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Immunization with recombinant macaque major histocompatibility complex class I and II and human immunodeficiency virus gp140 inhibits simian–human immunodeficiency virus infection in macaques

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Genetic, epidemiological and experimental evidence suggest that the major histocompatibility complex (MHC) is critical in controlling human immunodeficiency virus (HIV) infection. The objectives of this study were to determine whether novel recombinant Mamu MHC constructs would elicit protection against rectal challenge with heterologous simian–human immunodeficiency virus (SHIV) strain SF162.P4 in rhesus macaques. Mamu class I and II gene products were linked together with HIV gp140, simian immunodeficiency virus (SIV) p27 and heat-shock protein 70 to dextran. The vaccine was administered to two groups, each consisting of nine macaques, either subcutaneously (SC), or rectally and boosted by SC immunization. The controls were untreated or adjuvant-treated animals. Repetitive rectal challenges with up to ten doses of SHIV SF162.P4 showed a significant decrease in the peak and sequential viral RNA concentrations, and three macaques remained uninfected, in the nine SC-immunized animals, compared with infection in all nine controls. Macaques immunized rectally followed by SC boosters showed a less significant decrease in both sequential and peak viral loads compared with the SC-immunized animals, and all were infected following rectal challenge with SHIV SF162.P4. Plasma and mucosal IgG and IgA antibodies to Mamu class I alleles and HIV gp120, as well as to RANTES (regulated upon activation, normal T-cell expressed, and secreted; CCR5) were increased, and showed significant inverse correlations with the peak viral load. These results suggested that allo-immunization with recombinant MHC constructs linked to HIV–SIV antigens merits further investigation in preventing HIV-1 infection.

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INTRODUCTION

Xeno-immunization with inactivated simian immunodeficiency virus (SIV) grown in human $CD4^+$ T-cells consistently prevents SIV infection in ~90 % of rhesus

Two supplementary tables are available with the online version of this paper.

macaques (Arthur *et al.*, 1995; Carlson *et al.*, 1990; Desrosiers *et al.*, 1989; Dormont *et al.*, 1995; Murphey-Corb *et al.*, 1989; Stott *et al.*, 1994). However, attempts to reproduce protection against SIV by allo-immunization with inactivated SIV grown in macaque $CD4^+$ T-cells have been met with limited success or failure (Polyanskaya *et al.*, 1997; Stott *et al.*, 1994). The critical significance of human leukocyte antigen (HLA) in the development and prevention of human immunodeficiency virus type 1 (HIV-1) infection has been widely documented. HIV and SIV replication are significantly related to major histocompatibility complex (MHC) class I alleles (Carrington & O'Brien, 2003; Goulder & Watkins, 2008). Indeed, HLA-B*57 and HLA-B*27 with HIV in humans and Mamu-B*08, Mamu-B*17 and Mamu-A*01 with SIV in macaques control virus replication and progress in the development of AIDS. In contrast, HLA-B*3502 and HLA-B*3503 are associated with rapid progression to AIDS. Genome-wide single-nucleotide polymorphisms have been identified in HLAs (International HIV Controllers Study, 2010), with specific amino acids in the HLA-B peptide-binding groove that control HIV infection.

Epidemiological evidence suggests that transmission of HIV from mother to baby occurs more frequently among uniparous women (Kind *et al.*, 1995), and mother-to-child HLA class I concordance increases pre-natal HIV-1 transmission (MacDonald *et al.*, 1998). Slow progression is significantly associated with protective HLA-B alleles, especially when these are not shared between mother and child (Thobakgale *et al.*, 2009). Furthermore, systemic allo-immunization of women has revealed that HIV replication *ex vivo* in $CD4^+$ T-cells is inhibited, and this was correlated with a significant increase in CCR5 antibodies and CC chemokines and downmodulation of the CCR5 co-receptors (Leith *et al.*, 2003; Wang *et al.*, 1999, 2002). Unprotected heterosexual and homosexual intercourse also elicits allogeneic responses in both partners, which may contribute to protection against HIV infection (Kingsley *et al.*, 2009; Peters *et al.*, 2004). Allo-immunization as an AIDS vaccine was proposed early in vaccine development (Lehner *et al.*, 2000; Shearer *et al.*, 1993).

