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Mhc class I haplotypes associated with survival time in simian immunodeficiency virus (SIV)-infected rhesus macaques

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In both human immunodeficiency virus-infected humans and simian immunodeficiency virus (SIV)-infected macaques, genes encoded in the major histocompatibility complex (MHC) class I region are important determinants of disease progression. However, compared to the human human lymphocyte antigen complex, the macaque MHC region encodes many more class I genes. Macaques with the same immunodominant class I genes express additional Mhc genes with the potential to influence the disease course. We therefore assessed the association between of the Mhc class I haplotypes, rather than single gene variants, and survival time in SIV-infected rhesus macaques (Macaca mulatta). DNA sequence analysis and Mhc genotyping of 245 pedigreed monkeys identified 17 Mhc class I haplotypes that constitute 10 major genotypes. Among 81 vaccination-naive, SIV-infected macaques, 71 monkeys carried at least one Mhc class I haplotype encoding only MHC antigens that were incapable of inducing an effective anti-SIV cytotoxic T lymphocytes response. Study of these macaques enabled us to relate individual Mhc class I haplotypes to slow, medium and rapid disease progression. In a post hoc analysis, classification according to disease progression was found to explain at least 48% of the observed variation of survival time.
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Introduction

Simian immunodeficiency virus (SIV)-infected rhesus macaques represent the primary animal model for human immunodeficiency virus (HIV) infection.[1] After infection with SIV, rhesus macaques develop symptoms of immunodeficiency that are remarkably similar to those of HIV-infected humans. The course of disease varies between infected monkeys as much as between HIV-infected humans. Even among animals infected with the same virus and dose, the time until onset of AIDS-defining symptoms varies between 7 weeks and 8 years post infection, indicating that host factors play a major role in determining disease progression.

The highly polymorphic *Mhc* genes represent the most important host factor influencing disease progression in HIV-infected patients.[1,2] Major histocompatibility complex (MHC) class I antigens that present foreign peptides can activate cytotoxic T lymphocytes (CTL), which are crucial for containing viral replication. Several studies have shown (1) a temporal relationship between the decline of viral load and the emergence of CTL responses in both infected humans and infected monkeys,[3–6] (2) an increase of viral load after the appearance of CTL-escape HIV/SIV mutants during chronic and acute infection[7–13] and (3) a dramatic rise of viral load after $CD8^+$ T-cell depletion.[14–16] Additional indirect evidence for a CTL effect upon viral replication came from genetic association studies showing that *Mhc* class I alleles are strongly associated with survival time in HIV-infected humans [17–20].

The macaque *Mhc* region differs from the human lymphocyte antigen (HLA) complex by a larger number of *Mhc* class I genes.[21,22] Even *Mhc* homozygous macaques may carry two or three expressed *A* genes, and even up to 10 expressed *B* genes.[23,24] In contrast, only one *A* and one *B* gene exist in the human *HLA* class I region. This means that macaques sharing one *Mhc* allele triggering an immunodominant CTL response still

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express several other *Mhc* sequences. These other MHC molecules may also influence the disease course by inducing subdominant CTL responses, or by modulating killer immunoglobulin receptor (KIR)–MHC interactions. Several studies have shown that single MHC antigens such as *Mamu*-A*01 and -B*17 are significantly associated with prolonged survival in SIV-infected rhesus macaques.[13,25–27] However, monkeys sharing one or two of these MHC antigens may still exhibit a variable course of disease.[27,28]

We therefore assessed the association between *Mhc* class I haplotypes, rather than single gene variants, and survival time in SIV-infected rhesus macaques. In order to obtain detailed information on the *Mhc* class I haplotypes present in the German Primate Center (GPC) breeding colony, the monkeys which were of Indian descent, we first performed a low-resolution *Mhc* typing on monkeys derived from three generations. For high-resolution *Mhc* typing, we developed typing techniques for additional *Mhc* class I loci and retyped a cohort of 245 monkeys, which comprised the SIV-infected monkeys as well as their ancestors and siblings. Almost all SIV-infected monkeys in our study cohort were found to carry at least one *Mhc* class I haplotype that was associated with rapid disease progression and was thus incapable of inducing a strong anti-SIV CTL response. Functionally, the SIV-infected monkeys were therefore 'quasi-hemizygous' which allowed us to investigate the contribution of the second haplotype to disease progression. Since the genealogies of the monkeys were known, we could also control for the potential familial influence of other host genes. We identified haplotypes of the *Mhc* class I region clearly associated with a rapid and slow disease course, and configurations that were characterized by a particularly large variation in survival time.

Results

Mhc class I haplotypes

Initially, monkeys of Indian descent from three generations housed at the GPC were typed for *DQB1* and for 15 *Mhc* class I sequences or allele lineages, employing previously published techniques.[25,29,30] Segregation analysis revealed 19 *DQB1-Mhc* class I haplotypes. In order to obtain *Mhc* class I cDNA sequences of each haplotype, we subcloned reverse transcription (RT)–PCR products from 18 animals and sequenced approximately 1600 inserts. Transcribed *Mhc* class I sequences of the most frequent haplotypes were thus obtained from at least two unrelated animals. Sequence analysis revealed the presence of 45 distinct class I alleles. All except eight alleles had been published before.[24] Three of the novel alleles were highly similar to previously published sequences, namely *Mamu-A1*2102*, *-B*4503* and *-B*3003*.

The alleles *Mamu-B*7401* and *-B*7402* differed from each other by a nonsynonymous mutation in the cytoplasmic region, and both diverge by either one or two nucleotides from an annotated rhesus macaque genomic DNA sequence (GenBank accession no. XR014617). Alternatively spliced transcripts of *Mamu-B*74* were detected in seven individuals. They differ from *Mafa-B*390101* (GenBank accession no. AY969136[31]) by only one or two nucleotides. The splice variants are nonfunctional as they contain a frame shift mutation at the beginning of exon 3 due to a deletion of 74 bp. In two monkeys, both the spliced and the unspliced variant of either *Mamu-B*7401* or *-B*7402* were found. *Mamu-B*74* therefore seems to have been conserved, but may not represent a classical *B* gene. Since genotyping revealed the presence of *Mamu-B*74* in almost all animals, we did not investigate its association with disease progression or with specific *Mhc* class I haplotypes any further.

