed on many genotypes (like *Mamu-KIR3DL01*, *3DL05*, *3DL07*, *3DSW08*, *3DSW09*) encode receptors that could perhaps target the abundantly expressed class I molecules. In this context, the less frequent loci (e.g. *Mamu-KIR3DL02*, *3DL04*, *3DS01*, *3DS03*, *3DS04*, *3DSW07)* may encode products that have a more specialized function, or may perhaps recognize the low expressed 'minors'.

Our results concerning KIR gene frequencies in different populations suggest that differential evolution has occurred. Notably, origin-specific allelic variation of MHC class I is also observed between these three populations (17, 18, 54, 55). Therefore it may be relevant to functionally examine whether these origin-specific MHC and KIR alleles encode for receptor-ligand pairs.

The extreme KIR diversity we have observed is applicable to both the humber as well as ratio of inhibitory and activating loci. This suggests that diversifying selection has acted on both types of loci. It could well be that KIR diversity is favorably selected for within an individual as well a within a population. For instance because the MHC class I expression is hampered under certain pathogenic or by particular pathogens, and it proves beneficial to recognize 'missing-self' by inhibitory receptors. Whereas evasion of this mechanism of recognition may then lead to alternative recognition by activating receptors in the context of 'altered-self' or 'non-self'. Moreover, should a pathogen escape from the diverse KIR repertoire of one individual, it wil be confronted by even more diversity in the population. In this case, this extreme KIR diversity, as observed in rhesus macaques, may provide an effective strategy for surviving epidemic infections.

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REFERENCES

1. Di Santo JP. 2008. Functionally distinct NK-cell subsets: developmental origins and biological implications. *Eur J Immunol* 38: 2948-51
2. Lanier LL. 2008. Evolutionary struggles between NK cells and viruses. *Nat Rev Immunol* 8: 259-68
3. Diefenbach A, Raulet DH. 2002. The innate immune response to tumors and its role in the induction of T-cell immunity. *Immunol Rev* 188: 9-21
4. Moffett-King A. 2002. Natural killer cells and pregnancy. *Nat Rev Immunol* 2: 656-63
5. Lanier LL. 2008. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol* 9: 495-502
6. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. 2008. Functions of natural killer cells. *Nat Immunol* 9: 503-10
7. Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski J, Cheng J, et al. 2004. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* 305: 872-4
8. Martin MP, Qi Y, Gao X, Yamada E, Martin JN, Pereyra F, Colombo S, et al. 2007. Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nat Genet* 39: 733-40
9. Cooley S, Trachtenberg E, Bergemann TL, Saeteurn K, Klein J, Le CT, Marsh SGE, et al. 2009. Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. *Blood* 113: 726-32
10. Hiby SE, Walker JJ, O'shaughnessy KM, Redman CWG, Carrington M, Trowsdale J, Moffett A. 2004. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J Exp Med* 200: 957-65
11. Bontrop RE, Watkins DI. 2005. MHC polymorphism: AIDS susceptibility in non-human primates. *Trends Immunol* 26: 227-33
12. Vierboom MPM, Jonker M, Bontrop RE, 't Hart B. 2005. Modeling human arthritic diseases in nonhuman primates. *Arthritis Res Ther* 7: 145-54
13. Knechtle SJ, Burlingham WJ. 2004. Metastable tolerance in nonhuman primates and humans. *Transplantation* 77: 936-9
14. Cooley S, Weisdorf DJ, Guethlein LA, Klein JP, Wang T, Le CT, Marsh SGE, et al. 2010. Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood* 116: 2411-9
15. Otting N, Heijmans CMC, Noort RC, de Groot NG, Doxiadis GGM, van Rood JJ, Watkins DI, et al. 2005. Unparalleled complexity of the MHC class I region in rhesus macaques. *Proc Natl Acad Sci USA* 102: 1626-31
16. Bonhomme M, Doxiadis GGM, Heijmans CMC, Vervoort V, Otting N, Bontrop RE, Crouau-Roy B. 2008. Genomic plasticity of the immune-related Mhc class I B region in macaque species. *BMC Genomics* 9: 514
17. Doxiadis GGM, de Groot N, Otting N, Blokhuis JH, Bontrop RE. 2010. Genomic plasticity of the MHC class I A region in rhesus macaques: extensive haplotype diversity at the population level as revealed by microsatellites. *Immunogenetics*

18. Wiseman RW, Karl JA, Bimber BN, O'Leary CE, Lank SM, Tuscher JJ, Detmer AM, et al. 2009. Major histocompatibility complex genotyping with massively parallel pyrosequencing. *Nat Med* 15: 1322-6

19. Loffredo JT, Sidney J, Bean AT, Beal DR, Bardet W, Wahl A, Hawkins OE, et al. 2009. Two MHC class I molecules associated with elite control of immunodeficiency virus replication, Mamu-B*08 and HLA-B*2705, bind peptides with sequence similarity. *J Immunol* 182: 7763-75

20. Boyson JE, McAdam SN, Gallimore A, Golos TG, Liu X, Gotch FM, Hughes AL, et al. 1995. The MHC E locus in macaques is polymorphic and is conserved between macaques and humans. *Immunogenetics* 41: 59-68

21. Otting N, Bontrop RE. 1993. Characterization of the rhesus macaque (Macaca mulatta) equivalent of HLA-F. *Immunogenetics* 38: 141-5

22. Boyson JE, Iwanaga KK, Golos TG, Watkins DI. 1997. Identification of a novel MHC class I gene, Mamu-AG, expressed in the placenta of a primate with an inactivated G locus. *J Immunol* 159: 3311-21

23. Urvater JA, Otting N, Loehrke JH, Rudersdorf R, Slukvin II, Piekarczyk MS, Golos TG, et al. 2000. Mamu-I: a novel primate MHC class I B-related locus with unusually low variability. *J Immunol* 164: 1386-98

24. Trowsdale J, Barten R, Haude A, Stewart CA, Beck S, Wilson MJ. 2001. The genomic context of natural killer receptor extended gene families. *Immunol Rev* 181: 20-38

25. Vilches C, Parham P. 2002. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol* 20: 217-51

26. Colonna M, Samaridis J. 1995. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science* 268: 405-8

27. Döhring C, Scheidegger D, Samaridis J, Cella M, Colonna M. 1996. A human killer inhibitory receptor specific for HLA-A1,2. *J Immunol* 156: 3098-101

28. Martin MP, Single RM, Wilson MJ, Trowsdale J, Carrington M. 2008. KIR haplotypes defined by segregation analysis in 59 Centre d'Etude Polymorphisme Humain (CEPH) families. *Immunogenetics* 60: 767-74

29. Wilson MJ, Torkar M, Haude A, Milne S, Jones T, Sheer D, Beck S, et al. 2000. Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc Natl Acad Sci USA* 97: 4778-83

30. Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B, Tyan D, et al. 1997. Human diversity in killer cell inhibitory receptor genes. *Immunity* 7: 753-63

31. Uhrberg M, Parham P, Wernet P. 2002. Definition of gene content for nine common group B haplotypes of the Caucasoid population: KIR haplotypes contain between seven and eleven KIR genes. *Immunogenetics* 54: 221-9

32. Norman PJ, Abi-Rached L, Gendzekhadze K, Hammond JA, Moesta AK, Sharma D, Graef T, et al. 2009. Meiotic recombination generates rich diversity in NK cell receptor genes, alleles, and haplotypes. *Genome Res* 19: 757-69

33. Hershberger KL, Shyam R, Miura A, Letvin NL. 2001. Diversity of the killer cell Ig-like receptors of rhesus monkeys. *J Immunol* 166: 4380-90

34. Grendell RL, Hughes AL, Golos TG. 2001. Cloning of rhesus monkey killer-cell Ig-like receptors (KIRs) from early pregnancy decidua. *Tissue Antigens* 58: 329-34

35. Sambrook JG, Bashirova A, Palmer S, Sims S, Trowsdale J, Abi-Rached L, Parham P, et al. 2005. Single haplotype analysis demonstrates rapid evolution of the killer immunoglobulin-like receptor (KIR) loci in primates. *Genome Res* 15: 25-35

36. Blokhuis JH, van der Wiel MK, Doxiadis GGM, Bontrop RE. 2010. The mosaic of KIR haplotypes in rhesus macaques. *Immunogenetics* 62: 295-306

37. Kruse PH, Rosner C, Walter L. 2010. Characterization of rhesus macaque KIR genotypes and haplotypes. *Immunogenetics* 62: 281-93

38. Karl JA, Wiseman RW, Campbell KJ, Blasky AJ, Hughes AL, Ferguson B, Read DS, et al. 2008. Identification of MHC class I sequences in Chinese-origin rhesus macaques. *Immunogenetics* 60: 37-46

39. Blokhuis JH, van der Wiel MK, Doxiadis GGM, Bontrop RE. 2009. Evidence for balancing selection acting on KIR2DL4 genotypes in rhesus macaques of Indian origin. *Immunogenetics* 61: 503-12

40. Hollenbach JA, Meenagh A, Sleator C, Alaez C, Bengoche M, Canossi A, Contreras G, et al. 2010. Report from the killer immunoglobulin-like receptor (KIR) anthropology component of the 15th International Histocompatibility Workshop: worldwide variation in the KIR loci and further evidence for the co-evolution of KIR and HLA. *Tissue Antigens* 76: 9-17

41. Gonzalez-Galarza FF, Christmas S, Middleton D, Jones AR. 2011. Allele frequency net: a database and online repository for immune gene frequencies in worldwide populations. *Nucleic Acids Res* 39: D913-9

42. Parham P. 2005. MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol* 5: 201-14

43. Hiby SE, Apps R, Sharkey AM, Farrell LE, Gardner L, Mulder A, Claas FH, et al. 2010. Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. *J Clin Invest* 120: 4102-10

44. Parham P. 2004. NK cells and trophoblasts: partners in pregnancy. *J Exp Med* 200: 951-5

45. Hearn JP. 1986. The embryo-maternal dialogue during early pregnancy in primates. *J Reprod Fertil* 76: 809-19

46. Abi-Rached L, Moesta AK, Rajalingam R, Guethlein LA, Parham P. 2010. Human-specific evolution and adaptation led to major qualitative differences in the variable receptors of human and chimpanzee natural killer cells. *PLoS Genet* 6: e1001192

47. Gourraud P-A, Meenagh A, Cambon-Thomsen A, Middleton D. 2010. Linkage disequilibrium organization of the human KIR superlocus: implications for KIR data analyses. *Immunogenetics* 62: 729-40

48. Trichel AM, Rajakumar PA, Murphey-Corb M. 2002. Species-specific variation in SIV disease progression between Chinese and Indian subspecies of rhesus macaque. *J Med Primatol* 31: 171-8

49. Blokhuis JH, Doxiadis GGM, Bontrop RE. 2009. A splice site mutation converts an inhibitory killer cell Ig-like receptor into an activating one. *Mol Immunol* 46: 640-8

50. Rosner C, Kruse PH, Hermes M, Otto N, Walter L. 2011. Rhesus macaque inhibitory and activating KIR3D interact with Mamu-A-encoded ligands. *J Immunol* 186: 2156-63

51. Bostik P, Kobkitjaroen J, Tang W, Villinger F, Pereira LE, Little DM, Stephenson ST, et al. 2009. Decreased NK cell frequency and function is associated with increased risk of KIR3DL allele polymorphism in simian immunodeficiency virus-infected rhesus macaques with high viral loads. *J Immunol* 182: 3638-49

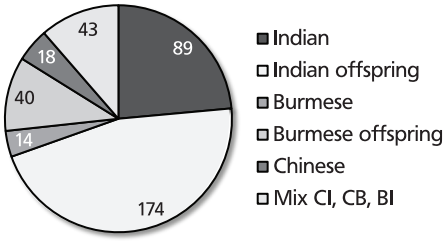
52. Colantonio AD, Bimber BN, Neidermyer WJ, Reeves RK, Alter G, Altfeld M, Johnson RP, et al. 2011. KIR Polymorphisms Modulate Peptide-Dependent Binding to an MHC Class I Ligand with a Bw6 Motif. *PLoS Pathog* 7: e1001316

53. Budde ML, Lhost JJ, Burwitz BJ, Becker EA, Burns CM, O'Connor SL, Karl JA, et al. 2011. Transcriptionally Abundant Major Histocompatibility Complex Class I Alleles Are Fundamental to Nonhuman Primate Simian Immunodeficiency Virus-Specific CD8+ T Cell Responses. *J Virol* 85: 3250-61

54. Naruse TK, Chen Z, Yanagida R, Yamashita T, Saito Y, Mori K, Akari H, et al. 2010. Diversity of MHC class I genes in Burmese-origin rhesus macaques. *Immunogenetics* 62: 601-11

55. Otting N, de Vos-Rouweler AJM, Heijmans CMC, de Groot NG, Doxiadis GGM, Bontrop RE. 2007. MHC class I A region diversity and polymorphism in macaque species. *Immunogenetics* 59: 367-75

SUPPLEMENTARY INFORMATION



Suplementary Figure 1 The composition of a cohort of 378 rhesus macaques. The animals' origin is either Indian, Burmese, Chinese, or a mix of these.

Supplementary Table 1
Plasmid library of Mamu-KIR alleles.