We have recently developed novel recombinant HLA class I and II proteins linked to dextran molecules (Schøller *et al.*, 2010). These have been combined with trimeric HIV gp140, SIV Gag p27 and heat-shock protein (HSP) 70 on dextran backbones and mixed with TiterMax adjuvant to immunize rhesus macaques (Mörner *et al.*, 2011). Intravenous challenge with a single large dose of simian–human immunodeficiency virus (SHIV) strain SF162.P4 resulted in a significant decrease in viral load or in prevention of infection.

The objectives of this study were: (i) to establish whether the allogeneic Mamu constructs were immunogenic in rhesus macaques; (ii) to establish whether protection could be induced by subcutaneous (SC) administration of the recombinant Mamu vaccine candidate and challenging macaques rectally with repeated doses of SHIV SF162.P4; and (iii) to attempt rectal mucosal-primed and SC booster immunization with the Mamu vaccine and to monitor the viral load following rectal challenge with SHIV SF162.P4. The results suggested that systemic allo-immunization with the Mamu vaccine candidate elicited significant protection against rectal challenge with SHIV SF162.P4.

RESULTS

Vaccine preparation and viral load in SC-immunized macaques

The purity of class I Mamu-A*01, trimeric HIV gp140 and the C-terminal fragment of HSP70 ($HSP_{359–609}$) were determined by Western blotting (Fig. 1a–c). A diagrammatic illustration of the structure of the vaccine components bound to a dextran backbone is shown in Fig. 1(d), with the CD8 molecule of the $CD8^+$ T-cell binding to Mamu class I, and T-cell receptors to the antigens of the construct. Systemic SC immunization (three times) with the Mamu vaccine candidate and adjuvant was followed by repeated doses (up to ten times) of rectal challenge with 25 $TCID_{50}$ SHIV SF162.P4 (group 1; Fig. 1e). The sequential viral load [measured as $\log_{10}$ RNA equivalents $(\text{ml plasma})^{-1}$] following challenge with SHIV SF162.P4 showed a significant decrease of up to 3 logs in the immunized macaques ($P<0.01$ to $P<0.0001$) compared with the untreated controls (group 4, Fig. 2a). The corresponding data with control group 3a treated with $HSP_{359–609}$ + dextran + TiterMax showed a less significant decrease in viral load ($P<0.05$) (Fig. 2b). The vaccine prevented SHIV infection in three out of nine macaques, and a significant decrease in the peak viral load after infection (2.8 ± 0.7) in the immunized compared with the untreated (3.96 ± 0.22, $P=0.048$; group 4, Fig. 2a) or adjuvant-treated (4.22 ± 0.3, $P=0.046$; group 3a, Fig. 2b) controls. However, analysis of the six infected animals (3.2 ± 0.4) in comparison with control group 3a (4.2 ± 0.3) showed a decrease of 1 log but failed to reach the 5 % level of significance, probably because of the small number of animals ($n=6$ and $n=4$, respectively). The peak viral load in the protected group 1 macaques at week 2 reached the same level as the untreated controls (group 4), but the viral load in the latter group increased by another log by week 3, whereas, at the same time, viral load in the immunized animals decreased by 1 log (Fig. 2a). This was not observed with the treated controls (group 3a), which reached the peak value at the same time as the immunized group 1 but with a 1 log higher viral load (Fig. 2b). The sequential viral loads for each animal in the three groups are shown in Fig. 2(c–e). Thus, it appeared that treatment with dextran + HSP70 + TiterMax exerted a limited non-specific protection against SHIV SF162.P4 compared with the untreated controls.