For additional genotyping purposes, 23 novel primer pairs were created which were sufficient to yield reliable results in our modestly diverse study population. A total of 245 animals, comprising the SIV-infected monkeys as well as their ancestors and siblings, were finally typed for 37 class I sequences or loci using the previously established and the novel primers. The genotyping results revealed that the initially assigned haplotypes were by and large correct. However, two haplotypes segregated into two and four related configurations. In total, 17 *Mhc* class I haplotypes encompassing the *A* and *B* regions were deduced and their frequencies were determined (Tables 1 and 2). They consisted of seven individual *A* and 14 *B* haplotypes. The number of expressed *A* genes varied between two and three. Notably, *Mamu-A*1403* was not present in all haplotypes, as was reported for other rhesus macaques of Indian origin.[24] The number of expressed *B* genes varied between two and eight per haplotype. As many as 18 transcribed class I sequences were observed in some heterozygous monkeys. Since we may not have detected all transcribed genes, the true number may have been even larger.

In 200 investigated descendants, two potential recombination events were observed between the *A* and *B* region, but none within the *B* region. This means that we did not observe an enhanced recombination rate in the *Mhc* class I region.

Haplotypes significantly associated with rapid disease progression

Four haplotypes (1.1–1.4; Table 2) were very similar in that they differed only by the presence or absence of one *B* gene. We combined them into a single group (group 1) because it was impossible to define the particular haplotypes, not only in monkeys with two different group 1 haplotypes, but also in other heterozygotes whose second haplotype carried *Mamu-B*38* or *-B*46*. The 11 monkeys carrying only group 1 configurations were all euthanized within 31 weeks post infection (w.p.i.) with symptoms of AIDS and had a mean survival time of 20.5 w.p.i. (s.d. 6.5 w.p.i.; Table 3; Figure 1a). Among them, the eight nonimmunized monkeys had an average survival time of 18.0 w.p.i. Thus, the group 1 configurations were significantly associated with rapid disease progression ($P = 0.0003$).

For analytical reasons, we also combined all haplotypes containing members of *Mamu-B*30*, *-B*07* loci/lineages and *Mamu-B*01* into group 2 (2.1, 2.2), regardless of which other genes were present, except *Mamu-A*02*. The six monkeys carrying both a group 1 and a group 2 haplotype also had to be euthanized with symptoms of AIDS soon after SIV infection, with a mean survival time of 18.0 w.p.i. (s.d. 5.1 w.p.i.; Figure 1b; Table 3). Group 2 configurations were thus also associated with rapid disease progression ($P = 0.001$).

Table 1 Primers used for genotyping

Mhc *sequence*	*Forward/reverse primer*	*Anneal* (°C)	*Specific primer* (μM)	*Internal control*	*Internal control* (μM)
A2*05	AAAACGACGGCCAGTGACCAGAACACACGGATCTG/ GAAACAGCTATGACCCCTCCAGGTAGGCTCTCCA	62	0.8	DRB	0.12
A1*21	AAAACGACGGCCAGTCGGAACACACGGATCTACA/ GAAACAGCTATGACCGTCCTCGTTCAGGGCGAA	60	0.8	Actin	0.08
A2*24	AAAACGACGGCCAGTACAGACTCTCCGAGCGGA/ CAGGAAACAGCTATGACCGTCGTAGGCGTACTGGTA	62	1.0	DRB	0.08
B*21	AAAACGACGGCCAGTAACACACGGATCTCCAAGGC/ GAAACAGCTATGACCAGCGACTCCATGCACGCC	62	0.8	DRB	0.08
B*22	AAAACGACGGCCAGTCCGAGGGAACCTGAGGACA/ GAAACAGCTATGACCCTGCTGCTCTGCCTCCG	62	0.8	DRB	0.08
B*26	AAAACGACGGCCAGTGTCTCACACCCTCCAGAC/ GAAACAGCTATGACCCCTCATGGTCAGAGACGGA	64	0.72	DRB	0.1
B*27	AAAACGACGGCCAGTGGCCCACGCACAGAGTCA/ CAGGAAACAGCTATGACCGTCGTAGGCGTACTGGTC	62	0.8	Actin	0.08
B*28	AAAACGACGGCCAGTGGAAGACGTGACACGGAGA/ GAAACAGCTATGACCCAGGTCGCAACCGTACAA	62	0.8	Actin	0.08
B*29	AAAACGACGGCCAGTGCCGGGAACCCTGGTATG/ GAAACAGCTATGACCGAACCGCTCCGCATAACGGT	61	0.8	DRB	0.08
B*30	AAAACGACGGCCAGTGAGGCCCACGCACAGAATC/ GAAACAGCTATGACCCTTGCCGTCGTAGGCATA	62	1	Actin	0.1
B*38	AAAACGACGGCCAGTGACCGGAACACACGGAGA/ GAAACAGCTATGACCTTCAGGGTGTATAATCCTTA	62	1	DRB	0.08
B*43	AAAACGACGGCCAGTATGAGGTATTTCAGCACTGCA/ GAAACAGCTATGACCCTTCGCTGCTCCGCCTCG	62	0.8	DRB	0.08
B*45	AAAACGACGGCCAGTCACGCAGTTCGTGCGGTA/ GAAACAGCTATGACCCCAGGTAGGCTCTCTGCC	60	0.8	DRB	0.08
B*46	AAAACGACGGCCAGTACAGGGCTCGCCAAGGAG/ GAAACAGCTATGACCCTCCACTGCTCTGCCTCG	62	0.8	DRB	0.08
B*47	AAAACGACGGCCAGTCACGGAAAGCAACGGCCCA/ GAAACAGCTATGACCTCGTAGGCGTCCTGCCGG	60	0.8	DRB	0.08
B*48	AAAACGACGGCCAGTACGGATCGCCAAGGACGC/ GAAACAGCTATGACCCAGCCATACATCCACTGGTA	62	0.8	Actin	0.08
B*49	AAAACGACGGCCAGTGAGACACAGAAAGCCAAGGA/ GAAACAGCTATGACCCTGAGCCGCCTCATCTGCA	60	0.8	DRB	0.08
B*57	AAAACGACGGCCAGTTCCGAGAGAGGAGCCGCA/ GAAACAGCTATGACCGGGGTGGTGGATCACATG	62	0.8	DRB	0.08
B*65	AAAACGACGGCCAGTGAGCCCTGGCATCTCGAA/ GAAACAGCTATGACCCTCCACTGCTCCGCCTCA	62	0.8	DRB	0.08
B*68	AAAACGACGGCCAGTCCGGGAACCCTGGTATG/ GAAACAGCTATGACCCTCCACTGCTCTGCCTCG	60	1	DRB	0.08
B*6901	AAAACGACGGCCAGTACTCCATGAGGTATTTCACC/ GAAACAGCTATGACCAGCCAGACATCCTCTGGTA	60	0.8	DRB	0.08
B*6903	AAAACGACGGCCAGTACTCCATGAGGTATTTCACC/ GAAACAGCTATGACCCCGTTCTCCATGTGTCTGA	60	0.8	DRB	0.08
B*75	AAAACGACGGCCAGTCAAGGACGTCACACAGTCC/ GAAACAGCTATGACCCTTGCCGTCGTAGGCG	64	0.8	DRB	0.08