Allele designation	Accession number
*Mamu-KIR3DL20*013*	GU112304
*Mamu-KIR1D*002*	GU112257
*Mamu-KIR2DL04*001*	EU702486
*Mamu-KIR3DL01*003*	GU112305
*Mamu-KIR3DL01*012*	GU112286
*Mamu-KIR3DL02*008*	GU112281
*Mamu-KIR3DL04*00102*	GU112311
*Mamu-KIR3DL05*008*	GU112291
mmKIR3DL06-like-JHB	JF730318
*Mamu-KIR3DL07*00902*	GU112282
*Mamu-KIR3DL08*008*	GU112285
*Mamu-KIR3DL10*00201*	GU112259
*Mamu-KIR3DL11*001*	GU112271
*Mamu-KIR3DS01*00102*	GU112307
*Mamu-KIR3DS02*008*	GU112278
*Mamu-KIR3DS03*00101*	GU112312
*Mamu-KIR3DS04*002*	EU419028
*Mamu-KIR3DS05*00201*	GU112262
*Mamu-KIR3DS06*00201*	GU112298
*Mamu-KIR3DSW07*001*	GU112272
*Mamu-KIR3DSW08*009*	GU112328
*Mamu-KIR3DSW09*004*	GU112328

Supplementary Table 2 Primer pairs used to detect absence/presence of specific KIR gene groups

KIR gene group	5'-sequence-3'	domain	alleles
Mamu-KIR3DL20	FW CCAGAGCTCGTTTGACATTTACCG RV GGGTGTGACCACACATAGGGCAG	D2	*001-*014
Mamu-KIR1D	FW CTGCAGCTCCTGGCGCTCC RV ATCTGTAGGTCCCTCCATGGGTCC	D2	*001-*002
Mamu-KIR2DL04	FW CTTAGGCTCCCTGCAGTGCCG RV ACCACTCGTAGGGAGAGTCACGGAG	D2	*001-*020
Mamu-KIR3DL01	FW CCTTGTCCTGCAGCTCCCAGAC RV GGGTGGCAGGGCCCAGAA	D2	*001-*027
Mamu-KIR3DL02	FW GCCCTCCCAGGTCCCCTC RV CTGACAACACATAGGGGGAGTGAGTAAA	D1	*001,*002,*004,*008 not *003,*005-*007
Mamu-KIR3DLW03	FW CTTGTCCTGCAGCTCCCGGT RV CCRAAGCATCTGTAGGTTCCTCCA	D2	*001-*004
Mamu-KIR3DL04	FW GGTGTTACTATCGTCGTGGGCTTC RV ACCACTCAGTGGGGGAGTGCT	D0	*001-*002
Mamu-KIR3DL05	FW CCGACTTCCCTCTGGGCCCTGTG RV GAAACGTGCAGTGGGTCACTKGGGA	D2	*001-*011
Mamu-KIR3DL06	FW CCCAGAACTCGTTTGACATGTACCATCTATA RV GTGACCACTTGTAGGGTGCGGTACA	D2	*001-*002
Mamu-KIR3DL07	FW CTTCTTTCTGCACAGTGAGGTGAA RV CACTGGGAGCTGACAACACATAGTC	D1	*001-*005,*007,*009 not *006,008,*010
Mamu-KIR3DL08	FW AGAAAACCTTCCCTCCTGGCCT RV TGGGAGCTGACAACACATAGGGA	D1	*001-*011 not *004
Mamu-KIR3DL10	FW GCAGCTCCCAGAACTCGTTTGACATGTAT RV CAGAAACGAGCAGTGGGTCACTTGAGTT	D2	*001-*006
Mamu-KIR3DL11	FW ACAGTGAGGTGACCTTTGAGGAGCT RV TTCTCATATTTACCTGTGATCACGATGTTT	D1	*001-*007
Mamu-KIR3DS01	FW CCACGGTGCAGGCAGGAGAGG RV TCTGACCACTGGTAGGGTGCGGA	D2	*001-*003
Mamu-KIR3DS02	FW CTTGCACCTTGTTGGAGAGCG RV GTCACTGGGAGCTGACAACACATAGT	D1	*001-*011
Mamu-KIR3DS03 (#21, [37])	FW GGTGCCTCAGGGAGGACACA RV GGTCCCTGCGTCTGCCGGA	D0	*001-*003
Mamu-KIR3DS04	FW AACCTTCTCTCTCAGCCCAGCCA RV CGCTTGGCACTGCAGAGAGACTAAT	D2	*001-*004
Mamu-KIR3DS05	FW GTGTCAACGGAACATTCCAGGA RV CTCGGGTGTGACCACTTGTAGGA	D2	*001-*003
Mamu-KIR3DS06	FW TGTTGGAGAGCTCCATGGTGGA RV ACACATAGGGGGAGTGAGTGACAGAG	D1	*001-*006
Mamu-KIR3DSW07	FW CGTGACTCTTCGGTGTTACTATCGTGA RV ATCTGTAGGTCCCTGCGTGTGCT	D0	*001-*002
Mamu-KIR3DSW08 (1)	FW ACTTCTTTCTGCACAGTGAGGTGAA RV CACGATGTCCAGGGTGTCACTC	D1	*001-*004,*009
Mamu-KIR3DSW08 (2)	FW CAGTGAGGTGACCTTTGAGGAGCT RV GTCACTGGGAGCTGACAACACATC	D1	*005-*008
Mamu-KIR3DSW09 (1)	FW CCTTCTTGTTTGCCCGGCCT RV CCGACATCTGTAGGTCCCTGCA	D0	*001-*003
Mamu-KIR3DSW09 (2)	FW GCACAGTGAGGTGAACTTTGAGAAG RV TGTGATCACGATGTCCAGGGT	D1	*004,*005

Supplementary Table 3 KIR genotypes of 378 rhesus macaques. Animals have been sorted by origin and digital genotype. Highlighted are animals featured in the main text.

					Mamu-KIR																																	
	NAME	ORIGIN	FATHER	MOTHER	3DL20	1D	2DL04	3DL01	3DL02	3DLW03	3DL04	3DL05	3DL06	3DL07	3DL08	3DL10	3DL11	3DS01	3DS02	3DS03	3DS04	3DS05	3DS06	3DSW07	3DSW08 (1)	3DSW08 (2)	3DSW08 (all)	3DSW09 (1)	3DSW09 (2)	3DSW09 (all)	total # KIR	# inhib. KIR	# activ. KIR	I:A ratio
1	BB202	I	96012	9165	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	0	1	0	0	0	1	1	0	1	1	16	8	5	1.6
2	R02029	I	94016	94064	1	1	1	1	1	1	0	1	0	1	1	0	0	0	1	1	0	1	0	0	0	1	1	1	0	1	14	6	5	1.2
3	8676	I	2957	3739	1	1	1	1	1	1	0	0	0	1	1	1	0	1	0	0	0	0	0	0	0	1	1	1	1	1	12	6	3	2
4	8669B	I	3991	3837	1	1	1	1	1	1	0	0	0	1	1	0	1	0	0	0	1	0	1	0	0	1	1	1	1	1	13	6	4	1.5
5	8769	I	2957	JB	1	1	1	1	1	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	1	1	1	1	1	11	5	3	1.67
6	94008	I	1GR	3760	1	1	1	1	1	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	1	1	1	1	1	11	5	3	1.67
7	8902	I	ME	2378	1	1	1	1	1	0	0	1	0	1	1	1	0	1	1	0	0	1	1	0	1	1	1	0	0	0	14	6	5	1.2
8	1GT	I	3441	KB	1	1	1	1	1	0	0	1	0	1	1	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	11	6	2	3
9	9265	I	3643	3061	1	1	1	1	1	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	11	5	3	1.67
10	94077	I	3643	D27	1	1	1	1	1	0	0	1	0	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	10	4	3	1.33
11	D49	I	2777	3633	1	1	1	1	1	0	0	0	0	1	1	0	0	1	0	0	0	1	1	0	0	1	1	1	1	1	12	4	5	0.8
12	1RY	I	2794	3159	1	1	1	1	1	0	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	9	4	2	2
13	1GX	I	3441	2838	1	1	1	1	1	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	8	4	1	4
14	98007	I	8840	D52	1	1	1	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	7	3	1	3
15	TA	I	EE	2455	1	1	1	1	0	1	0	1	0	1	1	1	0	0	1	0	0	1	0	0	0	1	1	1	1	1	13	6	4	1.5
16	2CP	I	2774	WC	1	1	1	1	0	1	0	1	0	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	1	1	13	6	4	1.5
17	8740	I	HQ	2A	1	1	1	1	0	1	0	1	0	1	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0	0	13	6	4	1.5
18	1ZC	I	3991	JC	1	1	1	1	0	1	0	1	0	1	1	0	0	0	0	1	1	0	0	0	0	1	1	1	1	1	12	5	4	1.25
19	96072	I	8934	1YF	1	1	1	1	0	1	0	1	0	1	1	0	0	0	0	1	0	1	1	0	0	1	1	0	1	1	13	5	5	1
20	9252	I	1AJ	1HI	1	1	1	1	0	1	0	1	0	1	1	0	0	0	0	0	1	1	0	0	0	1	1	1	1	1	12	5	4	1.25
21	9119	I	H34	2AR	1	1	1	1	0	1	0	1	0	1	0	1	1	0	1	0	0	1	0	0	0	1	1	0	1	1	13	6	4	1.5
22	BB207	I	96012	96027	1	1	1	1	0	1	0	1	0	1	0	1	1	0	1	0	0	1	0	0	0	1	1	0	1	1	13	6	4	1.5
23	Q068	I			1	1	1	1	0	1	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	1	1	1	1	1	11	5	3	1.67
24	1BM	I	2315	1644	1	1	1	1	0	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	1	1	1	1	10	4	3	1.33
25	C6	I	3441	2838	1	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	1	1	0	1	1	11	4	4	1
26	D71	I	2777	2560	1	1	1	1	0	1	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	1	1	1	1	1	10	3	4	0.75
27	1XL	I	3026	2AP	1	1	1	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	7	3	1	3
28	1VU	I	2957	3036	1	1	1	1	0	0	0	1	0	1	1	1	1	0	1	0	0	1	1	0	0	1	1	0	0	0	13	6	4	1.5
29	1KO	I	EE	2351	1	1	1	1	0	0	0	1	0	1	1	1	0	0	1	0	1	1	0	0	0	1	1	0	1	1	13	5	5	1
30	1GI	I	2957	JK	1	1	1	1	0	0	0	1	0	1	1	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	10	5	2	2.5
31	9133	I	4043	3238	1	1	1	1	0	0	0	1	0	1	0	1	0	0	1	0	1	1	1	0	0	1	1	0	1	1	13	4	6	0.67
32	8872	I	EE	2559	1	1	1	1	0	0	0	1	0	1	0	1	0	0	1	0	1	1	0	0	0	1	1	0	1	1	12	4	5	0.8
33	99025	I	8840	8839	1	1	1	1	0	0	0	1	0	1	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	10	3	4	0.75
34	R03023	I	97004	97059	1	1	1	1	0	0	0	1	0	0	1	1	0	0	1	0	0	1	0	1	0	1	1	0	1	1	12	4	5	0.8
35	94032	I	3836	BB5	1	1	1	1	0	0	0	1	0	0	0	1	0	0	1	0	1	1	1	0	0	1	1	0	1	1	12	3	6	0.5
36	R03020	I	97004	97025	1	1	1	1	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	8	3	2	1.5
37	A46	I	2794	3613	1	1	1	1	0	0	0	0	0	0	1	1	0	0	1	0	0	1	1	1	0	1	1	0	1	1	12	3	6	0.5
38	R03008	I	97004	97028	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	1	1	1	0	0	0	8	2	3	0.67
39	1WS	I	2W	2407	1	1	1	0	1	0	1	1	0	1	0	0	1	1	1	0	0	0	0	0	0	1	1	0	1	1	12	5	4	1.25
40	9125	I	3836	OC	1	1	1	0	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	9	4	2	2
41	2H	I	V	BL	1	1	1	0	0	1	0	1	0	1	0	0	1	1	1	0	0	0	0	0	0	1	1	0	1	1	11	4	4	1
42	1XR	I	3991	1DE	1	1	0	1	1	0	0	1	0	1	1	1	0	0	1	1	0	0	0	0	0	1	1	0	1	1	12	6	4	1.5
43	2BH	I	2774	VA	1	0	1	1	1	1	0	1	0	1	1	1	0	0	1	1	0	0	1	1	0	0	1	1	0	1	15	7	6	1.17
44	8727	I	ME	YL	1	0	1	1	1	1	0	1	0	1	1	1	0	1	1	0	0	0	1	0	0	1	1	1	1	1	14	7	5	1.4
45	2CD	I	2957	XV	1	0	1	1	1	1	0	1	0	1	1	0	1	1	1	1	0	0	0	0	0	1	1	1	0	1	13	7	4	1.75
46	94071	I	3643	YJ	1	0	1	1	1	1	0	1	0	1	1	0	0	0	1	0	1	1	0	0	0	1	1	0	1	1	13	6	5	1.2
47	D17	I	2777	2801	1	0	1	1	1	1	0	1	0	1	1	0	1	1	1	0	0	1	1	0	0	0	0	0	0	0	12	6	4	1.5
48	9066	I	ME	UG	1	0	1	1	1	1	0	1	0	1	0	1	0	1	1	0	0	0	1	0	0	1	1	0	1	1	13	6	5	1.2
49	B72	I	2957	1JX	1	0	1	1	1	1	0	0	0	1	1	1	0	1	0	0	0	0	0	0	0	1	1	1	1	1	11	6	3	2
50	D48	I	2777	2453	1	0	1	1	1	1	0	0	0	1	1	1	0	0	1	0	0	0	1	0	0	1	1	1	1	1	12	6	4	1.5
51	95061	I	C13	8926	1	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	1	1	10	6	2	3
52	98024	I	8798	8646	1	0	1	1	1	1	0	0	0	1	1	0	0	1	0	0	0	1	0	0	0	1	1	1	1	1	11	5	4	1.25
53	94061	I	ZN	1WQ	1	0	1	1	1	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	10	5	3	1.67
54	1KX	I	2774	2837	1	0	1	1	1	0	0	1	0	1	1	1	0	1	1	1	0	0	1	0	0	1	1	0	1	1	14	6	6	1
55	9056	I	ME	LS	1	0	1	1	1	0	0	1	0	1	1	1	0	1	1	1	0	0	1	0	0	1	1	0	1	1	14	6	6	1
56	D10	I	2777	3136	1	0	1	1	1	0	0	1	0	1	1	1	0	1	1	0	0	0	1	0	0	1	1	1	1	1	13	6	5	1.2
57	8633	I	2957	1CE	1	0	1	1	1	0	0	1	0	1	1	0	1	1	1	0	1	0	0	0	0	1	1	1	1	1	13	6	5	1.2
58	2CK	I	2957	XV	1	0	1	1	1	0	0	1	0	1	1	0	1	1	0	0	0	0	0	0	0	1	1	1	1	1	11	6	3	2
59	95060	I	YN	1FV	1	0	1	1	1	0	0	1	0	1	1	1	0	1	0	1	0	0	0	0	0	1	1	0	1	1	11	6	3	2
60	8719	I	MG	3674	1	0	1	1	1	0	0	1	0	1	1	0	0	0	0	0	0	1	0	0	0	1	1	0	1	1	10	5	3	1.67
61	4032	I			1	0	1	1	1	0	0	1	0	1	0	1	0	0	1	0	1	1	0	0	0	1	1	0	1	1	12	5	5	1
62	8909	I	BB24	1PG	1	0	1	1	1	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	9	5	2	2.5
63	9017	I	3070	2989	1	0	1	1	1	0	0	1	0	1	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	9	5	2	2.5
64	8710	I	EE	1MX	1	0	1	1	1	0	0	1	0	1	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	9	5	2	2.5
65	9068	I	3070	1GG	1	0	1	1	1	0	0	1	0	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	9	5	2	2.5
66	8935	I	3991	1HG	1	0	1	1	1	0	0	1	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	8	4	2	2
67	96001	I	8827	1BS	1	0	1	1	1	0	0	0	0	1	1	1	0	1	0	0	0	1	0	0	0	1	1	1	1	1	11	5	4	1.25
68	1OL	I	3441	3029	1	0	1	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1	1	0	1	1	9	5	2	2.5
69	1QK	I	2777	3005	1	0	1	1	1	0	0	0	0	1	0	1	1	0	1	0	0	0	1	0	1	1	1	0	1	1	11	5	4	1.25
70	1WL	I	3991	3228	1	0	1	1	1	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	8	4	2	1
71	1HQ	I	2777	2861	1	0	1	1	1	0	0	0	0	1	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	9	3	4	0.75
72	EAW	I			1	0	1	1	0	1	1	1	0	1	1	1	0	0	1	0	0	1	1	0	0	1	1	1	1	1	14	7	5	1.4
73	C68	I	BB24	LN	1	0	1	1	0	1	0	1	0	1	1	1	0	0	1	1	0	1	1	0	0	1	1	0	1	1	13	6	5	1.2
74	BB227	I	98041	97038	1	0	1	1	0	1	0	1	0	1	1	1	0	0	1	1	0	1	1	0	0	1	1	0	1	1	13	6	5	1.2

Supplementary Table 3 continued 2 of 5.