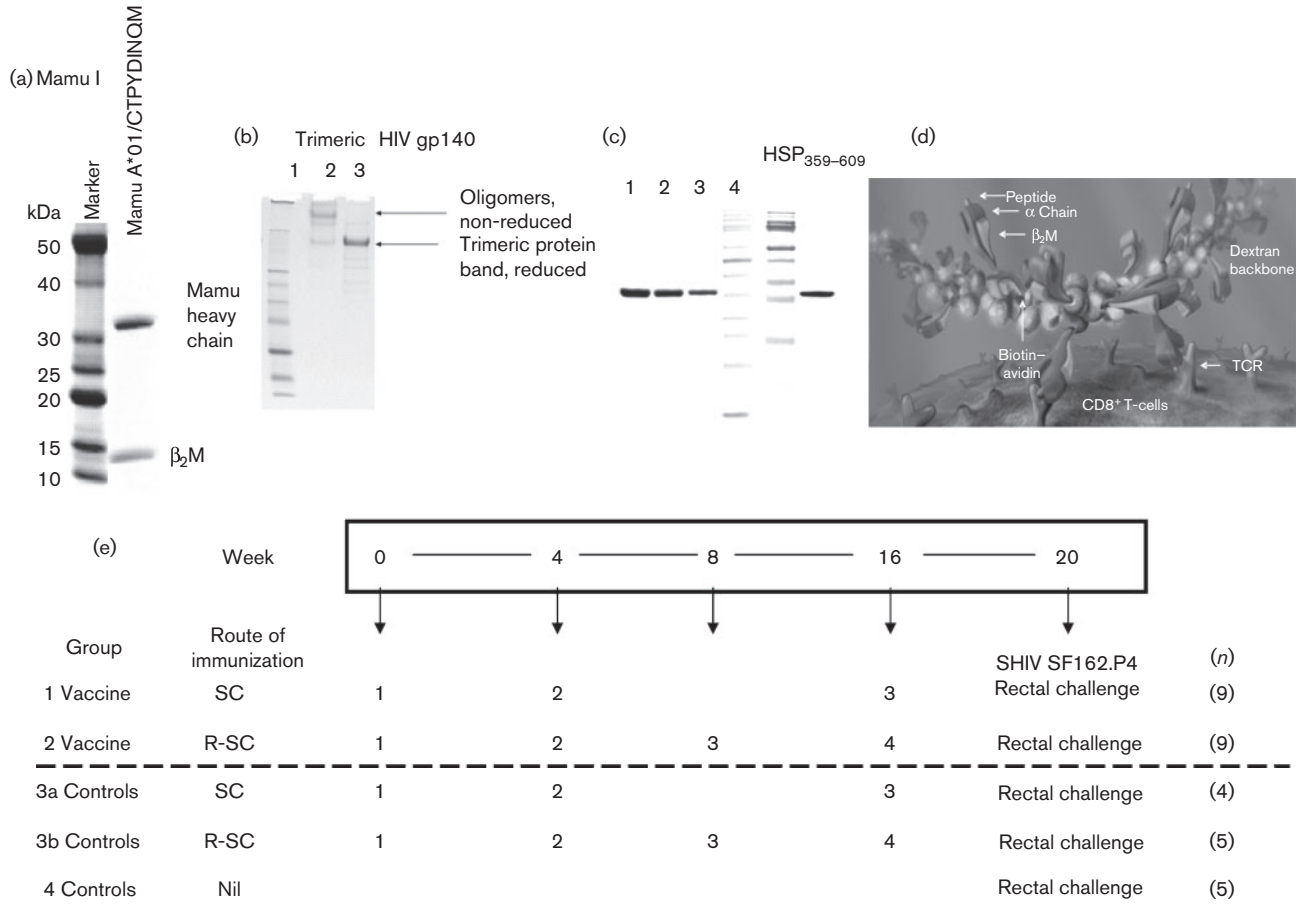

Fig. 1. (a) SDS-PAGE of the folded and purified MHC Mamu class I molecule used in vaccine preparation. β2M, β_2-Microglobulin. (b) SDS-PAGE of purified trimeric HIV gp140. Lanes: 1, marker; 2, non-reduced oligomers; 3, reduced trimeric protein band. (c) SDS-PAGE of purified 28 kDa $HSP_{359-609}$ (lanes 1–3 and 6). Lanes 4 and 5, size markers. (d) Diagram showing MHC class I attached to dextran by avidin–biotin, and binding to T-cell receptor (TCR) of $CD8^+$ T-cells. (e) Immunization schedule and virus challenge carried out in 32 macaques. The vaccine consisted of recombinant MHC class I and II + trimeric HIV gp140 + SIV p27 + $HSP_{359-609}$ + TiterMax in group 1. In group 2, the same vaccine was used, but CpG-C was used instead of TiterMax and the vaccine was administered twice rectally (R), followed by two SC boosters with TiterMax. The three control groups of 14 macaques in total were as follows. Groups 3a and 3b were SC controls: group 3a was treated with $HSP_{359-608}$ + dextran + TiterMax, whilst group 3b was treated twice rectally, followed by twice SC, treated similarly except that CpG-C was exchanged for TiterMax for the rectal treatment. Group 4 was an untreated control group.

Viral load in rectally immunized animals boosted by SC immunization