One monkey with a group 1 and two monkeys with a group 2 haplotype on one chromosome, and type 19 on the other also displayed rapid disease progression with a mean survival of 19.0 w.p.i. ($P = 0.04$; s.d. 2.0 w.p.i.; Figure 1c; Table 3). Since no significant difference between the respective mean survival times was observed (logrank $\chi^2 = 1.617$, two degrees of freedom (d.f.), $P = 0.446$), groups 1, 2 and 19 haplotypes will henceforth be referred to as 'rapid progressor' haplotypes. Altogether, the monkeys carrying only rapid progressor haplotypes had a mean survival time (19.5 w.p.i., s.d. 5.7 w.p.i.) that was highly significantly shorter than that of the remaining genotypes (logrank $\chi^2 = 45.619$, one d.f., $P < 0.0001$).

The viral load was determined in five rapid progressors and was revealed to be higher than in monkeys with an intermediate or slow disease course (Figure 2). Moreover, none of the three tested macaques displayed Elispot responses against Tat, Gag or Nef-peptide pools beyond 12 w.p.i. (Table 4). In none of the rapid progressors were anti-SIV antibodies detected (data not shown). We therefore concluded that none of the *Mhc* class I genes encoded by the rapid progressor haplotypes supported an effective, long-lasting anti-SIV immune response.

Altogether, 20 monkeys carried rapid progressor haplotypes on both chromosomes. Of the 61 remaining monkeys, 50 carried a rapid progressor genotype on one chromosome. These 'quasi-hemizygous' macaques enabled us to relate individual *Mhc* class I haplotypes to medium and slow disease progression. Two SIV-infected monkeys carried an unknown genotype since samples from the parents were unavailable. Nine monkeys carried one or two of the configurations described in Table 2, but did not possess a rapid progressor genotype.

Table 2 *Mhc* class I haplotypes: association with disease progression in SIV-infected macaques and frequency in the GPC cohort

Haplotype Mamu A-	*Mamu B-*	*Frequency*
Mhc *class I haplotypes associated with rapid disease progression*		
Group 1		45.1
1.1 *A1*04–A4*1403*	*B*12–B*22–B*3001–B*38–B*46–B*49–B*57*	
1.2 *A1*04–A4*1403*	*B*12–B*3001–B*38–B*46–B*49–B*57*	
1.3 *A1*04–A4*1403*	*B*12–B*22–B*3001–B*46–B*49–B*57*	
1.4 *A1*04–A4*1403*	*B*12–B*22–B*3001–B*38–B*49–B*57*	
Group 2		
2.1 *A1*04–A4*1403–A2*05*	*B*01–B*07–B*3002*	4.3
2.2 *A1*21–A2*24–A3*1303*	*B*01–B*07–B*3002–B*57*	2.5
19 *A1*08–A3*1303–A2*05*	*B*21–B*28–B*45*	6.2
Mhc *class I haplotypes associated with intermediate survival times*		
1.2+A*02 *A1*02–A1*04–A3*1403*	*B*12–*3001/2–B*38–B*46–B*49–B*57*	0.6
1.3+A*02 *A1*02–A1*04–A4*1403*	*B*12–B*22–B*3001/2–B*46–B*49–B*57*	1.8
2.2+A*02 *A1*02–A3*1302*	*B*01–B*07–B*3002–B*57*	1.8
24 *A1*02–A1*04–A4*1403*	*B*17–B*2901*	1.2
26 *A2*05 - A4*1403*	*B*17–B*2901*	2.5
27 *A1*08–A3*1303–A2*05*	*B*6802–B*6903–B*7501*	9.3
30 *A1*08–A3*1303–A2*05*	*B*65–B*6901–B*46*	4.9
8 *A1*08–A3*1303–A2*05*	*B*26–B*27–B*3003–B*43–B*49–B*57*	5.5
Mhc *class I haplotypes associated with slow disease progression*		
4 *A1*01–A2*05*	*B*4701*	5.5
18 *A1*08–A3*1303–A2*05*	*B*38–B*46–B*4701*	4.9

Table 3 Survival analysis of SIV-infected rhesus macaques carrying on one chromosome a 'rapid progressor haplotype', stratified by the second *Mhc* class I haplotype

Mhc *haplotype*	*No. animals*	*Mean (w.p.i.)*	*s.d. (w.p.i.)*	*Disease progression*
Group 1	11	20.5	6.5	Rapid
Group 2	6	18.0	5.1	Rapid
19	3	19.0	2.0	Rapid
1.2/1.3/ 2.2+A*02	6	31.3[a]	19.5[a]	Intermediate
30	7	52.0	15.4	Intermediate
27	10	52.4	22.3	Intermediate
24, 26 (B*17)	6	61.2[a]	16.3[a]	Intermediate
8	8	64.8[a]	55.1[a]	Intermediate
18	7	85.5	22.3	Slow
4 (A*01)	6	89.1	15.6[a]	Slow

[a]Mean and standard deviation (s.d.) of the survival time may be systematically underestimated because the largest observation was censored (i.e., euthanized without symptoms of AIDS).

These monkeys were included to assess the influence of *Mhc* genotypes on disease progression in a larger cohort.

