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

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NK cells are essential in shaping immune responses and play an important role during pregnancy and in controlling infections. Killer cell immunoglobulin-like receptors (KIRs) 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 in the rhesus macaque, 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.

Key words: Comparative immunology · Evolution · KIR · NK cells · Rhesus macaque

Supporting Information available online

Introduction

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 (KIRs) may influence this balance through interactions with their ligands, the MHC molecules, which are called human leukocyte antigens

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(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.

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 (Supporting Information Table 1). 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.

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.

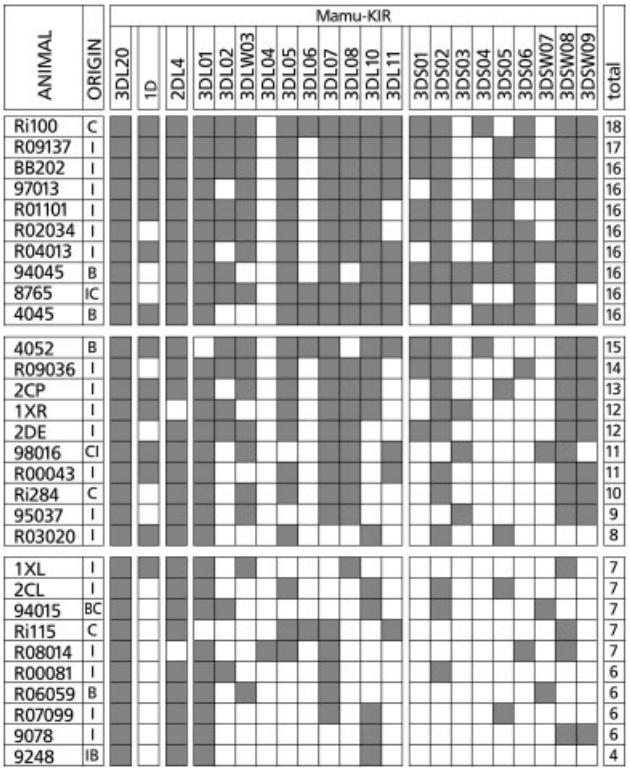

Figure 1. KIR genotypes in a panel of rhesus macaques. Depicted for each animal is its origin, the presence (*filled*) or absence (*empty*) of different Mamu-KIR loci, and the total number of loci present on its genotype. The panel is divided into three groups; at the top are the ten animals with the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

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.

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

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

♯ loci	♯ animals	Average I:A
4	1	No activ.
6	4	2.5
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	1.5

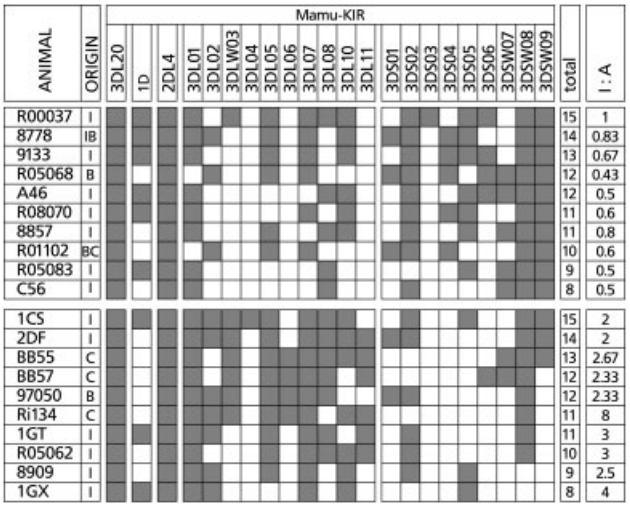

Figure 2. Extremes in inhibitory and activating genotypes. Depicted for each animal is its origin, the presence (*filled*) or absence (*empty*) of different *Mamu-KIR* loci, the total number of loci present on its genotype, and the ratio (I:A) between inhibitory and activating KIRs. The panel is divided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