Rectal mucosal immunization was carried out with the Mamu vaccine (twice) but exchanging TiterMax for the CpG-C mucosal adjuvant, followed by SC immunization (twice) with the vaccine + TiterMax. After challenge (up to ten times) rectally with SHIV SF162.P4, all immunized and untreated control macaques became infected (Fig. 3c). However, a significant decrease in the sequential viral load (up to 2 logs) was observed between the immunized and untreated controls ($P<0.05$ to $P\leqslant0.001$; Fig. 3a). The peak viral load was also significantly lower in the immunized than in the untreated animals (2.91 ± 0.2 vs 3.9 ± 0.22; $P=0.011$), but this was not found in the adjuvant-treated control animals (Fig. 3a, b). Surprisingly, in the five control macaques in group 3b treated with CpG-C + dextran + HSP70 (twice) followed by TiterMax + dextran + HSP70 (twice), one animal was uninfected and no difference in sequential or peak viral load was recorded (Fig. 3c, d). Hence, rectal mucosal followed by SC immunization resulted in a significant decrease in viral load but none of the animals was free of the viral infection, and four treatments with HSP70, dextran, CpG-C or TiterMax elicited some non-specific protection.

Acquisition of infection

The number of rectal challenges with 25 $TCID_{50}$ SHIV SF162.P4 required to infect the SC-immunized and two