Haplotypes associated with an intermediate survival time
Eight haplotypes present in monkeys which carried a rapid progressor haplotype on the other chromosome displayed an intermediate mean survival time between 31.3 and 64.8 w.p.i. (Table 3). The respective genotype-specific estimates were significantly larger than the mean survival time of the rapid progressors (all $P<0.05$), but were not found to differ significantly from one another (logrank $\chi^2=4.673$, three d.f., $P=0.2$). Their mean survival (50.5 w.p.i., s.d. 20.7 w.p.i.) was significantly different from that of both the rapid progressors (logrank $\chi^2=39.981$, one d.f., $P<0.001$) and the remaining genotypes (logrank $\chi^2=10.161$, one d.f., $P=0.001$), except configuration 8 (see below).

Three haplotypes differed from the rapid progressor ones 1.2, 1.3 or 2.2, only by the presence of *Mamu-A*02* (Table 2), so that we were able to investigate the influence of this allele upon survival against a genetic background significantly associated with rapid disease progression (Figure 1d). *Mamu*-A*02 presents an epitope from each of Nef[32] and Gag in a dominant fashion, and several other epitopes subdominantly.[33] The estimated mean survival time of six monkeys from five independent breeding groups was 31.3 w.p.i., which represents an underestimate because the largest observation was censored. Nevertheless, the presence of *Mamu-A*02* significantly prolonged survival in the respective animals (logrank $\chi^2=7.426$, one d.f., $P=0.006$). Notably, the survival times and viral load of the *Mamu-A*02* animals were found to vary considerably (Table 3; Figure 2).

Seven animals, obtained from five breeding groups, with haplotype 30 on one chromosome and a rapid progressor haplotype on the other had a mean survival time of 52.0 w.p.i. (s.d. 15.4 w.p.i.; Figure 1e; Table 3). Two *Mhc* identical sibling pairs had similar survival times (33 vs 44 w.p.i. and 45 vs 48 w.p.i.). Two immunized monkeys, not contributing to the survival analysis, showed strong Nef Elispot responses with peak values of 1600 spot-forming cells (s.f.c.) per 1 million PBMC indicating that haplotype 30 can induce strong CTL responses (Table 4).

Haplotype 27 was found in 10 individuals from four families together with a group 1 or group 2 haplotype. The SIV-infected monkeys had a mean survival of 52.4 w.p.i. (s.d. 22.3 w.p.i.; Table 4 ; Figure 1j). Two *Mhc* class I and class II identical sibling pairs from two families had

a discordant disease course (26 vs 83 w.p.i., and 39 vs 69 w.p.i.). Interferon-γ (IFN-γ) Elispot activity was measured for two unrelated naive SIV-infected monkeys with a differing disease course. Both monkeys showed only sporadic Nef-, Tat- and Gag-responses over a time period of 40 w.p.i. (Table 4).

An association with slow disease progression has been reported for *Mamu-B*17*.[25,27] In our study cohort, like in other populations,[24,27] *Mamu-B*17* was linked to *Mamu-B*2901* (Table 2). This composite *B* configuration was linked to two different *A* regions, one of which carried *Mamu-A*02*. No notable differences were observed between the survival times of the *Mamu-B*17* carriers with or without *Mamu-A*02*. The joint mean survival time of haplotypes 24 and 26 was 61.2 w.p.i. (s.d. 16.3 w.p.i.; Table 3; Figure 1f). Although this may still represent an underestimate due to censoring of the largest observation, we can assume that *Mamu-B*17* was associated with an intermediate, rather than a slow, disease progression in our study cohort.

Haplotype 8 was inherited by six monkeys. Two *Mhc* class I identical full siblings were euthanized at 50 w.p.i. with symptoms of immunodeficiency, but not AIDS, whereas two of their half siblings with the same *Mhc* class I genes survived for only 6 and 24 w.p.i., respectively. Another pair of *Mhc* class I-identical siblings had greatly discordant survival times of 20 and 145 w.p.i. Thus, although the estimated mean survival time of haplotype 8 animals was an intermediate of 64.8 w.p.i., the s.d. (55.1 w.p.i.) appeared to be remarkably larger than that of the other genotypes (Table 3, Figure 1i).

Haplotypes associated with slow disease progression

Haplotype 18 was significantly associated with slow disease progression when compared to the intermediate progressors (logrank $\chi^2 = 4.795$, one d.f., $P = 0.03$). Notably, except for *Mamu-B*4701*, haplotype 18 carries only genes that are either present in haplotypes associated with rapid disease progression, or that are only weakly