					Mamu-KIR																																			
	NAME	ORIGIN	FATHER	MOTHER	3DL20	1D	2DL04	3DL01	3DL02	3DLW03	3DL04	3DL05	3DL06	3DL07	3DL08	3DL10	3DL11	3DS01	3DS02	3DS03	3DS04	3DS05	3DS06	3DSW07	3DSW08 (1)	3DSW08 (2)	3DSW08 (all)	3DSW09 (1)	3DSW09 (2)	3DSW09 (all)	total # KIR	# inhib. KIR	# activ. KIR	I:A ratio
75	BXJ	I			1	0	1	1	0	1	0	1	0	1	1	1	0	0	0	0	0	1	0	0	0	1	1	0	1	1	11	6	3	2
76	1DL	I	EE	2414	1	0	1	1	0	1	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	1	1	0	1	1	11	5	4	1.25
77	95037	I	BB24	2419	1	0	1	1	0	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	1	1	1	1	1	9	4	3	1.33
78	98011	I	B59	8964	1	0	1	1	0	0	0	1	0	1	1	1	1	0	1	0	0	1	0	0	0	1	1	0	1	1	12	6	4	1.5
79	8933	I	2W	1ID	1	0	1	1	0	0	0	1	0	1	1	0	1	0	1	1	0	0	0	0	0	1	1	0	1	1	11	5	4	1.25
80	8949	I	2521	FX	1	0	1	1	0	0	0	1	0	1	1	0	1	0	1	1	0	0	0	0	0	1	1	0	1	1	11	5	4	1.25
81	96004	I	YN	YJ	1	0	1	1	0	0	0	1	0	1	1	0	1	0	1	0	0	1	0	0	1	1	1	0	0	0	10	5	3	1.67
82	R02062	I	8798	9222	1	0	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	8	4	2	2
83	8857	I	2W	1FI	1	0	1	1	0	0	0	1	0	0	1	1	0	0	1	0	0	1	0	1	0	1	1	0	1	1	11	4	5	0.8
84	2CL	I	EE	ZY	1	0	1	1	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	7	3	2	1.5
85	A53	I	2794	2817	1	0	1	1	0	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	0	1	1	0	0	0	8	3	3	1
86	C56	I	3441	1W	1	0	1	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	1	0	1	1	8	2	4	0.5
87	9078	I	ZN	2T	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	1	1	6	2	2	1
88	D39	I	2777	NS	1	0	0	1	1	1	0	1	0	1	1	1	0	0	1	1	0	0	0	0	0	1	1	0	1	1	12	7	4	1.75
89	1UE	I	HQ	ZE	1	0	0	1	0	1	0	1	0	1	1	1	1	0	1	1	0	0	1	0	0	1	1	0	1	1	13	7	5	1.4
90	1CS	I	4032	L61	1	1	1	1	1	1	1	1	0	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	1	1	15	8	4	2
91	96077	I	1WS	LB	1	1	1	1	1	1	1	1	0	1	1	0	0	1	0	1	0	0	0	0	0	1	1	0	1	1	14	7	4	1.75
92	R01101	I	D48	8689	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	1	1	0	0	0	1	1	1	0	1	16	7	6	1.17
93	R00022	I	8902	8630	1	1	1	1	1	0	0	1	0	1	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	0	11	5	3	1.67
94	97013	I	1WS	2CE	1	1	1	1	0	1	0	1	0	1	1	1	1	0	1	0	0	1	1	1	0	1	1	1	1	1	16	7	6	1.17
95	R00037	I	D39	8813	1	1	1	1	0	1	0	1	0	1	1	1	0	0	1	1	0	1	1	0	0	1	1	0	1	1	15	6	6	1
96	95055	I	8902	1LD	1	1	1	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	0	1	1	1	1	0	1	1	11	4	4	1
97	R00069	I	C56	B72?62	1	1	1	1	0	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	10	4	3	1.33
98	97004	I	8902	8617	1	1	1	1	0	0	0	1	1	1	1	1	1	0	1	1	0	0	1	1	0	1	1	0	1	1	15	7	5	1.4
99	94056	I	A46	8813	1	1	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	10	4	3	1.33
100	95023	I	A46	8853	1	1	1	1	0	0	0	1	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	9	3	3	1
101	96084	I	C56	A24	1	1	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	1	1	0	1	1	10	3	4	0.75
102	95031	I	8902	8617	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	1	1	1	0	1	1	9	2	4	0.5
103	R05077	I	98008	98059	1	0	1	1	1	1	0	1	0	1	1	1	0	0	1	1	0	0	0	0	0	1	1	1	1	1	13	7	4	1.75
104	98008	I	8769	8853	1	0	1	1	1	1	0	1	0	1	1	0	0	1	0	1	1	0	0	0	0	1	1	1	1	1	13	6	5	1.2
105	98003	I	D17	8662	1	0	1	1	1	0	0	1	0	1	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	0	10	5	3	1.67
106	R03025	I	D17	97072	1	0	1	1	1	0	0	1	0	1	0	1	0	0	1	1	0	1	1	0	0	0	0	0	0	0	11	5	4	1.25
107	95020	I	1WS	2AZ	1	0	1	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	1	1	0	1	1	10	5	3	1.67
108	R00081	I	8935	1GJ	1	0	1	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	6	3	1	3
109	95013	I	2BZ	2BV	1	0	1	1	0	1	0	1	0	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	1	1	12	6	4	1.5
110	94054	I	1GT	1JX	1	0	1	1	0	0	0	1	0	1	1	1	1	0	1	0	0	1	0	0	0	1	1	0	1	1	12	6	4	1.5
111	96060	I	1GT	8686	1	0	1	1	0	0	0	1	0	1	1	1	1	0	1	0	0	0	0	0	0	1	1	0	1	1	11	6	3	2
112	R02084	I	8934	98003	1	1	1	1	1	0	0	1	0	1	0	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	12	5	4	1.25
113	R00041	I	BB66	D10	1	1	1	1	1	0	0	0	0	1	1	1	1	0	1	1	0	0	0	1	0	1	1	1	0	0	12	6	3	2
114	B62	I	2957	1BM	1	1	1	1	0	1	0	1	0	1	1	0	1	0	1	0	0	0	0	0	0	1	1	0	1	1	12	6	3	2
115	BB212	I	96012	9119	1	1	1	1	0	1	0	1	0	1	0	1	1	0	1	0	0	1	0	0	0	1	1	0	1	1	13	6	4	1.5
116	99036	I	C13	1BM	1	1	1	1	0	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	10	4	3	1.33
117	9011	I	2521	1GI	1	1	1	1	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	9	3	3	1
118	2DF	I	1GR	1VU	1	0	1	1	1	1	0	1	0	1	1	1	1	1	1	0	0	0	0	0	0	1	1	1	1	1	14	8	4	2
119	2BZ	I	2774	1CS	1	0	1	1	1	1	0	1	0	1	1	1	0	1	1	0	0	1	1	0	0	1	1	0	1	1	15	7	6	1.17
120	R09036	I	R04066	96023	1	0	1	1	1	1	0	1	0	1	1	1	0	1	1	0	0	0	1	0	0	1	1	1	1	1	14	7	5	1.4
121	2DE	I	1GR	1GI	1	0	1	1	1	0	1	0	1	1	0	0	1	1	1	0	0	0	0	0	0	1	1	1	1	1	12	6	4	1.5
122	R04016	I	R00042	97047	1	0	1	1	0	1	0	1	0	1	1	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	13	6	5	1.2
123	R04074	I	R00005	9243	1	0	1	1	0	0	0	1	0	1	1	1	0	0	0	0	0	1	0	0	0	1	1	0	1	1	10	5	3	1.67
124	R09115	I	EAW??	R04016	1	0	0	1	0	1	1	1	0	1	1	0	0	0	0	1	0	0	1	0	0	1	1	0	1	1	11	6	4	1.5
125	R04035	I	96077	98024	1	1	1	1	1	1	1	1	0	1	1	0	0	1	0	0	0	0	0	0	0	1	1	1	1	1	13	7	3	2.33
126	R09137	I	EAW	bb202	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	0	1	1	0	0	1	1	1	1	1	17	8	6	1.33
127	R06043	I	R00069	R02084	1	1	1	1	1	1	0	1	0	1	0	1	0	1	1	0	0	0	1	0	0	1	1	0	1	1	14	6	5	1.2
128	R06049	I	R00069	98003	1	1	1	1	1	1	0	1	0	1	0	1	0	1	1	0	0	0	1	0	0	1	1	0	1	1	14	6	5	1.2
129	R06105	I	8769	8669B	1	1	1	1	1	0	0	0	1	1	0	0	1	0	0	1	0	0	0	1	0	1	1	1	1	1	12	5	4	1.25
130	R02041	I	8719	1BM	1	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	1	1	1	1	11	5	3	1.67
131	R06072	I	R00069	R01050	1	1	1	1	1	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	10	4	3	1.33
132	R07119	I	R00069	R01050	1	1	1	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	10	4	3	1.33
133	R01103	I	95055	95041	1	1	1	1	1	0	1	1	0	1	1	0	0	0	1	0	0	1	0	0	1	1	1	0	1	1	13	6	4	1.5
134	R07064	I	95055	95041	1	1	1	1	1	0	1	1	0	1	1	0	0	0	1	0	0	1	0	0	1	1	1	0	1	1	13	6	4	1.5
135	R01011	I	1WS	2CL	1	1	1	1	1	0	1	1	0	1	1	0	1	0	1	1	0	0	1	0	0	1	1	0	0	0	13	6	4	1.5
136	BB220	I	C68	9125	1	1	1	1	1	0	1	1	0	1	0	1	0	1	1	0	0	1	0	0	0	1	1	0	0	0	13	6	4	1.5
137	95041	I	2BZ	1CS	1	1	1	1	0	1	1	0	1	0	1	0	1	1	1	0	0	0	1	0	0	1	1	0	0	0	13	6	4	1.5
138	R03015	I	EAW	94077	1	1	1	1	1	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	0	11	5	3	1.67
139	R03068	I	95055	95041	1	1	1	1	1	0	0	1	0	1	1	1	0	1	1	0	0	1	1	0	1	1	1	0	1	1	15	6	6	1
140	R07018	I	R02082	R03015	1	1	1	1	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	1	1	0	1	1	12	5	4	1.25
141	R04111	I	9133	8909	1	1	1	1	1	0	0	0	0	1	0	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	12	4	5	0.8
142	R05053	I	95020	94032	1	1	1	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	10	4	3	1.33
143	R03054	I	EAW	Q068	1	1	1	1	0	1	1	1	0	1	1	1	0	0	0	0	0	0	1	0	0	1	1	1	1	1	13	7	3	2.33
144	R05030	I	EAW	Q068	1	1	1	1	0	1	1	1	0	1	1	1	0	0	0	0	0	0	1	0	0	1	1	1	1	1	13	7	3	2.33
145	R01051	I	EAW	Q068	1	1	1	1	0	1	1	1	0	1	1	1	0	0	0	0	0	0	1	0	0	1	1	1	1	1	13	7	3	2.33
146	R04013	I	94054	97013	1	1	1	1	0	1	0	1	0	1	1	1	1	0	1	0	0	1	1	1	0	1	1	1	1	1	16	7	6	1.17
147	R05006	I	94054	97013	1	1	1	1	0	1	0	1	0	1	1	1	1	0	1	1	0	0	1	1	1	0	1	1	0	1	16	7	6	1.17
148	R02017	I	EAW	Q068	1	1	1	1	0	1	0	1	0	1	1	1	0	0	1	0	0	1	1	0	0	1	1	1	1	1	14	6	5	1.2
149	R01099	I	9068	9243	1	1	1	1	0	1	0	1	0	1	1	1	0	0	1	0	0	1	0	0	1	1	1	0	1	1	13	6	4	1.5
150	R08097	I	C68	R01099	1	1	1	1	0	1	0	1	0	1	1	1	0	0	1	0	0	1	0	0	1	1	1	0	1	1	13	6	4	1.5

Supplementary Table 3 continued 3 of 5.