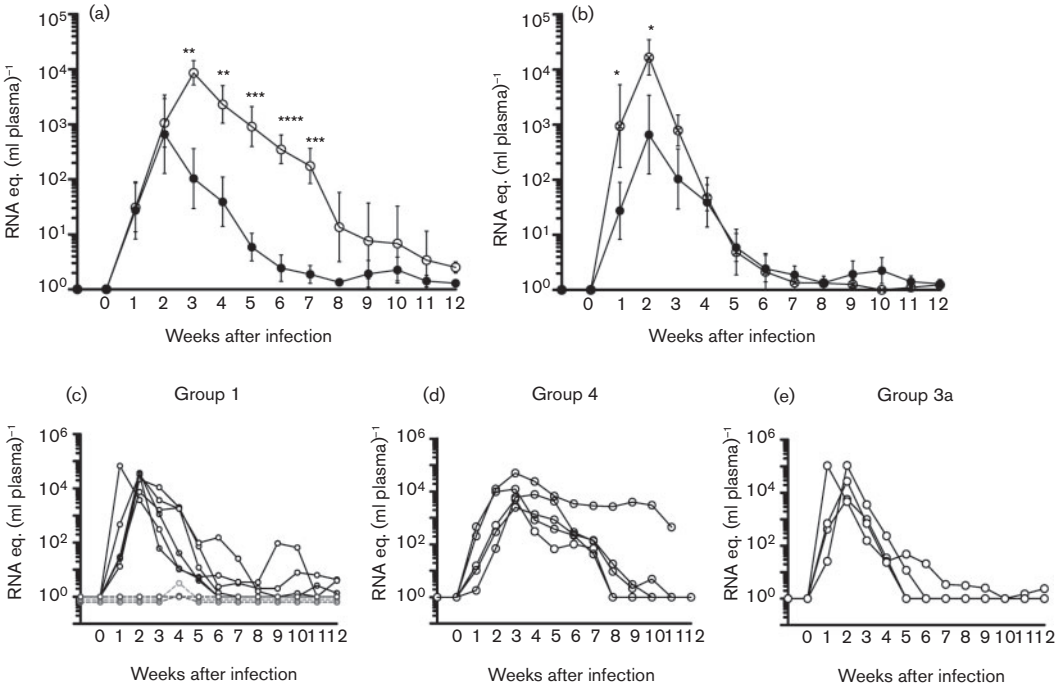

Fig. 2. (a, b) Analysis of sequential viral load in animals SC immunized with the vaccine (●, group 1), compared with the untreated control group (○, group 4) (a) and the adjuvant-treated animals (○, group 3a) (b). (c–e) Sequential viral load in individual macaques in the three groups. Viral load is presented as RNA equivalents (eq.) (ml plasma)$^{-1}$, adjusted by subtracting the baseline non-specific level from all groups. *$P<0.05$; **$P<0.01$; ***$P=0.001$; ****$P=0.0001$.

control groups of macaques is presented in Table S1 (available in JGV Online). The first three challenges failed to show a significant difference between the immunized (three out of nine) and the combined controls (one out of nine; $P=0.271$), but they were higher in group 1 than in the combined controls. However, the next three challenges (immunizations 4–6) resulted in a log-rank χ^2 value of 6.728, which was highly significant ($P=0.0095$). All nine controls were infected but only five of the nine immunized animals were infected after six challenges; one of the remaining four uninfected animals became infected by week 7, leaving three uninfected animals out of nine by the end of the experiment at week 14. We have not excluded the possibility that the rectal and related lymphoid tissue may have harboured the virus.

Plasma IgG and IgA antibodies to Mamu class I antigens and HIV gp120

All antibodies detected were from the plasma or mucosal fluids collected just before immunization and 4 weeks after the last immunization, just before the animals were first challenged rectally with SHIV SF162.P4. Antibodies to the recombinant Mamu-A*01 and Mamu-A*08 were assayed by ELISA and analysed by analysis of variance (ANOVA) with the Bonferroni multiple comparison test. Antibodies to Mamu-A*01 in the two immunized and two adjuvant-treated control groups were highly significantly different ($F=28.55$, $P<0.0001$ or $P=0.0002$; Fig. 4a, b). The SC-immunized animals (group 1) showed significantly higher IgG antibody levels [measured as area under the curve (AUC): 12.3 ± 1.2] than the rectal/SC-immunized (group 2, 5.9 ± 0.62; $P<0.05$) (Fig. 4a), whilst the control group 3a and untreated controls showed no change in antibody levels. Rectal/SC-immunized (group 2) animals (5.9 ± 0.62) also showed significantly higher serum IgG antibody levels than the control group 3b (1.34 ± 0.2). The results of antibodies to Mamu-A*08 were similar to those of Mamu-A*01, except that no significant difference was found between groups 1 and 2 (Fig. 4c). The pre-immunization absorbance values were negligible. A similar analysis of IgA antibodies also showed highly significant ANOVA results for both Mamu-A*01 ($F=10.03$, $P=0.0002$) and Mamu-A*08 ($F=17.6$, $P<0.0001$), with higher antibody levels in the immunized groups compared with the corresponding control groups ($P<0.05$) (Fig. 4b, d), but there was a negligible difference between groups 1 and 2 to Mamu-A*01 (Fig. 4b).