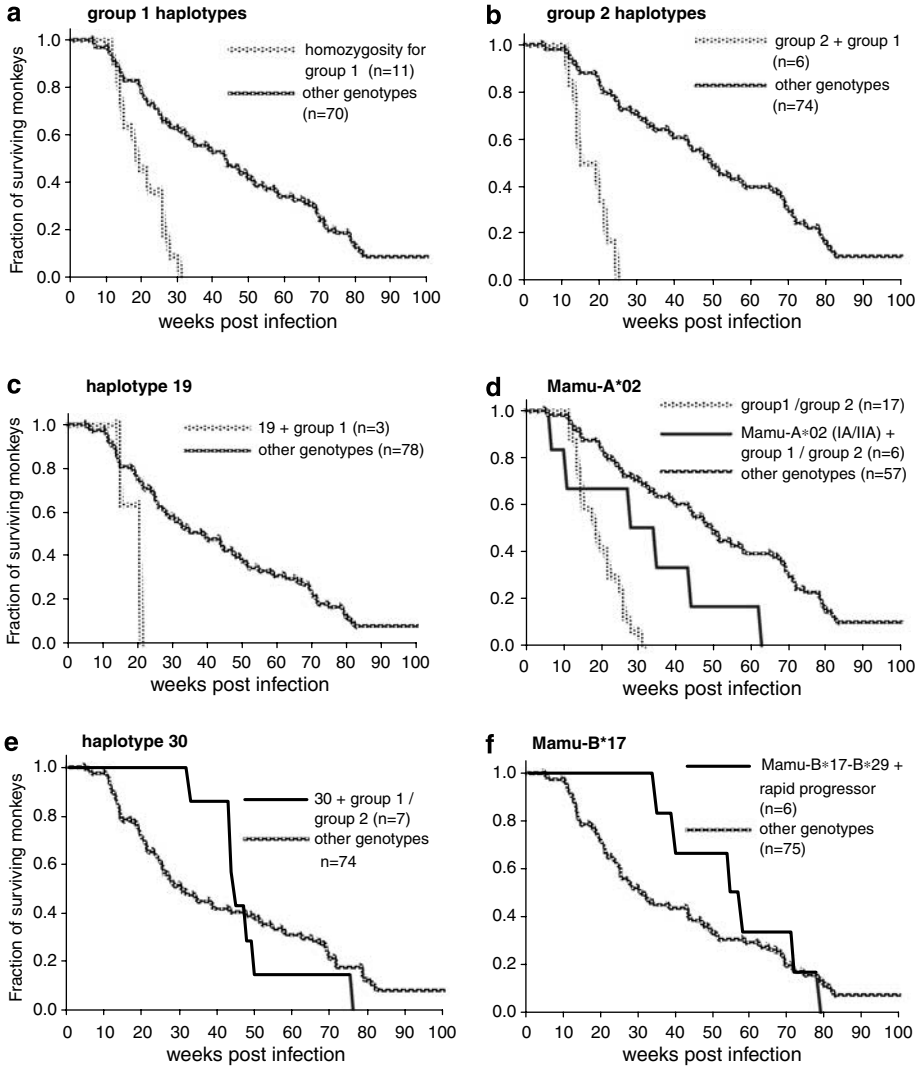

Figure 1 Survival curves of 81 SIV-infected rhesus monkeys, stratified by *Mhc* class I genotype. (**a**–**j**) Survival curves of monkeys carrying only haplotypes associated with rapid disease progression (light hatched line) or carrying one haplotype associated with rapid disease progression and the specified haplotype (solid black line) or carrying any other pair of haplotypes (dark hatched line).

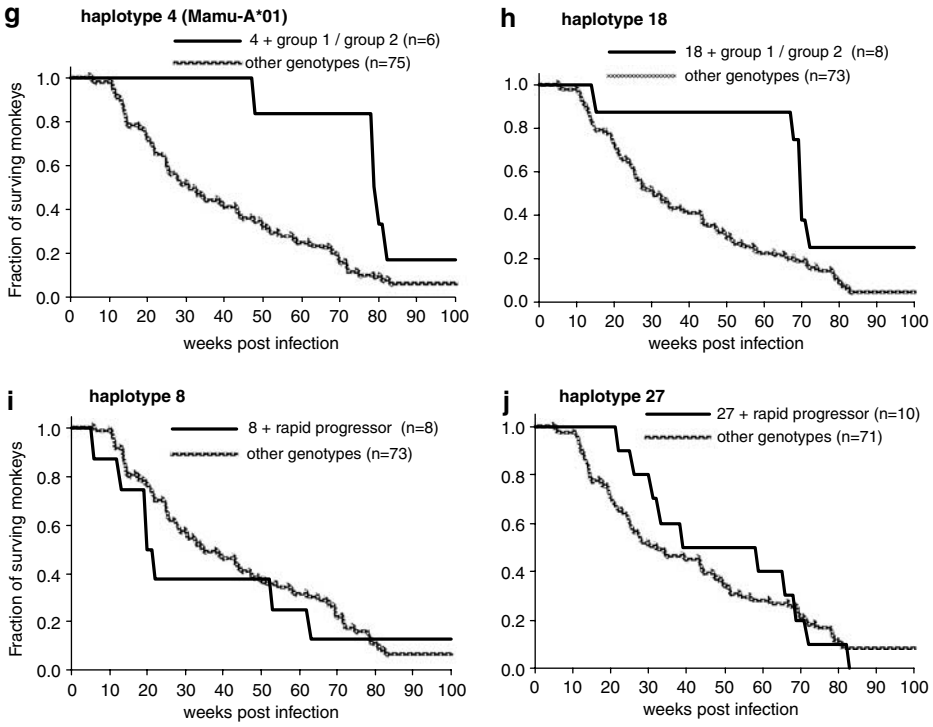

Figure 1 Continued.

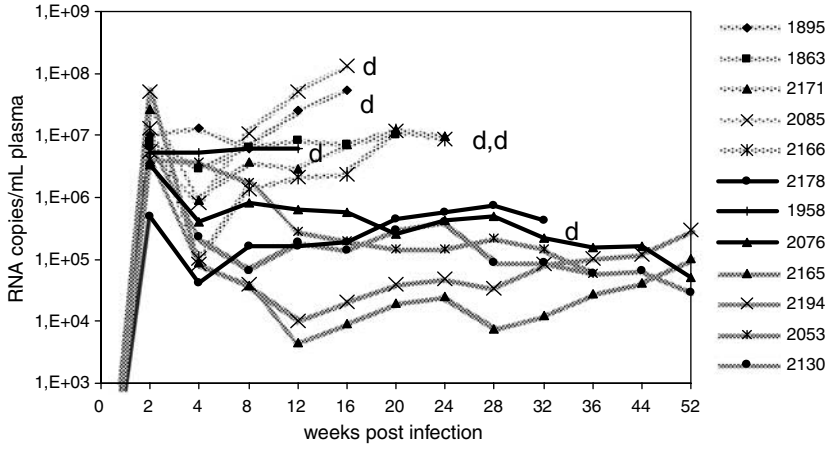

Figure 2 Plasma viral RNA copies per ml after SIV infection in monkeys stratified by their *Mhc* genotype. The viral load of individual monkeys carrying only rapid progressor haplotypes (light hatched line), *Mamu-A*02* on a background of *Mhc* genes associated with rapid disease progression (solid black line) or haplotype 18 in addition to a rapid progressor genotype (dark hatched line). Animal numbers are indicated (d, death with AIDS-related symptoms).

transcribed, such as *Mamu-A*1303*. The seven animals carrying haplotype 18 had a mean survival of 85.5 w.p.i. (s.d. 22.3 w.p.i.; Figure 1h; Table 3), a lower viral load and longer-lasting Elispot responses compared to more rapidly progressing animals (Figure 2; Table 4).

*Mamu-A*01*, which represents the best-studied rhesus *Mhc* allele,[34,35] was encoded in haplotype 4. Six monkeys carrying this and group 1 haplotypes were derived from three breeding groups. Their mean survival time of 89.1 w.p.i. (s.d. 15.6 w.p.i.; Figure 1g; Table 4) was significantly longer than that of the intermediate progressors (logrank $\chi^2 = 6.177$, one d.f., $P = 0.013$). *Mamu-B*4701* is also present in the haplotype 4 encoding *Mamu-A*01*, which may explain to some extent the significant association of *Mamu-A*01* with slow disease progression in our study cohort.