					Mamu-KIR																																
	NAME	ORIGIN	FATHER	MOTHER	3DL20	1D	2DL04	3DL01	3DL02	3DLW03	3DL04	3DL05	3DL06	3DL07	3DL08	3DL10	3DL11	3DS01	3DS02	3DS03	3DS04	3DS05	3DS06	3DSW07	3DSW08 (1)	3DSW08 (2)	3DSW08 (all)	3DSW09 (1)	3DSW09 (2)	3DSW09 (all)	total # KIR	# inhib. KIR	# activ. KIR	I:A ratio
151	R07057	I	C68	R01099	1	1	1	1	0	1	0	1	0	1	1	1	0	0	1	0	0	1	0	0	1	1	1	0	1	1	13	6	4	1.5
152	98039	I	C6	9056	1	1	1	1	0	1	0	1	0	1	1	1	0	0	0	1	0	1	1	0	0	1	1	0	1	1	14	6	5	1.2
153	BB218	I	C68	9119	1	1	1	1	0	1	0	1	0	1	1	0	1	0	1	1	0	0	0	0	0	1	1	0	1	1	13	6	4	1.5
154	9243	I	1GT	1XL	1	1	1	1	0	1	0	1	0	1	1	1	0	1	0	1	0	0	0	0	0	1	1	1	0	1	12	6	3	2
155	R06077	I	C68	9252	1	1	1	1	0	1	0	1	0	1	1	0	0	0	0	1	1	0	0	0	0	1	1	1	1	1	12	5	4	1.25
156	R04080	I	95055	96072	1	1	1	1	0	1	0	1	0	1	1	0	0	0	0	1	0	0	0	0	0	1	1	0	1	1	11	5	3	1.67
157	R06112	I	8719	1ZC	1	1	1	1	0	1	0	1	0	1	1	0	0	0	0	0	1	1	0	0	0	1	1	1	1	1	12	5	4	1.25
158	R08113	I	R00069	8933	1	1	1	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	1	1	0	1	1	11	5	3	1.67
159	R08062	I	R00069	R02084	1	1	1	1	0	1	0	1	0	1	0	0	0	0	1	0	0	1	1	0	0	1	1	0	1	1	12	4	5	0.8
160	R03059	I	95055	96072	1	1	1	1	0	1	0	1	0	1	0	0	0	0	0	0	0	1	1	1	0	1	1	0	1	1	12	4	5	0.8
161	R08056	I	C68	9265	1	1	1	1	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	1	1	0	1	1	10	4	3	1.33
162	R04109	I	94061	98021	1	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	1	1	9	3	3	1
163	R06103	I	A46	9119	1	1	1	1	0	1	0	0	0	1	1	0	1	0	1	0	0	0	0	0	0	1	1	0	1	1	11	5	3	1.67
164	R05039	I	R00069	R01050	1	1	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	1	1	0	1	1	10	3	4	0.75
165	96023	I	C6	1BM	1	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	1	1	1	1	1	11	4	4	1
166	R03002	I	95031	1XL	1	1	1	1	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	1	1	0	1	1	9	3	3	1
167	R05103	I	95031	1XL	1	1	1	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	8	3	2	1.5
168	R06036	I	C68	9265	1	1	1	1	0	0	0	1	0	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	1	1	12	5	4	1.25
169	R07029	I	C68	9265	1	1	1	1	0	0	0	1	0	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	1	1	12	5	4	1.25
170	R00043	I	8769	2CK	1	1	1	1	0	0	0	1	0	1	1	0	1	0	1	0	0	0	0	0	0	1	1	0	1	1	11	5	3	1.67
171	R03100	I	95020	94056	1	1	1	1	0	0	0	1	0	1	0	1	1	0	1	0	0	1	1	0	0	1	1	0	1	1	13	5	5	1
172	R07108	I	95020	94032	1	1	1	1	0	0	0	1	0	1	0	1	1	0	1	0	0	1	1	0	0	1	1	0	1	1	13	5	5	1
173	R01087	I	9133	8909	1	1	1	1	0	0	0	1	0	1	0	1	0	0	1	0	1	1	0	0	0	1	1	0	1	1	12	4	5	0.8
174	R02039	I	9133	8909	1	1	1	1	0	0	0	1	0	1	0	1	0	0	1	0	1	1	0	0	0	1	1	0	1	1	12	4	5	0.8
175	R00007	I	9133	8909	1	1	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	10	4	3	1.33
176	97026	I	C68	2CP	1	1	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	9	4	2	2
177	BB213	I	C68	2CP	1	1	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	9	4	2	2
178	BB228	I	C68	2CP	1	1	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	9	4	2	2
179	R04017	I	C68	9017	1	1	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	9	4	2	2
180	98041	I	C68	2CP	1	1	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	9	4	2	2
181	R00060	I	C6	1DL	1	1	1	1	0	0	0	1	0	0	1	1	0	0	0	0	0	1	0	1	0	1	1	0	1	1	11	4	4	1
182	R05064	I	A46	9119	1	1	1	1	0	0	0	1	0	0	1	1	0	0	0	0	0	1	0	1	0	1	1	0	1	1	11	4	4	1
183	R04044	I	D17	R00041	1	1	1	1	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	1	1	1	0	0	0	11	4	4	1
184	R08070	I	9133	R02039	1	1	1	1	0	0	0	0	0	1	0	1	0	0	1	0	1	1	0	0	0	1	1	0	1	1	11	3	5	0.6
185	R03080	I	95020	94032	1	1	1	1	0	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	9	3	3	1
186	R09153	I	95055	95026	1	1	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1	1	1	1	0	1	1	10	3	4	0.75
187	R05083	I	95055	96084	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	1	1	1	0	1	1	9	2	4	0.5
188	R07115	I	95055	96084	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	1	1	1	0	1	1	9	2	4	0.5
189	R04021	I	95031	1XL	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	1	1	0	1	1	8	2	3	0.67
190	R04054	I	C68	9125	1	1	1	0	1	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	9	4	2	2
191	R02034	I	EAW	97023	1	0	1	1	1	1	0	1	0	1	1	1	0	1	1	0	1	1	1	0	0	1	1	1	1	1	16	7	7	1
192	R06037	I	R01081	R03025	1	0	1	1	1	1	0	1	0	1	1	1	0	0	1	1	0	0	1	0	0	1	1	1	1	1	14	7	5	1.4
193	R02008	I	EAW	94077	1	0	1	1	1	1	0	1	0	1	1	1	0	0	1	0	0	1	1	0	0	1	1	1	1	1	14	7	5	1.4
194	R09148	I	EAW	9017	1	0	1	1	1	1	0	1	0	1	1	1	0	0	1	1	0	0	1	1	0	0	1	1	1	1	14	7	5	1.4
195	R02091	I	96077	98024	1	0	1	1	1	1	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	1	1	1	1	1	12	6	4	1.5
196	R08003	I	96077	98024	1	0	1	1	1	1	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	1	1	1	1	1	12	6	4	1.5
197	R08104	I	98008	R05077	1	0	1	1	1	1	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	1	1	1	1	1	12	6	4	1.5
198	R04082	I	8719	1ZC	1	0	1	1	1	1	0	1	0	1	1	0	0	0	0	1	0	0	0	0	0	1	1	0	1	1	11	6	3	2
199	R06010	I	R02041	R01021	1	0	1	1	1	1	0	1	0	1	1	0	0	0	0	1	0	0	0	0	0	1	1	0	1	1	11	6	3	2
200	R04020	I	8719	1UE	1	0	1	1	1	1	0	1	0	1	0	1	1	0	1	1	0	0	1	0	0	1	1	0	0	0	13	7	4	1.75
201	R00052	I	8769	1OL	1	0	1	1	1	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	1	1	1	1	1	10	5	3	1.67
202	R04103	I	8769	8727	1	0	1	1	1	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	1	1	1	1	1	10	5	3	1.67
203	R04051	I	EAW	95061	1	0	1	1	1	0	1	1	0	1	1	0	0	0	0	0	0	0	1	0	0	1	1	0	1	1	11	6	3	2
204	R05014	I	R00069	98003	1	0	1	1	1	0	0	1	0	1	1	1	0	1	1	0	0	0	1	0	0	1	1	0	1	1	13	6	5	1.2
205	R07058	I	R00069	R02084	1	0	1	1	1	0	0	1	0	1	1	1	0	1	1	0	0	0	1	0	0	1	1	0	1	1	13	6	5	1.2
206	R07092	I	R01102	9066	1	0	1	1	1	0	0	1	0	1	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	0	10	5	3	1.67
207	R06107	I	95020	94032	1	0	1	1	1	0	0	1	0	1	1	0	1	0	1	0	1	1	0	0	0	1	1	0	1	1	12	5	5	1
208	R04098	I	8935	1WL	1	0	1	1	1	0	0	1	0	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	8	4	2	2
209	R08135	I	R00069	98003	1	0	1	1	1	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	9	4	3	1.33
210	R08129	I	R00069	R01050	1	0	1	1	1	1	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	1	1	0	1	1	9	4	3	1.33
211	R02035	I	8935	1WL	1	0	1	1	1	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	8	4	2	2
212	R01050	I	D17	98003	1	0	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	8	3	3	1
213	R07046	I	EAW	R02017	1	0	1	1	0	1	1	1	0	1	1	1	0	0	1	0	0	1	1	0	0	1	1	1	1	1	13	7	4	1.75
214	R07045	I	R03054	R02034	1	0	1	1	0	1	1	1	0	1	1	1	0	0	1	0	0	1	0	0	0	1	1	1	1	1	13	7	4	1.75
215	R02019	I	EAW	95061	1	0	1	1	0	1	1	1	0	1	0	1	0	0	0	0	0	0	1	0	0	1	1	0	1	1	11	6	3	2
216	R03004	I	8719	1UE	1	0	1	1	0	1	0	1	0	1	1	1	1	0	1	1	0	1	1	0	0	1	1	0	1	1	15	7	6	1.17
217	R02060	I	EAW	95060	1	0	1	1	0	1	0	1	0	1	1	1	1	0	1	0	0	1	1	0	0	1	1	1	1	1	14	7	5	1.4
218	97047	I	C68	9017	1	0	1	1	0	1	0	1	0	1	1	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	13	6	5	1.2
219	BB209	I	C68	97047	1	0	1	1	0	1	0	1	0	1	1	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	13	6	5	1.2
220	BB217	I	C68	97026	1	0	1	1	0	1	0	1	0	1	1	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	13	6	5	1.2
221	R04060	I	C68	2CP	1	0	1	1	0	1	0	1	0	1	1	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	13	6	5	1.2
222	R04036	I	C68	97026	1	0	1	1	0	1	0	1	0	1	1	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	13	6	5	1.2
223	R03018	I	EAW	95061	1	0	1	1	0	1	0	1	0	1	1	1	1	0	0	1	0	0	1	1	0	0	1	1	1	1	13	6	5	1.2
224	R05048	I	EAW	95061	1	0	1	1	0	1	0	1	0	1	1	1	0	0	1	0	0	1	1	0	0	1	1	1	1	1	13	6	5	1.2
225	R05058	I	EAW	94071	1	0	1	1	0	1	0	1	0	1	1	1	0	0	1	0	0	1	1	0	0	1	1	1	1	1	13	6	5	1.2
226	R03061	I	BXJ	95013	1	0	1	1	0	1	0	1	0	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	1	1	12	6	4	1.5

Supplementary Table 3 continued 4 of 5.

	NAME	ORIGIN	FATHER	MOTHER	Mamu-KIR 3DL20	1D	2DL04	3DL01	3DL02	3DLW03	3DL04	3DL05	3DL06	3DL07	3DL08	3DL10	3DL11	3DS01	3DS02	3DS03	3DS04	3DS05	3DS06	3DSW07	3DSW08 (1)	3DSW08 (2)	3DSW08 (all)	3DSW09 (1)	3DSW09 (2)	3DSW09 (all)	total # KIR	# inhib. KIR	# activ. KIR	I:A ratio
227	R04069	I	BXJ	95013	1	0	1	1	0	1	0	1	0	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	1	1	12	6	4	1.5
228	R05082	I	BXJ	95013	1	0	1	1	0	1	0	1	0	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	1	1	12	6	4	1.5
229	R02028	I	BXJ	95037	1	0	1	1	0	1	0	1	0	1	1	1	0	0	0	1	0	1	0	0	0	1	1	0	1	1	12	6	4	1.5
230	R05015	I	BXJ	95037	1	0	1	1	0	1	0	1	0	1	1	1	0	0	0	1	0	1	0	0	0	1	1	0	1	1	12	6	4	1.5
231	R03029	I	BXJ	95037	1	0	1	1	0	1	0	1	0	1	1	1	0	0	0	1	0	0	0	0	0	1	1	0	1	1	11	6	3	2
232	R06066	I	R00069	8933	1	0	1	1	0	1	0	1	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	0	1	1	11	5	4	1.25
233	R07093	I	R00069	8933	1	0	1	1	0	1	0	1	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	0	1	1	11	5	4	1.25
234	R01021	I	8719	1UE	1	0	1	1	0	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	0	1	1	0	1	1	11	5	4	1.25
235	R02011	I	8719	1ZC	1	0	1	1	0	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	0	1	1	0	1	1	11	5	4	1.25
236	R05056	I	R00060	98039	1	0	1	1	0	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	0	1	1	0	1	1	11	5	4	1.25
237	R07084	I	C68	9252	1	0	1	1	0	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	0	1	1	0	1	1	11	5	4	1.25
238	BB215	I	C68	9078	1	0	1	1	0	1	0	1	0	1	1	0	0	0	0	1	0	0	0	0	0	1	1	0	1	1	10	5	3	1.67
239	R02093	I	95031	9029	1	0	1	1	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	1	0	1	1	0	1	1	11	5	4	1.25
240	R02082	I	EAW	94071	1	0	1	1	0	0	1	1	0	1	1	0	0	0	0	0	0	1	1	0	0	1	1	0	1	1	11	5	4	1.25
241	R05042	I	EAW	94077	1	0	1	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	8	4	2	2
242	R04087	I	97058	98011	1	0	1	1	0	0	0	1	0	1	1	1	1	0	1	0	0	1	0	0	0	1	1	0	1	1	12	6	4	1.5
243	R04099	I	94054	96001	1	0	1	1	0	0	0	1	0	1	1	1	1	0	1	0	0	1	0	0	0	1	1	0	1	1	12	6	4	1.5
244	R00053	I	9068	9243	1	0	1	1	0	0	0	1	0	1	1	1	1	0	1	0	0	1	0	0	0	1	1	0	0	0	11	6	3	2
245	R04081	I	94054	95026	1	0	1	1	0	0	0	1	0	1	1	1	1	0	1	0	0	0	0	0	0	1	1	0	1	1	11	6	3	2
246	R05062	I	94054	95026	1	0	1	1	0	0	0	1	0	1	1	1	1	0	1	0	0	0	0	0	0	1	1	0	0	0	10	6	2	3
247	R08089	I	C68	9252	1	0	1	1	0	0	0	1	0	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	1	1	11	5	4	1.25
248	R03016	I	94054	97013	1	0	1	1	0	0	0	1	0	1	1	0	1	0	1	0	0	0	0	0	0	1	1	0	1	1	10	5	3	1.67
249	R04104	I	94054	96060	1	0	1	1	0	0	0	1	0	1	1	0	1	0	1	0	0	0	0	0	0	1	1	0	1	1	10	5	3	1.67
250	R07120	I	97058	98011	1	0	1	1	0	0	0	1	0	1	1	0	1	0	1	0	0	0	0	0	0	1	1	0	1	1	10	5	3	1.67
251	R06031	I	C68	R01099	1	0	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	8	4	2	2
252	R06086	I	R00053	2CL	1	0	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	8	4	2	2
253	BB219	I	C68	9078	1	0	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	8	4	2	2
254	R04094	I	C68	9078	1	0	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	8	4	2	2
255	R05018	I	R00053	9248	1	0	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	8	4	2	2
256	R06118	I	C68	9248	1	0	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	8	4	2	2
257	R07006	I	BB212	BB207	1	0	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	8	4	2	2
258	R07099	I	BXJ	R02028	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	6	3	1	3
259	R07121	I	95020	98016	1	0	1	0	0	1	0	1	0	1	0	0	1	0	1	0	0	0	0	1	0	1	1	0	1	1	10	4	4	1
260	R03078	I	EAW	94071	1	0	0	1	1	0	1	1	0	1	1	0	0	0	1	0	0	0	1	0	0	1	1	0	1	1	11	6	4	1.5
261	R07032	I	R02008	R01051	1	0	0	1	1	0	1	1	0	1	0	1	0	0	1	0	0	0	1	0	0	1	1	0	0	0	10	6	3	2
262	R09048	I	EAW	97047	1	0	0	1	0	1	1	1	0	1	1	0	0	0	0	1	0	0	1	0	0	1	1	0	1	1	11	6	4	1.5
263	R08014	I	R03054	R01051	1	0	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	7	4	2	2
264	4067	B			1	1	1	1	1	0	0	1	0	0	0	1	1	0	1	1	0	1	0	0	0	1	1	0	0	0	12	5	4	1.25
265	4050	B			1	1	1	1	0	1	0	1	0	1	1	1	1	0	1	0	0	0	0	1	0	1	1	0	0	0	13	7	3	2.33
266	4074	B			1	1	1	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	8	3	2	1.5
267	4051	B			1	1	1	1	0	0	0	1	0	0	0	1	1	0	1	1	0	1	1	0	0	1	1	0	0	0	12	4	5	0.8
268	4052	B			1	1	1	0	1	1	0	1	1	1	0	1	1	1	1	0	1	0	0	0	0	1	1	0	1	1	15	7	5	1.4
269	4077	B			1	0	1	1	1	0	0	1	1	1	1	0	0	0	1	0	0	0	1	1	0	1	1	0	1	1	13	6	5	1.2
270	R00063	B	9312	4101	1	0	1	1	0	1	0	1	1	1	1	0	0	0	1	0	0	0	0	1	0	1	1	0	1	1	12	6	4	1.5
271	4049	B			1	0	1	1	0	1	0	1	1	0	1	1	1	0	1	0	0	1	0	0	0	1	1	0	1	1	13	7	4	1.75
272	R03060	B	98022	98047	1	0	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	8	4	2	2
273	4062	B			1	0	1	1	0	1	0	0	1	1	0	1	1	0	1	1	0	1	1	0	0	1	1	0	1	1	14	6	6	1
274	4065	B			1	0	1	1	0	0	0	1	0	1	1	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	12	5	5	1
275	4078	B			1	0	1	1	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	1	0	1	1	0	1	1	10	5	3	1.67
276	4106	B			1	0	1	1	0	0	0	1	0	1	0	1	1	0	1	1	0	1	0	1	0	0	0	0	0	0	11	5	4	1.25
277	4072	B			1	0	1	1	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	1	8	4	2	2
278	R01027	B	4049	4064	1	1	1	1	0	1	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	1	1	0	1	1	13	7	3	2.33
279	96006	B	4052	4109	1	1	1	1	0	1	0	1	1	1	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	12	6	3	2
280	95007	B	4052	4095	1	1	1	0	1	1	0	1	1	1	0	1	1	1	1	0	1	0	0	0	0	1	1	0	1	1	15	7	5	1.4
281	95007	B	4052	4095	1	1	1	0	1	1	0	1	1	1	0	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	13	5	5	1
282	97050	B	4049	4064	1	0	1	1	1	1	0	1	1	1	1	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	12	7	3	2.33
283	94045	B	4052	4109	1	0	1	1	1	0	0	1	0	1	0	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	16	6	8	0.75
284	R00018	B	4049	4064	1	0	1	1	1	0	0	1	0	1	0	1	0	1	1	0	1	1	0	0	0	1	1	0	1	1	13	5	6	0.83
285	R06045	B	95007	96089	1	1	1	1	0	1	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	1	1	1	1	1	13	7	3	2.33
286	R07077	B	R01027	R01030	1	1	1	1	0	1	0	1	1	1	1	0	0	0	1	0	0	0	0	0	1	1	1	0	1	1	12	6	3	2
287	R04091	B	95007	4065	1	1	1	1	0	1	0	1	1	1	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	12	6	3	2
288	R04009	B	95007	96089	1	1	1	1	0	1	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	1	1	0	1	1	11	6	2	3
289	R01030	B	4049	4074	1	1	1	1	0	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	1	1	1	0	1	1	11	5	3	1.67
290	R08049	B	R00063	96006	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1	0	0	0	0	0	0	12	5	4	1.25
291	R07059	B	R01027	96091	1	1	1	1	0	1	0	0	1	1	1	1	0	0	1	0	0	0	0	0	1	1	1	0	1	1	12	6	3	2
292	R06014	B	R00063	96006	1	1	1	1	0	1	0	0	1	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	10	5	2	2.5
293	R04046	B	95007	96091	1	0	1	1	1	1	0	1	1	1	1	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	14	7	5	1.4
294	R05023	B	95007	96089	1	0	1	1	1	1	0	1	1	1	1	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	14	7	5	1.4
295	R04090	B	95007	4077	1	0	1	1	1	1	0	1	1	1	1	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	14	7	5	1.4
296	R05002	B	R00063	94045	1	0	1	1	1	1	0	1	1	0	1	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	13	6	5	1.2
297	R09055	B	R05024	R02049	1	0	1	1	1	1	0	1	1	0	1	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	13	6	5	1.2
298	R05024	B	95007	96091	1	0	1	1	1	1	0	1	0	1	1	0	0	1	1	0	1	0	0	0	1	1	1	0	1	1	13	6	5	1.4
299	R08077	B	R03060	R02049	1	0	1	1	0	1	0	1	1	1	1	1	0	0	1	0	0	0	0	1	1	1	1	0	1	1	13	7	4	1.75
300	R02049	B	4049	4074	1	0	1	1	0	1	0	1	1	1	1	1	0	0	1	0	0	0	0	0	1	1	1	0	1	1	12	7	3	2.33
301	R05031	B	R01027	R02049	1	0	1	1	0	1	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	1	1	0	1	1	12	7	3	2.33
302	R06034	B	R00063	96076	1	0	1	1	0	1	0	1	1	1	1	0	0	0	1	0	0	0	0	1	0	1	1	0	1	1	12	6	4	1.5