As with Mamu, IgG and IgA antibodies to HIV (strain YU2) gp120 showed a significant ANOVA result ($P<0.0001$) and higher IgG and IgA antibody levels were found in group 1 than in group 2 immunized macaques (Fig. 4e, f). Altogether, these data suggested that rectal immunization followed by SC immunization was less effective than SC immunization alone in eliciting IgG and IgA antibodies to the allo-antigens and to HIV gp140. Antibodies to SIV p27 were elicited but failed to show significant differences in antibody levels between the

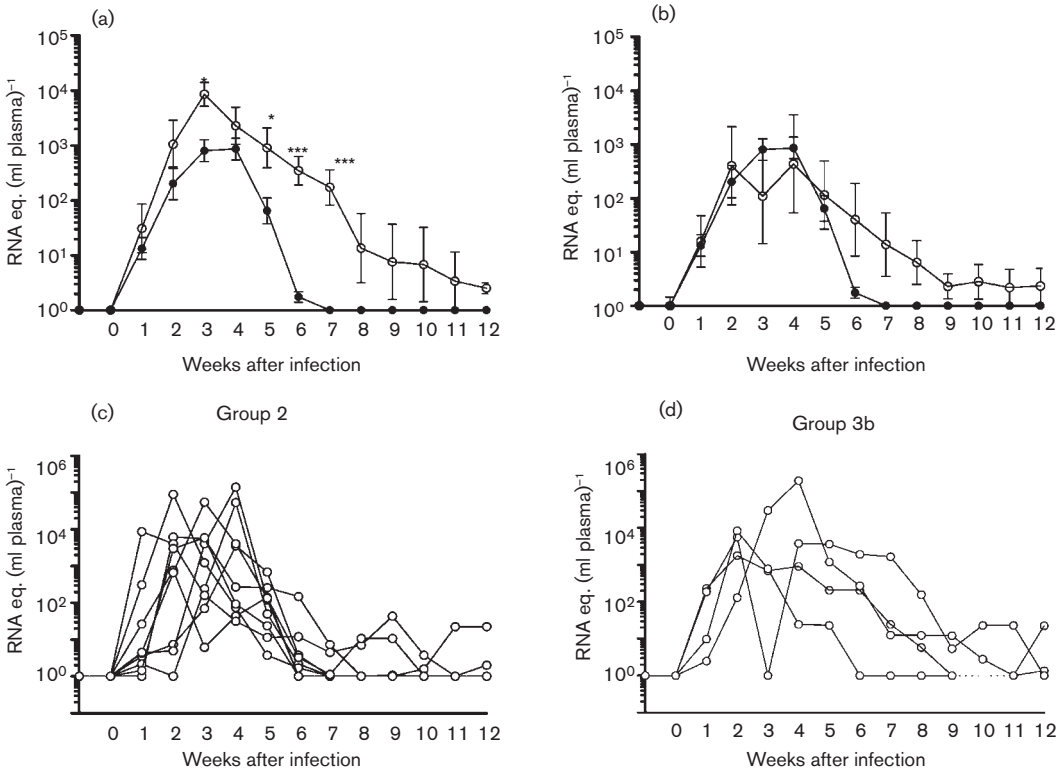

Fig. 3. (a, b) Sequential viral load [RNA eq. (ml plasma)$^{-1}$] in animals immunized rectally, followed by SC immunization with the vaccine but using CpG-C in the mucosal immunization and TiterMax in the SC immunization (●, group 2), compared with untreated controls (○, group 4) (a) and adjuvant-treated macaques (●, group 3b) (b). (c, d) Sequential viral load as described in Fig. 2 is presented for individual macaques in groups 2 (c) and 3b (d). *$P<0.05$; ***$P\leqslant 0.001$.

groups and were not analysed further (data not shown). There was no obvious difference in IgG or IgA titre to the allo-antigens or to HIV gp120 between the three protected and six infected macaques in group 1.