Influence of Mhc *gene copy number variation on survival time*
The number of transcribed *Mhc* class I genes in an individual may affect T-cell receptor repertoire and may thus indirectly determine the quality of CTL responses of the respective carrier. When the total number of

Table 4 SIV Elispot results from *Mhc*-typed SIV-infected rhesus monkeys

Genotype	*No. monkeys*	*Response against Gag*	*Response against Tat*	*Response against Nef*	*Survival (w.p.i.)*
Groups 1, 2	2, ni	++ (2–12)	++ (2–12)	+ (2–6)	15, 22
Groups 1, 1	1, ni	+ (2–8)	—	—	22
19, 30	2, i	++ (2–41)	+ (2–16)	+++ (2–41)	44, 70
Groups 1, 18	1, ni	++ (2–28)	+ (12–28)	+ (2–40)	70
19, 18	1, ni	++ (2–48)	—	++ (4–52)	70
18, 18	1, i	++ (2–48)	++ (2–48)	+++ (2–52)	70
Groups 1, 27[a]	2, ni	++	++	++	39, 72

Abbreviation: w.p.i, weeks post infection.
+++, peak values >10-fold above background.
++, peak values >3.1- to 10-fold above background.
+, peak values two- to threefold above background.
Duration of the responses in brackets (w.p.i.). i, immunized with an SIV vaccine. ni, SIV vaccine naive.
[a]All responses appeared only sporadically.

transcribed *Mhc* genes per genotype was related to individual survival time using a Cox proportional hazards model, the presence of more *Mhc* genes was found to correlate significantly with shorter survival (hazard ratio 1.19, $P = 0.002$). This result indicates that mere copy number variation at the *Mhc* locus may already influence disease progression. The number of different *Mhc* genes expressed in an individual did not affect survival time significantly.

Contribution of Mhc *class II genotypes to survival time*
We have previously described *Mhc* class II genotypes associated with rapid disease progression in rhesus macaques of different origin.[36] Reinspection of these data revealed that, in the GPC cohort, *Mhc* class II genotype *Mamu-DQB1*0601-DRB1*0309-DRB*W201* was linked to *Mhc* group 1 haplotypes and to those containing *Mamu-A*02* (1.3-*A*02* and 2-*A*02*; Table 2), all of which are associated with a more rapid disease progression. Because of this strong linkage we were unable to quantify the potential contribution of this *Mhc* class II genotype to disease progression. However, homozygous monkeys express only one *DRB* gene, which may be disadvantageous. *Mhc* class I group 1 and group 2 configurations were also linked to *Mhc* class II haplotypes expressing two *DRB* genes, namely *Mamu-DQB1*1801-DRB1*0303-DRB1*1007* and *Mamu-DQB1*1811-DRB1*0406-DRB5*0301*.[37] One of them (*Mamu-DQB1*1801-DRB1*0303-DRB1*1007*) was also linked to class I configurations associated with slow disease progression, including configuration 4. This finding indicates that *Mhc* class I genotypes have a much more marked influence upon disease progression than class II genotypes, thereby corroborating previous reports on HIV-infected humans[20] and SIV-infected macaques.[38]

Mhc *region configurations explain at least 48% of the variation in disease progression*
In order to somehow quantify the overall influence of the *Mhc* genotype on disease progression in our study cohort, we performed a *post hoc* unbalanced one-way analysis of variance (ANOVA) of the 60 observed event times (that is, euthanizations with AIDS). Classification of a genotype as either 'slow', 'intermediate', 'rapid progressor' or 'unknown' was found to be a highly significant explanatory variable for onset of AIDS-defining symptoms ($F = 53.2$, $P < 0.0001$). The coefficient of determination of the fitted model was $R^2 = 0.478$, which implies that the classified genotype explained 47.8% of the variation in survival time.

Discussion

The present study reports upon the relationship between survival time in SIV-infected monkeys and 17 *Mhc* class I region haplotypes, which were deduced by cDNA sequencing and extensive typing of three generations of rhesus macaques housed at the GPC. Our data validate and extend previous descriptions of the rhesus macaque *Mhc* class I region. Although the internal variability of a single locus generally appears to be low, the *Mhc* class I *A* and *B* regions display considerable variation in terms of the number and combination of transcribed *Mhc* class I genes.[21–24]

We detected two or three transcribed *A* genes per *Mhc* region and three novel *A* haplotypes in addition to the four that had been published before.[24] Only one configuration displayed allelic polymorphism (*Mamu-A1*2102-A2*2401-A3*1303*) when compared to a published haplotype.[24] In contrast to one report,[24] we failed to detect *Mamu-A4*14* alleles in eight of the 17 haplotypes analyzed here. For genotyping *Mamu-A4*14*, we used primers specific for *Mamu-A4*1403* as well as published primers amplifying all *Mamu-A4*14* alleles.[24]

In the *B* region, we found two to seven transcribed genes per haplotype. Nine *B* region haplotypes are novel; five had been published before.[24] Furthermore, we identified one *Mhc* class I gene, *Mamu-B*74*, which is conserved between cynomolgus and rhesus macaques and which seems to be subject to alternative splicing. The variability of the *Mhc* region is exemplified by the four 'group 1' haplotypes (Table 2) which differ from each other only by the presence or absence of one of the seven *B* genes examined here. In addition, some genes are present on several *B* region haplotypes. Although these results indicate a great plasticity of the rhesus *Mhc*, we

have not obtained any evidence for an enhanced recombination rate in that region. This is in agreement with a similar lack of evidence for an enhanced recombination rate in the human MHC region.[39]

The most important aim of our study was to assess the impact of *Mhc* class I-genotypes on disease progression in SIV-infected rhesus macaques. Therefore we studied the influence of *Mhc* class I haplotypes upon disease progression in SIV infection, rather than single gene alleles. This was feasible because of the relatively low genetic variation of both the GPC rhesus monkey cohort and the infecting virus.