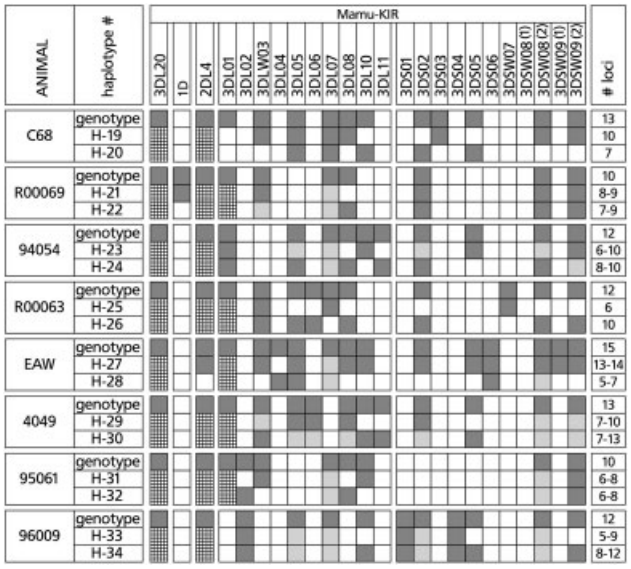

Figure 3. Plasticity of haplotypes. Depicted for each animal is the KIR content of its genotype as well as haplotypes. The presence (*dark-grey*) or absence (*empty*) of different *Mamu-KIR* loci is shown. A cell is filled *light-grey* when segregation analysis was inconclusive for this particular locus, and depicted as a *mesh* when it is highly likely that 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.

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-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. 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 with 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 a 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 display highly significant differences in distribution between populations.

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

Origin	♯ animals	Av. ♯ loci	Av. ♯ inhibitory	Av. ♯ activating	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 populations as determined by a Welch's *t*-test.

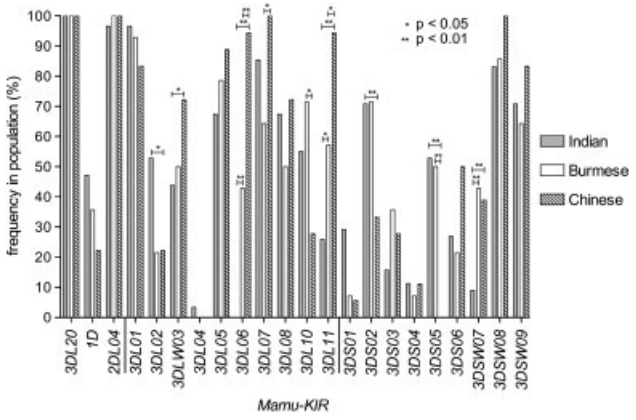

Figure 4. Frequencies of different *Mamu-KIR* genes observed in parental animals that originate in India, Burma, and China. On the x-axis, the three centromeric loci (*Mamu-KIR3DL20*, *-KIR1D*, and *-KIR2DL04*) are depicted, followed by ten inhibitory loci (*Mamu-KIR3DL*) and nine activating loci (*Mamu-KIR3DS*). The *y*-axis depicts the frequencies of these loci as observed in different populations. Statistical significant differences between populations are shown. *$p<0.05$; **$p<0.01$; as determined by a Yates' chi-square test with one degree of freedom.

First, *KIR3DS05* was not observed in Chinese animals. Second, *KIR3DSW07* is more frequent in Chinese and Burmese animals as compared with Indian animals. Finally, *KIR3DS02* is present more than twice in Burmese and Indian animals as compared with Chinese animals. Although *KIR3DS01* appears to be enriched in Indian animals, this is below the threshold value for significance.

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 nonhuman 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].

Table 3. KIR genotype statistics of humans and rhesus macaques

	Human[a)]	Rhesus macaque
Populations	108	3
Individuals	12741	378
KIR genotypes	396	272

[a)] Information derived from www.allelefrequencies.net.

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). 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 undoubtedly 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 the telomeric lineage II genes collapsed, leaving no room for telomeric variability [46]. In humans, allocation of lineage II and III genes continued both centromerically and telomerically, and at one 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. 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 (Figs. 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 division 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 rapid differential evolution has occurred. Notably, origin-specific allelic variation of MHC class I is also observed between these three populations [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 number 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 as within a population. For instance because the MHC class I expression is hampered under certain pathogenic conditions 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, in the population it will be confronted by even more diversity. As such, this extreme KIR diversity may provide an effective strategy for surviving epidemic infections.

Materials and methods

Animals and cells

The composition of a cohort of 378 rhesus macaques is depicted (Supporting Information Fig. 1). The cohort contains animals that are of either Indian, Burmese, Chinese, or mixed origin, as well as their offspring. BPRC housing and animal care procedures are in compliance with Dutch law on animal experiments and European directive 86/609/EEC. For the purpose of this particular study, whole blood was obtained during regular health checks. Peripheral blood mononuclear cells (PBMCs) 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 (Supporting Information Table 2). 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 *-KIR2DL02* was designed, which however does not cover all alleles. For *Mamu-3DL07* and *-3DL08* several alleles could also 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 (Supporting Information Table 3).

Quantitative PCR and melt curve analysis

To perform quantitative PCR, a 20 μL reaction was prepared containing: 1× PCR buffer, 1× 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 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 in triplicate 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's *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.

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Abbreviation: **KIR:** killer cell immunoglobulin-like receptor

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