Rectal and vaginal IgG and IgA antibodies to Mamu class I antigens and HIV gp120

An ELISA of rectal washings showed modest increases in IgG and IgA antibodies to Mamu-A*01 (Fig. 5a, b) and HIV gp120 (Fig. 5c, d), but these were significant only for IgA antibodies to Mamu-A*01 (Fig. 5b). However, vaginal IgG and IgA antibodies to both Mamu-A*01 (Fig. 5e, f; $P=0.01$ and $P<0.05$, respectively) and HIV gp120 (Fig. 5g, h; both $P<0.001$) were significantly raised in SC-immunized (group 1) animals compared with the group 3a controls. In the rectal/SC-immunized (group 2) animals, a significant increase in antibodies was not found.

Plasma and mucosal fluid CC chemokines

Plasma and mucosal fluid CC chemokines were assayed before immunization and 4 weeks after the last immunization and before the first rectal challenge. Three CC chemokines were assayed first in plasma, which showed significant upregulation of regulated upon activation, normal T-cell expressed, and secreted (RANTES; $P<0.05$) and macrophage inflammatory protein-1 (MIP-1α; $P<0.05$ or $P\leqslant 0.01$) after immunization in groups 1 and 2, but, with the exception of group 3b, in none of the controls (Table 1a, b). Only RANTES was significantly increased in the rectal and vaginal fluid ($P<0.01$ and $P<0.05$, respectively) (Table 1c, d) in group 1 but not in group 2, although group 3b showed a significant increase in RANTES and MIP-1α. Overall, RANTES was significantly upregulated in all three fluids of the protected group 1 animals but only in the plasma of group 2 animals, showing limited protection. MIP-1α was also increased only in the plasma of both immunized groups of animals, and MIP-1β failed to show a significant increase in any of the animals or fluids.

Correlation between peak viral load and plasma IgG or IgA antibodies to the allo-antigens and HIV gp120

SC immunization elicited a high inverse correlation coefficient (r) between the peak viral load and antibodies to the Mamu-A*01 and Mamu-A*08 allo-antigens (Fig. 6a–d), but only IgG antibodies reached significance (Fig. 6a,