Supplementary Table 3 continued 5 of 5.

					Mamu-KIR																																			
	NAME	ORIGIN	FATHER	MOTHER	3DL20	1D	2DL04	3DL01	3DL02	3DLW03	3DL04	3DL05	3DL06	3DL07	3DL08	3DL10	3DL11	3DS01	3DS02	3DS03	3DS04	3DS05	3DS06	3DSW07	3DSW08 (1)	3DSW08 (2)	3DSW08 (all)	3DSW09 (1)	3DSW09 (2)	3DSW09 (all)	total # KIR	# inhib. KIR	# activ. KIR	1:A ratio
303	96091	B	4049	4074	1	0	1	1	0	1	0	1	1	1	1	0	0	0	1	0	0	0	0	0	1	1	1	0	1	1	11	6	3	2
304	R05043	B	R01027	R01030	1	0	1	1	0	1	0	1	1	1	1	0	0	0	1	0	0	0	0	0	1	1	0	1	1	1	11	6	3	2
305	R07075	B	R00063	96076	1	0	1	1	0	1	0	1	1	1	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	11	6	3	2
306	R06100	B	R00063	94045	1	0	1	1	0	1	0	1	1	1	1	0	1	1	0	1	1	0	1	1	1	0	0	0	0	0	14	7	5	1.4
307	96089	B	4049	4065	1	0	1	1	0	1	0	1	1	0	1	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	13	6	5	1.2
308	R01048	B	97050	96089	1	0	1	1	0	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	10	5	3	1.67
309	R07111	B	R00063	4078	1	0	1	1	0	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	10	5	3	1.67
310	R08132	B	R00063	4078	1	0	1	1	0	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	10	5	3	1.67
311	R05081	B	R00063	4078	1	0	1	1	0	1	0	1	0	1	1	1	0	0	0	0	0	0	0	1	0	1	1	0	1	1	11	6	3	2
312	R08051	B	R03060	96089	1	0	1	1	0	1	0	1	0	0	0	1	0	0	1	1	0	1	0	1	0	0	0	0	0	0	10	4	4	1
313	R08060	B	R00063	96076	1	0	1	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	1	1	9	4	3	1.33
314	R05049	B	R00063	96076	1	0	1	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	1	1	9	4	3	1.33
315	R06059	B	R00063	4078	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	6	3	1	3
316	96076	B	4049	4072	1	0	1	1	0	0	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	9	4	3	1.33
317	R05068	B	95007	4077	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	1	0	1	1	0	1	1	0	1	1	12	3	7	0.43
318	Ri100	C			1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	0	1	0	0	1	1	0	1	1	18	9	6	1.5
319	Ri176	C			1	1	1	1	0	1	0	1	1	1	1	0	1	0	1	1	0	0	0	1	0	1	1	0	1	1	15	7	5	1.4
320	Ri388	C			1	1	1	1	0	1	0	1	1	1	1	0	1	0	0	0	0	0	1	0	0	1	1	0	1	1	13	7	3	2.33
321	4003	C			1	1	1	1	0	0	0	1	1	1	1	0	1	0	1	1	0	0	0	1	0	0	1	1	0	1	13	6	4	1.5
322	Ri134	C			1	0	1	1	1	1	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	11	8	1	8
323	Ri228	C			1	0	1	1	1	0	0	1	1	1	1	0	1	0	0	1	0	0	1	1	0	1	1	0	1	1	14	7	5	1.4
324	Ri136	C			1	0	1	1	1	0	0	1	1	1	1	0	1	0	0	1	0	0	1	1	0	1	1	0	1	1	14	7	5	1.4
325	BB71	C			1	0	1	1	0	1	0	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	1	0	1	1	15	8	5	1.6
326	Ri329	C			1	0	1	1	0	1	0	1	1	1	1	1	1	0	0	1	0	0	0	1	1	1	1	0	1	1	14	8	4	2
327	BB55	C			1	0	1	1	0	1	0	1	1	1	1	1	1	0	0	0	0	0	0	1	1	1	1	0	1	1	13	8	3	2.67
328	Ri189	C			1	0	1	1	0	1	0	1	1	1	1	0	1	0	1	0	0	0	1	0	0	1	1	0	1	1	13	7	4	1.75
329	BB57	C			1	0	1	1	0	1	0	1	1	1	1	0	1	0	0	0	0	0	1	1	0	1	1	0	0	0	12	7	3	2.33
330	4006	C			1	0	1	1	0	1	0	1	1	1	1	0	1	0	0	0	0	0	1	0	0	1	1	0	1	1	12	7	3	2.33
331	Ri284	C			1	0	1	1	0	1	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	10	5	3	1.67
332	Ri184	C			1	0	1	1	0	0	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	1	9	5	2	2.5
333	Ri172	C			1	0	1	0	0	1	0	0	1	1	0	0	1	0	0	1	0	0	0	0	0	1	1	0	1	1	9	4	3	1.33
334	4133	C			1	0	1	0	0	1	0	0	1	1	0	0	1	0	0	1	0	0	0	1	1	1	1	0	1	1	9	4	3	1.33
335	Ri115	C			1	0	1	0	0	0	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	7	4	1	4
336	9242	IB	3643	4109	1	1	1	1	1	0	0	1	0	1	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	11	5	3	1.67
337	R00077	IC	8932	1NS	1	1	1	1	1	0	0	0	1	1	1	0	0	1	0	1	1	1	0	0	1	1	0	0	0	0	13	5	5	1
338	R00035	CI	D55	8669B	1	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	8	3	2	1.5
339	R01079	CI	D55	8669B	1	1	1	1	0	1	0	1	0	1	1	1	0	1	1	0	1	0	0	0	0	1	1	1	1	1	14	6	5	1.2
340	R05105	IxCI	95020	98016	1	1	1	1	0	1	0	1	0	1	1	0	1	0	1	1	0	0	0	0	0	1	1	0	1	1	13	6	4	1.5
341	98016	CI	B59	C67	1	1	1	1	0	1	0	0	0	1	1	0	1	0	0	1	0	0	0	1	0	1	1	0	0	0	11	5	3	1.67
342	R00065	CI	D55	TA	1	1	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0	1	1	1	1	1	11	4	4	1
343	BB10	CI	3945	2677	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0	0	0	1	1	1	0	0	0	0	1	1	12	5	4	1.25
344	8765	IC	ME	3942	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	0	0	1	1	0	0	0	16	9	5	1.8
345	98055	IC	2BZ	3941	1	0	1	1	1	1	0	1	1	1	0	1	0	1	1	0	0	0	1	0	0	1	1	0	1	1	14	7	5	1.4
346	R04067	IxIC	96077	98055	1	0	1	1	1	1	0	1	0	1	1	0	1	1	1	0	0	0	1	0	0	1	1	0	1	1	15	7	6	1.17
347	D55	IC	2777	3946	1	0	1	1	1	1	0	1	0	1	1	1	0	1	1	0	0	1	1	0	0	1	1	0	1	1	15	7	6	1.17
348	R06038	IxIC	96077	98055	1	0	1	1	1	1	0	1	0	1	1	1	0	1	1	0	0	0	1	0	0	1	1	1	1	1	14	7	5	1.4
349	9029	CI	BB20	1CE	1	0	1	1	1	1	0	1	0	1	1	0	1	1	1	0	1	0	1	0	0	1	1	1	1	1	15	7	6	1.17
350	9245	IB	3643	4078	1	0	1	1	1	1	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	11	6	3	2
351	R04108	CI	95031	9029	1	0	1	1	1	1	0	0	0	1	1	0	0	1	1	0	1	0	0	0	0	1	1	1	1	1	12	5	5	1
352	R02048	IxBI	9068	9242	1	0	1	1	1	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	7	4	1	4
353	97058	CI	1WS	8944	1	0	1	1	0	0	0	1	0	1	0	1	1	0	1	0	0	1	0	0	0	1	1	0	1	1	11	5	4	1.25
354	R05098	CI	97058	98011	1	0	1	1	0	0	0	1	0	1	0	1	0	0	1	1	0	0	1	0	0	0	1	1	0	1	10	4	4	1
355	4030	I?			1	1	1	1	1	0	0	1	0	1	1	0	0	0	0	0	0	1	1	0	1	1	1	0	1	1	12	5	4	1.25
356	8778	IB?	ME	2412	1	1	1	1	1	0	0	1	0	1	0	1	0	1	1	0	1	1	0	0	0	1	1	0	1	1	14	5	6	0.83
357	98021	IBxI?	1AB	8657	1	1	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	1	1	0	1	1	10	4	3	1.33
358	4045	B			1	1	1	1	0	0	0	1	1	1	1	1	1	0	1	0	1	1	1	0	0	1	1	0	1	1	16	7	6	1.17
359	2871	?			1	1	1	1	0	0	0	1	1	1	0	1	0	0	1	1	0	0	1	1	0	0	1	1	0	1	13	5	5	1
360	4041	I?			1	0	1	1	1	1	0	1	0	1	1	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	14	7	5	1.4
361	97023	IB?xI?	1AB	9055	1	0	1	1	1	1	0	1	0	1	1	0	0	1	1	1	1	0	0	0	0	1	1	0	1	1	14	6	6	1
362	96425?	C?			1	0	1	1	1	1	0	1	0	1	1	0	0	0	1	1	0	0	1	0	0	1	1	0	1	1	12	6	4	1.5
363	R01094	IB?	94054	96009	1	0	1	1	1	0	0	1	0	1	1	1	1	1	1	0	1	0	0	0	0	1	1	0	1	1	14	7	5	1.4
364	R01031	IB?xI	1AB	8916	1	0	1	1	1	0	0	1	0	1	1	0	1	1	1	0	1	0	0	0	1	1	1	0	0	0	12	6	4	1.5
365	R01094	IB?	94054	96009	1	0	1	1	1	0	0	1	0	1	1	0	1	1	1	0	1	0	0	0	0	1	1	0	0	0	12	6	4	1.5
366	R03014	IB?	94054	96009	1	0	1	1	1	0	0	1	0	1	0	1	0	1	1	0	1	1	0	0	0	1	1	0	1	1	13	5	6	0.83
367	1AB	IB?	L	2412	1	0	1	1	1	0	0	1	0	1	0	1	0	1	1	0	1	0	0	0	0	1	1	0	1	1	12	5	5	1
368	2882	?			1	0	1	1	1	0	0	0	0	1	0	1	1	1	1	0	0	1	1	0	0	0	0	0	0	0	11	5	4	1.25
369	95026	IB?xI	1AB	8943	1	0	1	1	1	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	7	4	1	4
370	94015	BC?	1AB	8657	1	0	1	1	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	7	3	2	1.5
371	BB116	CC?	3945	BB75	1	0	1	1	0	1	0	1	1	1	1	1	1	0	1	0	0	0	0	1	1	1	1	0	1	1	14	8	4	2
372	4119	B?			1	0	1	1	0	1	0	1	0	1	1	1	1	0	0	0	0	1	0	0	1	1	1	0	1	1	12	7	3	2.33
373	R02014	IxIB?	BXJ	95013	1	0	1	1	0	1	0	1	0	1	1	1	0	0	0	0	0	1	0	0	0	1	1	0	1	1	11	6	3	2
374	BB22	CC?			1	0	1	1	0	0	0	1	0	1	1	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	11	5	4	1.25
375	R04089	IB?	94054	96009	1	0	1	1	0	0	0	1	0	1	0	1	0	0	1	0	1	1	0	0	0	1	1	0	1	1	11	4	5	0.8
376	9248	IB?	1AB	1TR	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	2	0	-
377	96009	IB?xI	1AB	B33	1	0	1	0	1	0	0	1	0	1	0	1	0	1	1	0	1	1	0	0	0	1	1	0	1	1	12	4	6	0.67
378	R01102	BC?	1AB	94015	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	10	3	5	0.6

CHAPTER 6

General Discussion

THE IMPORTANCE AND EVOLUTION OF THE KIR GENE SYSTEM

Since the discovery of NK cells in 1975 (1), a great deal of information has been gathered on their essential biological functions. These include the protection against pathogens, the eradication of malignancies, and the remodeling of spiral arteries during pregnancy (2-4). Moreover, NK cells are bridging gaps between the innate and adaptive immune system by positively or negatively modulating a scala of different functions controlled by various types of immunocompetent cells (5).