Initially, we identified three groups of *Mhc* class I haplotypes associated with rapid disease progression (Table 2). Even immunized macaques carrying these haplotypes died within 31 w.p.i. As a further indicator for fast disease course (1) Elispot data revealed short-lived responses in the rapid progressors and (2) viral load was highest in these animals. Additional evidence suggests that some MHC antigens are unable to induce an efficient anti-SIV immune response. In the case of *Mamu-B*01*, this concurs with previous reports.[25,40] Other sequences have been reported to be expressed only at low levels, such as *Mamu-B*57* and *-A*1303*.[24] Taken together, we have identified for the first time *Mhc* class I haplotypes associated with rapid disease progression. Furthermore, the respective haplotypes can be considered as recessive in relation to 'protective' *Mhc* genes/haplotypes which impede fast disease progression.

By investigating groups of monkeys that carried a haplotype associated with rapid disease progression on one chromosome, we identified two haplotypes significantly associated with slow disease progression (Table 2). One of the haplotypes carried only genes that are present on the rapid progressor genotypes, except *Mamu-B*4701*. *Mamu-B*4701* therefore appears to be a candidate gene to induce strong anti-SIV CTL responses. In addition, the presence of *Mamu-B*4701* on the haplotype 4, which also carries *Mamu-A*01*, may further explain why *Mamu-A*01* is most strongly associated with slow disease progression in our monkey colony.

We also identified region configurations potentially associated with an intermediate type of disease progression. The respective survival times differed significantly from those of the rapid and the slow progressors. Interestingly, the s.d. values of the survival time varied greatly in this intermediate progressor group, ranging from 29 to 65% of the mean. In contrast, the s.d. did not exceed 32% of the mean for the region configurations associated significantly with slow or rapid disease progression.

Genotypes displaying a lower variation of the survival time were configuration 30 and the configurations carrying *Mamu-B*17*. Configuration 30 uniquely carried *Mamu-B*65* and *-B*6901* (GenBank accession no. AY844601), which may potentially induce a consistent CTL response. *Mamu-B*6901* corresponds to the allele formerly termed *NB2*, which had been found to be associated with slow disease progression in SIV-infected monkeys of diverse origin.[25] Thus, in all animals carrying rapid progressor genotypes, slow progressor genotypes, *Mamu-B*17*, or configuration 30, the *Mhc* genotype was found to be broadly predictive of the principal disease course. Our study suggests that about 48% of the overall variation in disease progression in SIV-infected macaques can be explained by specific *Mhc* genotypes. Monkeys carrying these genotypes are therefore useful to examine the efficacy of any treatment against AIDS. The effects can be investigated in animals with differing immunogenetic background predicting different types of disease progression.

Several *Mhc*-sequences (*Mamu-A*02*, *-B*26*, *-B*27*, *-B*43*, *-B*68*, *-B*6903* or *-B*7501*) were associated with an intermediate albeit more variable survival time, characterized by an s.d. value above 40% of the mean. Possibly, these molecules can also induce anti-SIV CTL responses but they may be less potent than, for instance, those of *Mamu-A*01* carriers, as has been shown for *Mamu-A*02*.[41] The kinetics of the CTL response and stochastic events related to viral mutation rate and T-cell receptor repertoire may have a more profound impact upon survival in these animals than in those that induce a more consistent CTL response.[42]

Additionally, copy number variation of *Mhc* genes appears to influence disease progression. According to our results, the presence of more transcribed *Mhc* genes correlated with shorter survival time. These results are surprising as probably per haplotype no more than two or three *B* genes are highly transcribed.[24] The other *Mhc* genes are expressed at low to very low levels. It is unlikely that they induce a strong CTL response. However, processed peptides of these proteins may be bound by other MHC molecules and play a role in thymic education. Interestingly, three of the genotypes displaying a large variation of survival time encoded many transcribed *Mhc* genes. However, these findings merit further investigation.

Another explanation may be that the large variation in survival time is a specific attribute of some of the MHC antigens present on these genotypes. We may hypothesize that disease progression in these animals is influenced by a specific interaction between particular MHC antigens and polymorphic MHC receptors located outside of the *Mhc* region. We may speculate that the KIR, which are expressed on natural killer (NK) cells are likely candidates for the type of MHC receptors referred to above. Several reports have demonstrated that the presence of specific KIR–MHC pairs influence HIV-induced disease progression,[43–45] the onset of opportunistic infections in HIV-infected patients[46] and the probability of becoming HIV infected.[47] Results from *ex vivo* viral replication assays in SIV-infected rhesus macaques showed that NK cells can suppress viral replication substantially but to a variable extent.[48] As yet, no study of the interaction between particular macaque KIR and MHC antigens has been published. The results presented here may provide first clues as to the macaque MHC antigens interacting with KIRs. The macaque model of AIDS may become highly valuable for examining the interplay between innate and adaptive immune responses and, most notably, for determining their impact upon vaccine efficacy.

Materials and methods

Animals, genealogies and viruses

All rhesus monkeys analyzed in the present study were of Indian descent and were bred at the GPC, Göttingen. The 81 SIV-infected macaques represented descendants

of animals originally derived from the Caribbean Primate Research Center colony on Cayo Santiago, Puerto Rico. Parenthood of the monkeys was assessed as described.[49] All SIV-infected animals were housed at the GPC according to the German animal protection Act which complies with the European Union guidelines on the use of nonhuman primates for biomedical research. Experimental data from most of the animals have been published before.[25,36,50] All monkeys were naive with respect to immunization against SIV or any other antiviral treatment, except for the six immunized monkeys displaying the rapid progressor phenotype. A total of 30 animals were infected with $SIV_{mac}251$ grown on monkey peripheral blood mononuclear cells (PBMC). Altogether 21 animals were infected with $SIV_{mac}251$/32 H grown on the human T-cell line C8166. A total of 9 animals were infected with the *ex vivo* isolate $SIV_{mac}251$/32H/spl, and 21 monkeys were infected with $SIV_{mac}239$ grown on rhesus monkey PBMCs. The infecting virus did not significantly affect the survival time when the analysis was controlled for the presence or absence of a rapid progressor genotype (data not shown). None of the SIV-infected monkeys of this study was an elite controller.