It was discovered that the expression of MHC class I molecules on target cells was more or less essential for determining the manner in which the NK cell could be activated. The 'missing-self' hypothesis, posed in 1990, is considered a breakthrough that led to re-evaluation of past experiments, and opened up different avenues of NK cell research (6). Subsequently, many receptors that are responsible for the inhibition and activation of NK cells were discovered, as well as their cognate MHC and non-MHC ligands. Among these the receptors that played a key role in MHC class I-mediated regulation, are grouped into the killer cell immunoglobulin-like receptor (KIR) family. Molecular cloning revealed their characteristic immunoglobulin-like organisation, as well as motifs for an inhibitory and activating capacity (7-9). Over time, the ligand specificity of many of these receptors was determined, and could be linked to particular motifs on MHC class I molecules. Furthermore, cellular analyses revealed that KIR molecules and their interaction partners can not only determine the state of NK cell activation, they can also influence their education (10) – perhaps even influence the expansion of cells with a memory-like phenotype (11). Subsequent genetic analyses of these receptors, conducted on individuals from different human populations revealed that the genes are polymorphic in nature and that by means of copy number variation haplotypes can comprise different compositions of KIR genes (12, 13). As such, the KIR repertoire may differ greatly between individuals. This is highly relevant because specific epistatic interactions between HLA alleles and particular KIR genes are associated with susceptibility to or protection against diseases. (14). These associations include decreased susceptibility to viral infections, improved protection against certain types of cancer, susceptibility to autoimmunity, and reproductive success (15-18).

The evolution of KIR genes has been studied in many different mammalian species, and in most of them their organisation does not resemble the polymorphic, polygenic KIR gene system present in humans. Although two KIR genes are present in mice, for instance, these have been translocated to a different chromosome as compared to humans, and appear to have functions that are unrelated to those of human KIR. Moreover, KIR genes of other mammals – for instance, in rats, pigs, cattle, horses, and marine carnivores (19-24) – have remained relatively monomorphic, whereas structurally or functionally related gene families may have undergone significant expansion or diversification during evolution. Even in evolutionarily distant

'lower' primates like lemurs, it is observed that the KIR system is not comparable to humans; by way of convergent evolution a different gene family of receptors appears to achieve the same function by combinatorial diversification, whereas the KIR repertoire is condensed as compared to that observed in humans (25). More closely related primate species, such as the chimpanzee, the bonobo, and the gorilla, which possess a similar MHC achitecture as humans, show a comparable KIR architecture. However, as a result of rapid evolution, all the KIR genes have diverged in a species-specific way (26-28). On the other hand, primate species that have a more divergent MHC architecture, like orangutan and gibbon, show a more divergent KIR gene structure. Because of rapid species-specific evolution of both these genes, and KIR co-evolution with the MHC, the changes in the KIR organisation of these primates have been even more prominent (29, 30).

Rhesus macaques share a common ancestor with humans approximately 25 million years ago, indicating an evolutionary distance of 50 million years. Since rhesus macaques diverged from humans, their MHC architecture has expanded considerably (31, 32). Therefore, one wonders how the KIR gene system has evolved in this species.

TOWARDS A STANDARDISED NOMENCLATURE: FROM CHAOS TO ORDER

The first articles that reported rhesus macaque KIR sequences often made use of a differential nomenclature system (33-36). To complicate matters even further, gene names have frequently been assigned based on the chronological order of discovery (e.g. mmKIR3DL1 was the first cloned sequence, whereas mmKIR3DL20 was the 20th clone). This confusion in nomenclature continued and in one recent report, new allele names were assigned sequentially to each newly discovered KIR sequence (37).

To apply a more rationale approach to classification, based on phylogenetic clustering of sequences a preliminary classification of activating KIR genes into eight clades was made **(Chapter 3)**. In the mean time, more sequence data became available, which improved the power of the phylogenetic relationships. Therefore, at present, distinct clades of closely related alleles can now be recognised. As well as the lineage I, III, and V genes that cluster separately, the expanded lineage II genes are now subdivided into 10 inhibitory and 9 activating groups **(Chapter 4)**. As more high-resolution sequence data becomes available, new gene groups will most likely be discovered, while groups that are currently ambiguous may be unequivocally assigned.

In a recent collaborative effort, an official rhesus macaque KIR gene nomenclature has been proposed (manuscript in preparation). The available data are curated by a team of experts and will be made available through the Immuno Polymorphism Database (IPD;[38]). This effort has resulted in a classification of gene groups based on phylogenetic analysis, as well as in a standardised nomenclature for inhibitory KIR (*Mamu-KIR3DL*) and activating KIR (*Mamu-KIR3DS*) in which each of these names is

followed by two digits that identify and characterise the gene group.

GENETIC ANALYSES ELUCIDATE THE (DIS)ORGANISATION OF KIR HAPLOTYPES

It could be deduced from a particular sequenced haplotype that the rhesus macaque KIR complex is located on chromosome 19 in the leukocyte receptor complex (LRC) region between the genes of the leukocyte Ig-like receptors (*LILR*) and of the receptor for IgA (*FCAR*) (35). The architecture from centromeric to telomeric is a head-to-tail orientation of *Mamu-KIR3DL20*, *-1D*, the pseudogene *-DP*, and *-2DL04*, followed by a combination of inhibitory *-3DL* and activating *-3DS* genes. Of these genes, *Mamu-KIR3DL20* appears to be present in all genotypes that have been reported (**Chapter 5**). Although, its ligand binding domain is highly conserved, gene transcripts are not always observed by analysing peripheral blood mononuclear cells of individuals (**Chapter 4**). This may be because this gene product has an important function only under certain conditions. Phylogenetically this gene clusters in lineage V together with the enigmatic human *KIR3DL3*, which is also present on almost all recorded haplotypes and usually not transcribed. In both instances, the protein is thought to be important under particular developmental or pathogenic conditions, although no ligand has yet been defined (39).

The *Mamu-KIR1D* gene is observed in approximately 50% of the genotypes within animals of Indian origin, and detected less often in animals of either Burmese or Chinese origin (**Chapter 5**). Therefore, it was documented that this gene is absent from a majority of haplotypes (**Chapter 4 and 5**). Its genotype frequency suggests that some form of balancing selection might be operating on this gene. Moreover, because this gene displays very limited polymorphism, purifying selection may be effective as well. Currently the function of its product is unknown. Notably, *Mamu-KIR1D* has no homologue in humans but particular alleles of the *KIR2DS4* gene, which are present in 50% of the human individuals, may also encode for a truncated 1D molecule.

An extensive investigation of the conserved *Mamu-KIR2DL04* was performed in a large group of animals, and transcripts of one or several alleles were observed in most of them (**Chapter 2**). This allelic diversity was unexpected since human KIR2DL4 was considered to be highly conserved. However, a more detailed analysis revealed that the ligand binding Ig-like domain is still highly conserved, even more so than in humans, while the signal-transducing domain is polymorphic. This suggests that strong purifying selection has acted on the Ig-like domains. Polymorphism in the intracellular region is reflected in the observation that *Mamu-KIR2DL04* alleles can be divided into three forms. Two of these are present in high frequencies in the studied cohort, while the third is only observed in low numbers (**Chapter 2**). The fact that these forms have been maintained in the population points to balancing selection. Nevertheless, *Mamu-KIR2DL04* is not essential for life or reproduction, since rhesus macaques exist that lack the gene on both their haplotypes, and have succesfully re-

produced (**Chapters 4 and 5**). This is probably due to redundancy.

Based on a phylogenetic analysis of the lineage II genes, it can be concluded that these have expanded and diversified in the rhesus macaque. By comparing the published data, the number of reported inhibitory KIR gene groups has been extended from seven to ten, while the number of activating gene groups has been extended from four to nine, respectively. Moreover, many alleles have been documented within these gene groups (**Chapters 3 and 4**). A study of the gene content involving a large cohort of pedigreed animals has led to the conclusion that these genes may be present or absent on a given haplotype, and that any combination may in theory be possible (**Chapters 4 and 5**).

IS ALTERNATIVE SPLICING A MECHANISM TO DIVERSIFY RHESUS KIR GENES?

Many Mamu-KIR transcripts have been analysed, and one of the features that is noteworthy to discuss is that approximately 20% of the sequences represents some form of a splice variant. A more detailed analysis of intron and exon data showed that many cryptic alternative splice sites exist within the KIR genes, making them prone to this phenomenon of alternative splicing, which is not observed in, for instance, MHC class I transcripts.

In rhesus macaques, three drastic examples of splicing have been documented. First, a splice site mutation has been discovered that is responsible for truncating an activating *Mamu-KIR* and thereby removing the sequence that encodes for inhibitory motifs (**Chapter 3**). Second, the alternative splicing of *Mamu-KIR3DL20* results in a transcript that is highly homologous to the human lineage I *KIR2DL5A* and *-B* genes. This might be an elaborate form of convergent evolution by which a new gene product is generated. Finally, the alternative splicing of *Mamu-KIR1D* may result in a transcript that is no longer soluble but that comprises a stem and a transmembrane and cytoplasmic domain, including inhibitory motifs (**Chapter 4**).

Alternative splicing could be a diversifying mechanism to introduce alternative gene products at a low level. If a certain variant is favourably selected for, mutations that influence splicing may gradually accumulate until such a variant becomes fixed. This may be what happened to human lineage III KIR genes, which all contain a pseudo-exon with no obvious splice site mutations. Taken together, splicing seems to add an extra layer of polymorphism to the KIR system and may also be involved in generating novel types of KIR genes.

TO WHAT EXTENT HAVE MAMU-KIR AND MHC CLASS I CO-EVOLVED?

It is thought that primate KIR genes have co-evolved with the MHC class I genes (40). It may even be possible that the interaction with KIR molecules may have driven MHC-C evolution in hominids (41). In humans, KIR2DL4 is the ligand for HLA-G and therefore it is most likely that the rhesus KIR2DL04 may have co-evolved with Mamu-

AG, which is the functional homologue of HLA-G, since the Mamu-G orthologue of HLA-G has become inactivated. The observation that the ligand binding domain of 2DL04 is highly conserved suggests that it has co-evolved with an equally conserved interaction partner, such as this nonclassical MHC class I molecule (**Chapter 2**).

In rhesus macaques the expansion of lineage II KIR genes is hypothesised to be propelled by the expansion of the MHC class I genes. Indeed, through a study of the gene content in a large cohort of rhesus macaque of different origins, it has become evident that in this system many different haplotype configurations are present in this system (**Chapters 4 and 5**). All these phenomena strongly suggest co-evolution with the polymorphic and polygenic class I gene family. However, only by formally determining that these proteins are indeed interaction partners can this hypothesis be strengthened further. This is exactly what has been reported recently for a select combination of rhesus macaque KIR and MHC class I proteins (42, 43).

A comparison of gene frequencies between rhesus macaques of different origins yielded specific patterns of KIR gene distribution. These could be a reflection of the co-evolution that has taken place within a relatively short time-frame. Therefore, it is hypothesised that the *Mamu-KIR3DL04* gene that is observed primarily in Indian animals, and the *Mamu-KIR3DL06* gene that is reported in Burmese and Chinese animals may have co-evolved with MHC class I ligands that are specific for these populations (**Chapter 5**).

RHESUS MACAQUE KIR COMPLEXITY MAY EXCEED THAT OF HUMANS

Rhesus macaque haplotypes are characterised by a combination of gene content diversity and allelic variation; this combination is termed complexity. By analysing a number of KIR genotypes, it can be deduced that any lineage II gene can be either present or absent on a given haplotype. This suggests that haplotype compositions have been formed by recombination of the intergenic regions, hereby creating mosaic-like haplotype diversity (**Chapters 4 and 5**). Moreover, protein alignments of activating genes revealed that different gene groups can share highly similar domains (**Chapter 3**). It can be concluded from this that intragenic recombination is also a mechanism that can lead to the birth of a new gene group, and this mechanism may contribute to the generation of further haplotype diversity. Another example of this birth of new genes is the manner in which the novel sub-lineage of activating KIR genes has been generated by intragenic recombination between an ancestral KIR3DL and KIR2DL4 (**Chapter 3**).

Similar diversity-generating mechanisms have had an influence on the human KIR gene system (44, 45). In humans, a model of centromeric and telomeric recombination can be used to explain most of the observed haplotype configurations. Moreover, in humans there is a clear distinction between two types of haplotypes, group A and B, which is not observed in rhesus macaques. The current hypothesis is that

group A haplotypes have been favorably selected for by selective pressures of pathogens, whereas group B haplotypes favour reproductive success (46). The absence of this clear distinction between haplotype groups in rhesus macaques suggests that in this case different selective forces may have been applicable.

FORCES OF SELECTION: PATHOGENS, CANCER, REPRODUCTION, AND THE MHC

The rhesus macaque KIR gene system has been thoroughly characterised on many levels, and one of the main conclusions to be drawn is that different types of selection have led to extensive levels of complexity. The modes of selection that are operative on this system may be multifactorial. The highly conserved nature of the ligand binding domains of Mamu-KIR3DL20, -1D, and -2DL04 suggests that purifying selection has acted on them. Moreover, the different forms of *2DL04*, and the frequency at which the *1D* gene is present in a population suggests that balancing selection is applicable.

One can speculate as to which forces drive selection. If a receptor-ligand interaction has a selective advantage, and the ligand has remained conserved, purifying selection may then be acting on the receptor to ensure that it remains equally conserved. For instance, certain pathogen-associated molecular patterns are highly conserved, and possessing receptors that potentially can recognise these patterns would bestow a selective advantage on individuals. In a similar manner, receptor-ligand interactions that yield a selective advantage during developmental and reproductive processes will most likely remain conserved in a population. In this respect, Mamu-KIR2DL04 and its potential ligand Mamu-AG may be a similarly conserved receptor-ligand pair. However, in humans, the tolerogenic ability of HLA-G, which may be the functional homologue of Mamu-AG, is often exploited by cancer cells that express it to evade immune recognition. It is possible that its expression is also influenced or exploited by viruses. Perhaps Mamu-AG is abused in a similar manner, and therefore the signaling domain of Mamu-KIR2DL04 has a pleiotrophic character, since balancing selection has acted on its different forms. It is tempting to speculate that in the context of pregnancy, this molecule can transduce a tolerogenic signal, whereas in the context of infection an activating signal is transduced.

The inhibitory *Mamu-KIR* genes of lineage II have undergone expansion and diversification. It is hypothesised that this phenomenon resulted from co-evolution with the expanded MHC class I gene family. Therefore, the diversifying selection imposed on inhibitory KIR may have been a result of MHC class I molecules that have structurally diversified and perhaps functionally. Pathogens are constantly trying to evade and escape the immune system, and with dire consequences for their host. As a result, they may in some cases impose strong selective forces. For instance, many virusses disrupt the presentation pathway of pathogen-derived peptides via MHC class I to the T cell, and as a consequence may also weaken interactions with inhibi-

tory KIR. NK cells may subsequently kill the virus-infected cell. One strategy to rescue interactions with inhibitory KIR could be by expressing decoy MHC class I-like proteins, much like UL18, which is expressed by human cytomegalovirus to bind inhibitory LIR-1 on NK and T cells. Perhaps the occurrence of these types of viral molecules is what drove the generation of activating KIR through convergent evolution. In this respect, the diversified KIR repertoire of rhesus macaques may be the result of an arms race between the MHC, pathogens, and KIR. The observation that unique KIR genes are present in rhesus macaques of distinct origins, suggests that particular selective forces may have been restricted to geographical locations.

The discovered extreme diversity of KIR haplotypes seems to be counterintuitive, because in order to have functional interactions between KIR and MHC, both genes must be present in an individual. By putting against each other two complex gene systems that do not segregate together, the chances are lowered that suitable interaction partners will be present in the same individual. However, this paradox can be explained at the population level. In this case, individuals will be present that, depending on their MHC and KIR repertoires, may respond strongly against one pathogen, and weakly against another. Hence, a population is generated in such a way that its individuals all may react slightly differently to a similar pathogen. In a population this may be an effective strategy of hedging ones chances against dangerous pathogens that are able to escape from or evade the immune system of certain individuals. The plasticity of the KIR system would assure, for instance, that after a selective sweep by a pathogen, the diversity of the system may be rapidly restored by the birth of new genes by recombination.

Currently, NK cells and their KIR receptors in humans are being investigated therapeutically in the context of their anti-cancer capacities. Additionally, in the research fields of reproduction and vaccinology, a thorough understanding of KIR-ligand interactions may lead to novel therapies and vaccines. This is highly likely, given the discovery of NK cells with memory-like phenotypes, and the fact that more insight has been gained into the role NK cells play in achieving tolerance and priming of the adaptive immune system. Certainly in vaccinology it may be interesting to investigate whether specific pathogen-derived peptides, via MHC-KIR interactions, can induce an immune response. Although the rhesus macaque MHC class I has evolved in a species-specific manner, functional orthologues exist that can present the same peptides as particular humans molecules. The same may hold true for the Mamu-KIR system, which can comprise functional orthologues of human KIR molecules. In this respect, the naturally occurring KIR in rhesus macaques may model the human situation, as a relatively small cohort of animals may comprise a KIR complexity equal to that observed in all human populations combined.

REFERENCES

1. Kiessling R, Klein E, Pross H, Wigzell H. 1975. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol* 5: 117-21
2. Lanier LL. 2008. Evolutionary struggles between NK cells and viruses. *Nat Rev Immunol* 8: 259-68
3. Moretta A, Bottino C, Mingari MC, Biassoni R, Moretta L. 2002. What is a natural killer cell? *Nat Immunol* 3: 6-8
4. Parham P. 2004. NK Cells and Trophoblasts: Partners in Pregnancy. *J Exp Med* 200: 951-5
5. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, Yokoyama WM, et al. 2011. Innate or adaptive immunity? The example of natural killer cells. *Science* 331: 44-9
6. Ljunggren HG, Kärre K. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today* 11: 237-44
7. Colonna M, Samaridis J. 1995. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science* 268: 405-8
8. D'Andrea A, Chang C, Franz-Bacon K, McClanahan T, Phillips JH, Lanier LL. 1995. Molecular cloning of NKB1. A natural killer cell receptor for HLA-B allotypes. *J Immunol* 155: 2306-10
9. Wagtmann N, Biassoni R, Cantoni C, Verdiani S, Malnati MS, Vitale M, Bottino C, et al. 1995. Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra- and intracellular domains. *Immunity* 2: 439-49
10. Höglund P, Brodin P. 2010. Current perspectives of natural killer cell education by MHC class I molecules. *Nat Rev Immunol* 10: 724-34
11. Sun JC, Beilke JN, Lanier LL. 2010. Immune memory redefined: characterizing the longevity of natural killer cells. *Immunol Rev* 236: 83-94
12. Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B, Tyan D, et al. 1997. Human diversity in killer cell inhibitory receptor genes. *Immunity* 7: 753-63
13. Witt CS, Dewing C, Sayer DC, Uhrberg M, Parham P, Christiansen FT. 1999. Population frequencies and putative haplotypes of the killer cell immunoglobulin-like receptor sequences and evidence for recombination. *Transplantation* 68: 1784-9
14. Khakoo SI, Carrington M. 2006. KIR and disease: a model system or system of models? *Immunol Rev* 214: 186-201
15. Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski J, Cheng J, et al. 2004. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* 305: 872-4
16. Qi Y, Martin MP, Gao X, Jacobson L, Goedert JJ, Buchbinder S, Kirk GD, et al. 2006. KIR/HLA pleiotropism: protection against both HIV and opportunistic infections. *PLoS Pathog* 2: e79
17. Nelson GW, Martin MP, Gladman D, Wade J, Trowsdale J, Carrington M. 2004. Cutting edge: heterozygote advantage in autoimmune disease: hierarchy of protection/susceptibility conferred by HLA and killer Ig-like receptor combinations in psoriatic arthritis. *J Immunol* 173: 4273-6
18. Hiby SE, Apps R, Sharkey AM, Farrell LE, Gardner L, Mulder A, Claas FH, et al. 2010. Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. *J Clin Invest* 120: 4102-10

19. Hoelsbrekken SE, Nylenna Ø, Saether PC, Slettedal IO, Ryan JC, Fossum S, Dissen E. 2003. Cutting edge: molecular cloning of a killer cell Ig-like receptor in the mouse and rat. *J Immunol* 170: 2259-63

20. Sambrook JG, Sehra H, Coggill P, Humphray S, Palmer S, Sims S, Takamatsu H-H, et al. 2006. Identification of a single killer immunoglobulin-like receptor (KIR) gene in the porcine leukocyte receptor complex on chromosome 6q. *Immunogenetics* 58: 481-6

21. Dobromylskyj M, Ellis S. 2007. Complexity in cattle KIR genes: transcription and genome analysis. *Immunogenetics* 59: 463-72

22. Guethlein LA, Abi-Rached L, Hammond JA, Parham P. 2007. The expanded cattle KIR genes are orthologous to the conserved single-copy KIR3DX1 gene of primates. *Immunogenetics* 59: 517-22

23. Takahashi T, Yawata M, Raudsepp T, Lear TL, Chowdhary BP, Antczak DF, Kasahara M. 2004. Natural killer cell receptors in the horse: evidence for the existence of multiple transcribed LY49 genes. *Eur J Immunol* 34: 773-84

24. Hammond JA, Guethlein LA, Abi-Rached L, Moesta AK, Parham P. 2009. Evolution and survival of marine carnivores did not require a diversity of killer cell Ig-like receptors or Ly49 NK cell receptors. *J Immunol* 182: 3618-27

25. Averdam A, Petersen B, Rosner C, Neff J, Roos C, Eberle M, Aujard F, et al. 2009. A novel system of polymorphic and diverse NK cell receptors in primates. *PLoS Genet* 5: e1000688

26. Khakoo SI, Rajalingam R, Shum BP, Weidenbach K, Flodin L, Muir DG, Canavez F, et al. 2000. Rapid evolution of NK cell receptor systems demonstrated by comparison of chimpanzees and humans. *Immunity* 12: 687-98

27. Rajalingam R, Hong M, Adams EJ, Shum BP, Guethlein LA, Parham P. 2001. Short KIR haplotypes in pygmy chimpanzee (Bonobo) resemble the conserved framework of diverse human KIR haplotypes. *J Exp Med* 193: 135-46

28. Rajalingam R, Parham P, Abi-Rached L. 2004. Domain shuffling has been the main mechanism forming new hominoid killer cell Ig-like receptors. *J Immunol* 172: 356-69

29. Guethlein LA, Older Aguilar AM, Abi-Rached L, Parham P. 2007. Evolution of killer cell Ig-like receptor (KIR) genes: definition of an orangutan KIR haplotype reveals expansion of lineage III KIR associated with the emergence of MHC-C. *J Immunol* 179: 491-504

30. Abi-Rached L, Kuhl H, Roos C, ten Hallers B, Zhu B, Carbone L, de Jong PJ, et al. 2010. A small, variable, and irregular killer cell Ig-like receptor locus accompanies the absence of MHC-C and MHC-G in gibbons. *J Immunol* 184: 1379-91

31. Daza-Vamenta R, Glusman G, Rowen L, Guthrie B, Geraghty DE. 2004. Genetic divergence of the rhesus macaque major histocompatibility complex. *Genome Res* 14: 1501-15

32. Otting N, Heijmans CMC, Noort RC, de Groot NG, Doxiadis GGM, van Rood JJ, Watkins DI, et al. 2005. Unparalleled complexity of the MHC class I region in rhesus macaques. *Proc Natl Acad Sci USA* 102: 1626-31

33. Hershberger KL, Shyam R, Miura A, Letvin NL. 2001. Diversity of the killer cell Ig-like receptors of rhesus monkeys. *J Immunol* 166: 4380-90

34. Grendell RL, Hughes AL, Golos TG. 2001. Cloning of rhesus monkey killer-cell Ig-like receptors (KIRs) from early pregnancy decidua. *Tissue Antigens* 58: 329-34

35. Sambrook JG, Bashirova A, Palmer S, Sims S, Trowsdale J, Abi-Rached L, Parham P, et al. 2005. Single haplotype analysis demonstrates rapid evolution of the killer immunoglobulin-like receptor (KIR) loci in primates. *Genome Res* 15: 25-35

36. Andersen H, Rossio JL, Coalter V, Poore B, Martin MP, Carrington M, Lifson JD. 2004. Characterization of rhesus macaque natural killer activity against a rhesus-derived target cell line at the single-cell level. *Cell Immunol* 231: 85-95

37. Bostik P, Kobkitjaroen J, Tang W, Villinger F, Pereira LE, Little DM, Stephenson ST, et al. 2009. Decreased NK cell frequency and function is associated with increased risk of KIR3DL allele polymorphism in simian immunodeficiency virus-infected rhesus macaques with high viral loads. *J Immunol* 182: 3638-49

38. Robinson J, Mistry K, McWilliam H, Lopez R, Marsh SGE. 2010. IPD--the Immuno Polymorphism Database. *Nucleic Acids Res* 38: D863-9

39. Trundley AE, Hiby SE, Chang C, Sharkey AM, Santourlidis S, Uhrberg M, Trowsdale J, et al. 2006. Molecular characterization of KIR3DL3. *Immunogenetics* 57: 904-16

40. Abi-Rached L, Moesta AK, Rajalingam R, Guethlein LA, Parham P. 2010. Human-specific evolution and adaptation led to major qualitative differences in the variable receptors of human and chimpanzee natural killer cells. *PLoS Genet* 6: e1001192

41. Older Aguilar AM, Guethlein LA, Adams EJ, Abi-Rached L, Moesta AK, Parham P. 2010. Coevolution of killer cell Ig-like receptors with HLA-C to become the major variable regulators of human NK cells. *J Immunol* 185: 4238-51

42. Rosner C, Kruse PH, Hermes M, Otto N, Walter L. 2011. Rhesus macaque inhibitory and activating KIR3D interact with Mamu-A-encoded ligands. *J Immunol* 186: 2156-63

43. Colantonio AD, Bimber BN, Neidermyer WJ, Reeves RK, Alter G, Altfeld M, Johnson RP, et al. 2011. KIR Polymorphisms Modulate Peptide-Dependent Binding to an MHC Class I Ligand with a Bw6 Motif. *PLoS Pathog* 7: e1001316

44. Traherne JA, Martin M, Ward R, Ohashi M, Pellett F, Gladman D, Middleton D, et al. 2010. Mechanisms of copy number variation and hybrid gene formation in the KIR immune gene complex. *Hum Mol Genet* 19: 737-51

45. Pyo C-W, Guethlein LA, Vu Q, Wang R, Abi-Rached L, Norman PJ, Marsh SGE, et al. 2010. Different patterns of evolution in the centromeric and telomeric regions of group A and B haplotypes of the human killer cell Ig-like receptor locus. *PLoS ONE* 5: e15115

46. Parham P. 2005. MHC class I molecules and kirs in human history, health and survival. *Nat Rev Immunol* 5: 201-14

ABBREVIATIONS

AIDS	aquired immunodeficiency syndrome
APC	antigen-presenting cell
Asn	asparagine
BAC	bacterial artifical chromosome
BAT	HLA-B associated transcript
BPRC	Biomedical Primate Research Centre
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CEACAM	carcinoembryonic antigen-related cell adhesion molecule
CMV	cytomegalovirus
CRTAM	class I-restricted T-cell-associated molecule
Cs	Chlorocebus sabaeus
Ct	cycle threshold
CTL	cytotoxic T-lymphocyte
CYT	cytoplasmic
DAP	DNAX associated protein
DC	dendritic cell
DMSO	dimethylsulfoxide
dNTP	deoxyribonucleotide triphosphate
DNA	deoxyribonucleic acid
DNAM	DNAX accessory molecule
FASL	Fas ligand
FCAR	Fc fragment of IgA receptor
FceR	Fc fragment of IgE receptor
Fcg	Fc fragment of IgG
gDNA	genomic deoxyribonucleic acid
Gogo, Gg	Gorilla gorilla
GPVI	glycoprotein 6
HEK	human enbryonic kidney cell line
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
Hs	Homo sapiens
Ig	immunoglobulin
IL	interleukin
ILT	immunoglobulin-like transcript
IPD	immuno polymorphism database
ITAM	immunoreceptor tyrosine-bases activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif

JTT	Jones Taylor Thornton
K562	human erythroleukemia cell line
kD	kiloDalton
KIR	killer cell immunoglobulin-like receptor
KLRG	killer cell lectin-like receptor G
LAIR	leukocyte-associated immunoglobulin-like receptor
LIR, LILR	leukocyte immunoglobulin-like receptor
LLT	lectin like transcript
LRC	leukocyte receptor complex
Lys	lysine
Mamu, Mm	Macaca mulatta
MHC	major histocompatibility complex
M-MuLV	moloney murine leukemia virus
mRNA	messenger ribonucleic acid
MtDNA	mitochondrial deoxyribonucleic acid
MYA	millions years ago
NCR	natural cytotoxicity receptor
NECL	nectin-like protein
NK	natural killer
nkat	natural killer-associated transcript
NKG	natural killer group
NKR-P	natural killer cell receptor protein
Nole	Nomascus leucogenys
NTB-A	NK-T-B-antigen
Patr, Pt	Pan troglodytes
PBMC	peripheral blood mononuclear cell
PCR	polymerase chainreaction
PLGF	placental growth factor
Popy	Pongo pygmaeus
PSGL	p-selectin glycoprotein ligand
RNA	ribonucleic acid
Ser	serine
SIGLEC	sialic acid binding Ig-like lectin
SIV	simian immunodeficiency virus
SLAM	signaling lymphocyte activation molecule
SNP	single nucleotide polymorphism
SPF	specific pathogen free
TRAIL	tumor necrosis factor-related apoptosis inducing ligand
TM	transmembrane
VEGF	vascular endothelial growth factor
YFP	yellow fluorescent protein

SUMMARY

All nucleated cells in most vertebrate species investigated express Major Histocompatibility Complex (MHC) class I proteins. These proteins continuously present small peptides – derived from intracellularly degraded proteins – on the cell's outer membrane. Under particular conditions a cell may present an altered peptide repertoire; for instance, if it has been infected by a pathogen or has become malignant. Effector T-cells are specifically educated to discern between the peptide repertoire presented in a normal or an abberant state. In this manner, malignant or infected cells are targeted by the immune system and are subsequently eradicated. If an infected or malignant cell can prevent T-cell recognition, it may escape killing, and, as such, this provides a selective advantage. Therefore, in many pathogens mechanisms have evolved which either interfere with the expression of MHC class I molecules or the peptide presentation pathway itself. To guard against this form of immune evasion, a specialised set of effector cells, referred to as natural killer (NK) cells, scan with their receptors for the presence or absence of MHC class I cell surface expression. The family of NK cell receptors that is particularly important for this type of recognition are the killer cell immunoglobulin-like receptors (KIR). In essence these receptors, which are encoded by *KIR* genes, can be divided into ones with activating or inhibitory functions. In humans, the *KIR* genes are characterised by their allelic variation (polymorphism) and diversity (copy number variation). Therefore, individuals will usually differ in their KIR repertoire, which is reflected as differences of NK cell function.