Diagnosis of AIDS-like disease

The 48 animals that were euthanized before 47 w.p.i. had symptoms of AIDS-like disease, whereas only 12 of the 33 animals euthanized at a later time point had AIDS-related symptoms. Thus, the survival time of slow progressors may be underestimated and is indicated in the respective tables. Those monkeys which were euthanized with signs of AIDS-related symptoms suffered from anorexia, incurable diarrhoea, *Pneumocystis carinii* pneumonia infection or from neurological dysfunction evident by ataxia as judged from clinical as well as detailed necropsy findings, which were available for each monkey.[36] $CD4^+$ T-cell counts turned out to be an inadequate marker for the diagnosis of AIDS since many rapid progressors still had increasing $CD4^+$ counts by the time of euthanasia.[36,51,52] Viral RNA copy number could not be used as a proxy for AIDS as many monkeys were infected at a time when RNA copy number was not routinely analyzed. However, for most monkeys cell-associated viral load data were available.[53]

PCR amplification, cloning and sequence analysis of Mhc *class I sequences*

From 18 selected monkeys, RNA was isolated from *Herpes papio* virus-transformed B-cell lines or PHA-stimulated PBMCs using the Qiagen RNeasy Midi kit (Qiagen, Hilden, Germany). A toal of 10 μg RNA was used to produce cDNA employing a double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). The cDNA was purified by phenol–chloroform extraction. A toal of 1 μl of a tenfold dilution was used to amplify *Mhc* class I gene sequences. *Mamu-A* sequences were amplified using primers *Mamu-Afor* (5′-GATGGCGCCCCGAA CCCTCCTCCTGG) and *Mamu-Arev* (5′-GGCCTCG CAGTCCCACACAA), *Mamu-B*-sequences were amplified with primers *Mamu-Bfor* (5′-GATGGCGCCCC GAACCCTCCTCCTG) and *Mamu-Brev* (5′-GCTTTGCA GAAAGAGATGCCAGAG), each at a concentration of 0.4 μM. For each animal, *Mamu-A* and *-B* sequences were amplified twice using different heat-stable DNA polymerases (expand high-fidelity PCR System, Roche Diagnostics, Switzerland; high-fidelity PCR enzyme mix, Fermentas GmbH, St Leon, Germany). PCR reactions were performed in an initial denaturing step at 94 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 30 s, an annealing step at 60 °C and an elongation step at 72 °C for 72 s. The elongation step was prolonged by 4 s per cycle with a final elongation for 15 min. After agarose gel purification of the PCR products, ligation and cloning were performed with the TOPO-TA cloning kit for sequencing (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was prepared with the Qiaprep Spin Miniprep procedure (Qiagen). Per PCR reaction, 10–12 individual clones containing *A* sequences or 20–30 clones containing *B* sequences were sequenced using the Dye terminator chemistry (Applied Biosystems) on a 3730 × L DNA Analyzer (Applied Biosystems). DNA sequence alignment was performed using the BioEdit programme (www.mbio.ncsu.edu/BioEdit/bioedit.html). Identical novel DNA sequences obtained in at least three independent PCR reactions were registered at the Biomedical Primate Research Center, the Netherlands[54] and submitted to GenBank (accession no. EF219477–EF219486, EU157186).

Mhc *genotyping*

The rhesus macaques of the GPC were initially typed for *DQB1* and for *Mhc* class I genes using published techniques.[25,29,55] For the novel class I alleles, a total of 23 primer pairs were created (Table 1). Allele-specific PCR products ranged in size between 408 and ~1300 bp. *DRB*-specific primers (*MDRB-5′* and *3′-MDR*) or actin gene primers served as internal controls.[55,56] HPLC-purified primers were obtained from VBC-GENOMICS Bioscience Research (Vienna, Austria). Reaction mixtures of 25 μl contained 100–150 ng DNA, PCR buffer, Q-solution, 200 μM dNTP, 750–800 μM allele-specific forward and reverse primer, 80–120 nM of the internal control primers and 1.25 unit Taq polymerase (Qiagen). The samples were denatured initially at 95 °C for 3 min, followed by 38 cycles at 95 °C for 30 s, 58–64 °C for 40 s, 72 °C for 40 s and a final elongation step at 72 °C for 2 min. Allele-specific products were identified by electrophoresis of amplicons in 1% agarose gels containing ethidium bromide.

Elispot assay

Elispot assays were performed as described.[50] Briefly, purified PBMCs were resuspended in Elispot medium and seeded in triplicate in wells coated with anti-human IFN-γ monoclonal antibody. For antigenic stimulation, SIV Gag (15-mer, EVA7066, NIBSC) and Nef peptides (20-mer, EVA777, NIBSC), peptide pools or SEB were included in the medium. Cells were removed after incubation for 20 h. IFN-γ was determined by biotinylated anti-human IFN-γ detector antibody (Mabtech, Augustendalsvägen, Sweden) followed by the addition of strepavidin–alkaline phosphatase conjugate, and a final incubation with NBT/BCIP solution. Spots were counted using a BIOSYS2000 Elispot reader. All counts were normalized to a total of 10^6 cells. Samples yielding more than 100 s.f.c. per 10^6 PBMC above background, and twice that of the medium control, were scored as positive.

RNA copy numbers

Viral RNA was isolated from frozen plasma samples following the MagAttract Virus Mini M48 protocol (Qiagen). RNA copy number in infected monkeys was determined by quantitative real-time PCR as described[57] using TaqMan-based real-time PCR on an ABI-Prism 7500 sequence detection system (Applied Biosystems). Levels of amplified viral RNA were expressed as SIV-RNA copies per ml plasma.

Statistical analysis

Survival analysis was performed using the LIFETEST procedure of the SAS software package (SAS version 9.1, SAS Inc., Cary, NC, USA). In order to reduce bias in the estimation of the mean and standard deviation of genotype-specific survival times, the largest observation per genotype was consistently treated as an event time. It should be noted, however, that the results may still be systematically biased downward in cases where the largest observation was right censored. Differences between genotype-specific survival curves were assessed for statistical significance using a logrank test with the appropriate d.f. Unbalanced ANOVA of event times was performed using SAS procedure REG. The influence of the number of transcribed *Mhc* genes upon aids-free survival was assessed for statistical significance using a Cox proportional hazards model, as implemented in SAS procedure PHREG.

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Disclosure/conflict of interest

The authors declare that there are no conflicts of interest or any financial interest.

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Supplementary Information accompanies the paper on Genes and Immunity website (http://www.nature.com/gene)