The aim of this thesis is to characterise the complexity of the KIR gene system in rhesus macaques. Humans and rhesus macaques share a common ancestor that lived approximately 25 million years ago. Despite their evolutionary distance, however, many functional characteristics of important immune-related genes are shared by both species.

This thesis starts with an analysis of the *KIR2DL4* gene, which appears to be highly conserved in primates. In rhesus macaques, evidence was found that two types of selection have acted on this gene. First, the ligand binding domain of KIR2DL4 is highly conserved, suggesting purifying selection. Second, the signaling domains display polymorphism. Two forms are abundantly present in the cohort studied, suggesting that, as in humans, balancing selection is acting on different allotypes **(Chapter 2)**. Subsequently, activating *KIR* genes were investigated. Abundant allelic variation was observed, and phylogenetic analysis made it possible to define gene groups. Moreover, activating *KIR* genes may be generated *de novo* by splice-site mutations, a mechanism that in the KIR system appears to be uniquely reserved for Old World monkeys. Additionally, one activating gene group is characterised by an exon deletion that would yield a truncated gene product. At least two types of activating *KIR* genes have been generated independently of each other during rhesus macaque evo-

lution. Therefore, the hypothesis is put forward that directional selection has acted on its formation. Comparative analyses of the ligand binding domains show that new genes have been formed by exon shuffling, which together with point mutations has led to a further diversification of activating *KIR* **(Chapter 3)**.

To put the observed polymorphism into the context of haplotypic diversity, KIR transcripts were sequenced from a cohort of pedigreed animals. Based on their allelic variation, a robust distinction in gene groups was made, and the gene content of haplotypes was determined by segregation analysis. Because a relatively small number of animals were studied, the observed mosaic-like haplotype patterns suggest that an enormous level of complexity exists within the total rhesus macaque population, which at the very least might rival the situation in humans **(Chapter 4)**.

Subsequently, a large cohort of rhesus macaques originating from different geographical areas was examined. As expected a myriad of genotypes was observed, in which KIR genes may be absent or present. The exception to the rule may be *Mamu-KIR3DL20*, which seems to be present on all haplotypes and behaves like a framework gene. The plasticity of KIR haplotypes is illustrated by the fact that haplotypes with or without members of a particular gene group exist. Such plasticity can be explained if recombination is possible between all loci, and if diversifying selection is acting on haplotype content, which is different from the human situation in which balancing selection seems to have acted on haplotype composition **(Chapter 5)**.

The MHC class I gene repertoire has been expanded in Old World monkeys. There is evidence that this expansion also had a certain impact on the KIR gene repertoire and may have propelled the expansion of particular KIR gene groups **(Chapter 6)**. In conclusion, this thesis describes the evolution and complexity of the KIR gene system in rhesus macaques as compared to humans, and it presents evidence that this system has been subject to different types of selection, some of these are most likely due to co-evolution with an expanded MHC class I repertoire.

SAMENVATTING

In het meerendeel van de gewervelde dieren, waar tot op heden onderzoek naar is gedaan, brengen alle kernhoudende cellen klasse I eiwitten tot expressie die behoren tot het Major Histocompatibility Complex (MHC). Deze eiwitten bieden op de celmembraan voortdurend kleine peptiden aan, die zijn verkregen uit intracellulair afgebroken eiwitten. Onder bepaalde omstandigheden kan een cel een veranderd repertoire aan peptiden presenteren, bijvoorbeeld als deze is geïnfecteerd met een ziekteverwekker of kwaadaardig is geworden. Op basis van dit veranderde peptiderepertoire kunnen effector T-cellen onderscheid maken tussen gezonde en niet-gezonde cellen. Geïnfecteerde en kwaadaardige cellen worden op deze manier door het immuunsysteem herkend en vervolgens geëlimineerd. Wanneer een geïnfecteerde cel of een tumorcel herkenning door T-cellen weet te voorkomen, kan dit een selectievoordeel opleveren. Veel ziekteverwekkende organismen hebben daarom methoden ontwikkeld die de expressie van de MHC klasse I moleculen verstoren of de totstandkoming van de peptide-presentatie hinderen. De zogenoemde "natural killer" (NK) cellen waken ervoor dat door een dergelijk mechanisme ontsnapping aan het immuunsysteem kan plaatsvinden. Deze gespecialiseerde effector-cellen gebruiken specifieke receptoren om het celoppervlak te controleren op de aan- en afwezigheid van MHC klasse I eitwitten. De receptorfamilie van de "killer cell immunoglobulin-like receptors" (KIR) is hierbij van essentieel belang. Deze receptoren, die door *KIR* genen worden gecodeerd, kunnen onderverdeeld worden in eiwitten met een activerende of inhiberende functie. In de mens kenmerken *KIR* genen zich door hun allelische variatie (polymorfisme) en diversiteit (variatie in aantal kopieën). Zodoende zullen individuen dikwijls een verschillend KIR repertoire hebben, wat zich uit als een verschil in NK cel-functie.

De doelstelling van dit proefschrift is om de complexiteit van het KIR systeem in rhesusmakaken in kaart te brengen. Mensen en rhesusapen delen een gemeenschappelijke voorouder die ongeveer 25 miljoen jaar geleden leefde. Ondanks deze evolutionaire afstand worden veel functionele eigenschappen van belangrijke immuungenen door beide soorten gedeeld.

Dit proefschrift begint met een analyse van het *KIR2DL4* gen, dat zeer geconserveerd lijkt te zijn in primaten. In de rhesusmakaak zijn aanwijzingen gevonden dat dit gen onderhevig is aan twee vormen van selectie. Allereerst is het deel van 2DL4 dat liganden bindt zeer geconserveerd gebleven gedurende de evolutie. Ten tweede is er polymorfisme aanwezig in het gedeelte dat signalen doorgeeft in de cel. Hiervan zijn in een onderzochte groep dieren twee verschillende vormen volop aanwezig. Dit lijkt erop te duiden dat in de rhesusaap, net zoals in de mens, selectie heeft plaatsgevonden om een evenwicht te behouden tussen deze verschillende allotypen (**Hoofdstuk 2**).

Vervolgens werden de activerende *KIR* genen onderzocht. Een aanzienlijke hoeveelheid allelen werd gedetecteerd en met behulp van fylogenetische studies konden gengroepen worden gedefinieerd. Bovendien bleek dat activerende *KIR* genen in rhesusapen *de novo* zijn gevormd door mutaties in zogenaamde "splice-sites"; een mechanisme dat uniek voorbehouden lijkt te zijn aan Oude Wereld apen. Daarnaast kenmerkt één groep van activerende genen zich door een mutatie in een exon waardoor het genproduct wordt verkort. Tijdens de evolutie van de rhesusmakaak zijn ten minste twee soorten activerende *KIR* genen onafhankelijk van elkaar gevormd. Dit heeft geleid tot de hypothese dat directionele selectie op hun vorming van invloed is geweest. Vergelijkend onderzoek van de ligand-bindende domeinen toont dat door het uitwisselen van exonen nieuwe vormen van genen zijn gevormd. Samen met puntmutaties heeft dit geleid tot een verdere diversiteit aan activerende KIR (**Hoofdstuk 3**).

Om het waargenomen polymorfisme in de context van haplotype-diversiteit te plaatsen, werden KIR transcripten van een groep verwante dieren geanalyseerd. Op basis van allelische variatie kon een solide onderscheid in gengroepen worden gemaakt. Aan de hand van overervingspatronen in deze groepen werd vervolgens de samenstelling van verschillende haplotypen bepaald. Omdat dit onderzoek zich richtte op een relatief kleine groep individuen, kan op basis van de gevonden mozaïekachtige haplotype-patronen de verwachting uitgesproken worden dat in de gehele rhesusaap populatie, net als in de mens, een hoog niveau van complexiteit aanwezig is (**Hoofdstuk 4**).

Hierop aansluitend is een uitgebreid cohort van rhesusmakaken onderzocht, afkomstig uit verschillende geografische regio's. Geheel in lijn der verwachting werd een groot aantal genotypen waargenomen met aan- en afwezigheid van verschillende *KIR* genen. Een uitzondering hierop is waarschijnlijk het *Mamu-KIR3DL20* gen, dat tot dusver aanwezig is in alle haplotypen, en zich gedraagt als een "raamwerkgen". De plasticiteit (vormbaarheid) van het KIR gensysteem wordt verduidelijkt door het feit dat haplotypen inhoudelijk kunnen verschillen. Voor het meerendeel van KIR gengroepen geldt namelijk dat in een haplotype hun genen aan- of afwezig kunnen zijn. Dergelijke plasticiteit kan worden verklaard als tussen alle *KIR* genen recombinatie mogelijk is (**Hoofdstuk 5**).

Het MHC klasse I repertoire is in Oude Wereld apen aan expansie onderhevig gebleken. Er zijn aanwijzingen dat deze uitbreiding van invloed is geweest op het KIR repertoire en zelfs aan de basis heeft gelegen van de duplicatie van bepaalde KIR gengroepen (**Hoofdstuk 6**). Tot slot beschrijft dit proefschrift de evolutie en complexiteit van het KIR gensysteem van rhesusmakaken in vergelijking met mensen. Tevens levert het aanwijzingen dat dit gensysteem onderhevig is geweest aan verschillende vormen van selectiedruk.

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Ik hou van je.

LIST OF PUBLICATIONS

1. **J.H. Blokhuis**, M.K. van der Wiel, G.G.M. Doxiadis, R.E. Bontrop. The extreme plasticity of killer cell Ig-like receptor (KIR) haplotypes differentiates rhesus macaques from humans. European Journal of Immunology, *in press*, 2011.

2. S. A. Jagessar, N. Heijmans, E.L.A. Blezer, J. Bauer, **J.H. Blokhuis**, J.A.M. Wubben, J.W. Drijfhout, P.J. van den Elsen, J.D. Laman, B. A. 't Hart. Fine specificity and MHC restriction of CD4+CD8+CD56+ cytotoxic T cells mediating white and grey matter demyelination in the marmoset EAE model. *Submitted for publication, 2011.*

3. G.G.M. Doxiadis, N. de Groot, N. Otting, **J.H. Blokhuis**, R.E. Bontrop. Genomic plasticity of the MHC class I A region in rhesus macaques: extensive haplotype diversity at the population level as revealed by microsatellites. Immunogenetics 63 (2), 73-83, 2011.

4. **J.H.Blokhuis**, M.K. van der Wiel, G.G.M. Doxiadis, R.E. Bontrop. The mosaic of KIR haplotypes in rhesus macaques. Immunogenetics 62 (5), 295-306, 2010.

5. **J.H. Blokhuis**, M.K. van der Wiel, G.G.M. Doxiadis, R.E. Bontrop. Evidence for balancing selection acting on KIR2DL4 genotypes in rhesus macaques of Indian origin. Immunogenetics 61 (7), 503-512, 2009.

6. **J.H. Blokhuis**, G.G.M. Doxiadis, R.E. Bontrop. A splice site mutation converts an inhibitory killer cell Ig-like receptor into an activating one. Molecular Immunology 46, 640-648, 2009.

7. C.C. Posthuma, D.D. Nedialkova, J.C. Zevenhoven-Dobbe, **J.H. Blokhuis**, A.E. Gorbalenya, E.J. Snijder. Site-directed mutagenesis of the Nidovirus replicative endoribonuclease NendoU exerts pleiotrophic effects on the arterivirus life cycle. Journal of Virology 80 (4), 1653-61, 2006.

CURRICULUM VITAE

Jeroen Harry Blokhuis werd geboren op 3 oktober 1979 te Den Haag. Vanaf 1991 volgde hij middelbaar onderwijs aan het Christelijk Gymnasium Sorghvliet te Den Haag, waar hij op 10 juni 1997 zijn VWO-diploma behaalde. Aansluitend ging hij Biologie studeren aan de Universiteit Leiden. Het propaedeutisch examen werd op 28 augustus 1998 behaald. In 1999 werd als eerste specialisatie moleculaire biologie gekozen, en in 2000 een tweede specialisatie in de medische biologie. Zijn hoofdtentamen en stage deed hij op het gebied van de moleculaire virologie van RNA virussen onder begeleiding van Dr. C. Posthuma en Dr. E. Snijder aan de vakgroep Medische Microbiologie van het Leiden Universitair Medisch Centrum. Op 12 december 2002 studeerde hij af in de Biologie met als specialisatie Medische Biologie. Van 2003 tot 2005 was hij als freelancer werkzaam bij het Nederlandsch Octrooibureau te Den Haag. Op 1 mei 2005 startte hij zijn promotie onderzoek in de immunogenetica bij de afdeling Comparative Genetics and Refinement van het BPRC.

Jeroen Harry Blokhuis was born on October 3, 1979 in The Hague. He started his preuniversity education in 1991 at the Christelijk Gymnasium Sorghvliet in The Hague and graduated on June 10, 1997. He started his studies in Biology at Leiden University, and obtained his bachelors on August 28, 1998. As first specialisation he choose molecular biology in 1999, and medical biology in 2000. His masters thesis and practical training was performed on the subject of molecular virology of RNA viruses under supervision of Dr. C. Posthuma and Dr. E. Snijder in the department of Medical Microbiology in the Leiden University Medical Centre. With specialisation Medical Biology the title M.Sc. in Biology was obtained on December 12, 2002. From 2003 to 2005 he worked as a freelancer for a patent office (Nederlandsch Octrooibureau) in The Hague. On May 1, 2005 he started his PhD project on the topic of immunogenetics that was performed at the department of Comparative Genetics and Refinement of the BPRC.