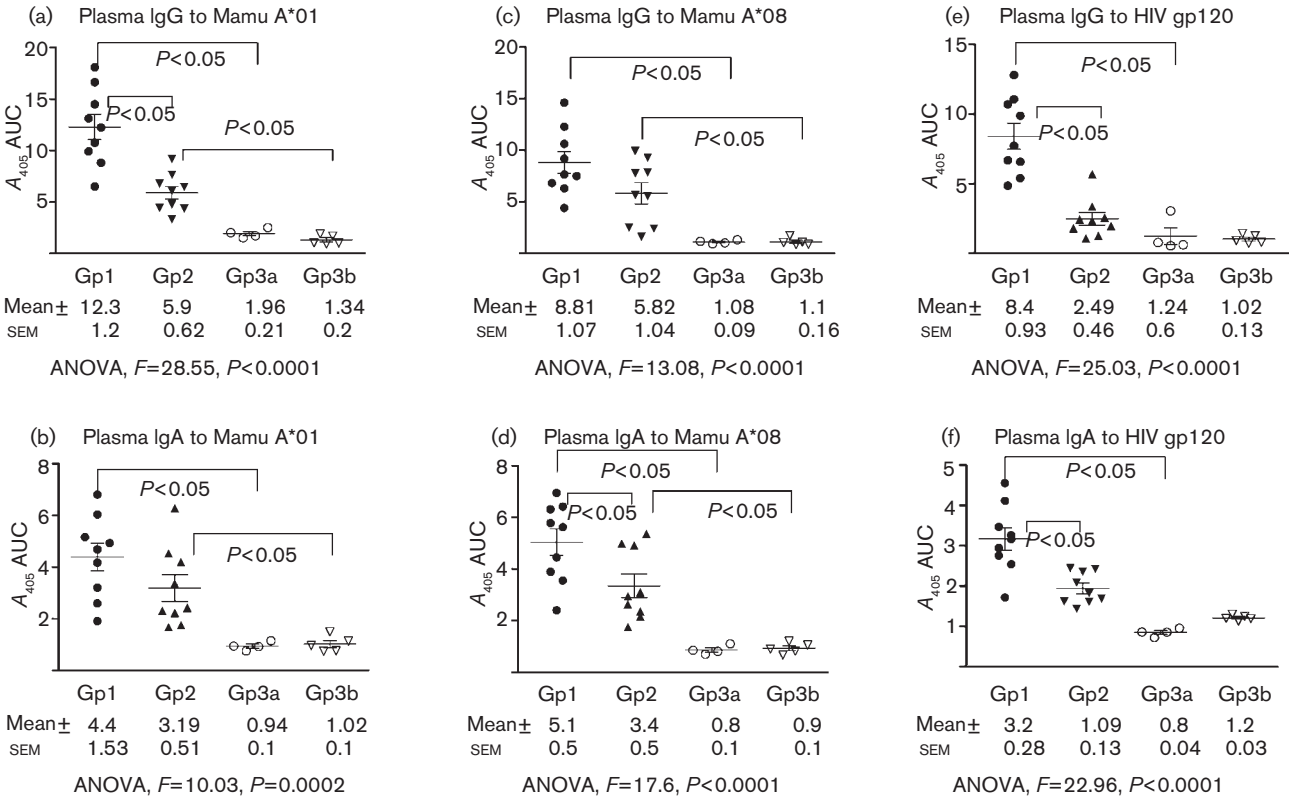

Fig. 4. Comparative plasma IgG (a, c) and IgA (b, d) levels against Mamu-A*01 (a, b) and Mamu-A*08 (c, d) and anti-HIV gp120 IgG (e) and IgA (f) antibodies in macaques SC-immunized macaques (group 1) or rectal/SC-immunized macaques (group 2) using CpG-C in the mucosal and TiterMax in the SC immunization. Control group 3a animals were SC-immunized with $HSP_{359-609}$ + dextran + TiterMax and group 3b were immunized rectally with CpG-C instead of TiterMax and SC boosted with the adjuvant used in group 3a. The results are expressed as means (± SEM) of the absorbance of the AUC after the last immunization, 1 or 2 days before challenge with SHIV SF162.P4.

b). Rectal/SC immunization (group 2) also showed high *r* values with IgG antibodies (−0.48 and −0.4, respectively), but only IgA antibodies to Mamu-A*01 and the peak viral load were significantly different (data not presented). However, the peak viral load failed to show an inverse correlation with plasma anti-HIV gp140 IgG or IgA antibodies after SC or rectal/SC immunization (data not presented).

Correlation between peak viral load and rectal or vaginal IgG and IgA antibodies to the allo-antigens and HIV gp120

SC immunization failed to show any correlation between peak viral load and rectal IgG antibodies to Mamu-A*01 or HIV gp120, unlike the strong trend of inverse correlation with the corresponding IgA antibodies (data not presented). However, vaginal IgG and IgA antibodies to Mamu-A*01 ($P=0.01$ and $P=0.002$, respectively; Fig. 6e, g) and HIV gp120 ($P=0.003$; Fig. 6h) showed a significant inverse correlation with the peak viral load after SC immunization, and a strong trend with IgG to HIV gp120 ($P=0.078$; Fig. 6f). Rectal/SC immunization (group 2) failed to show any correlation between peak viral load and either IgG or IgA to Mamu-A*01 or HIV gp120 in the two mucosal fluids (data not shown).

Correlation between CC chemokines and viral load

Significant inverse correlations were found between peak viral load and plasma MIP-1α levels in groups 1 ($P=0.01$) and 2 ($P<0.05$) (Fig. 7c, d), but not with RANTES (Fig. 7a, b) or with MIP-1β (results not shown). Vaginal fluid showed a significant inverse correlation between peak viral load and RANTES ($P=0.003$) only in group 1 (Fig. 7e) and not with MIP-1α. Rectal fluid failed to show a significant correlation (data not presented). These results are consistent with RANTES predominantly blocking the CCR5 co-receptors in the vaginal mucosa and plasma, preventing HIV-1 entry into $CD4^+$ T-cells in the SC-immunized animals.

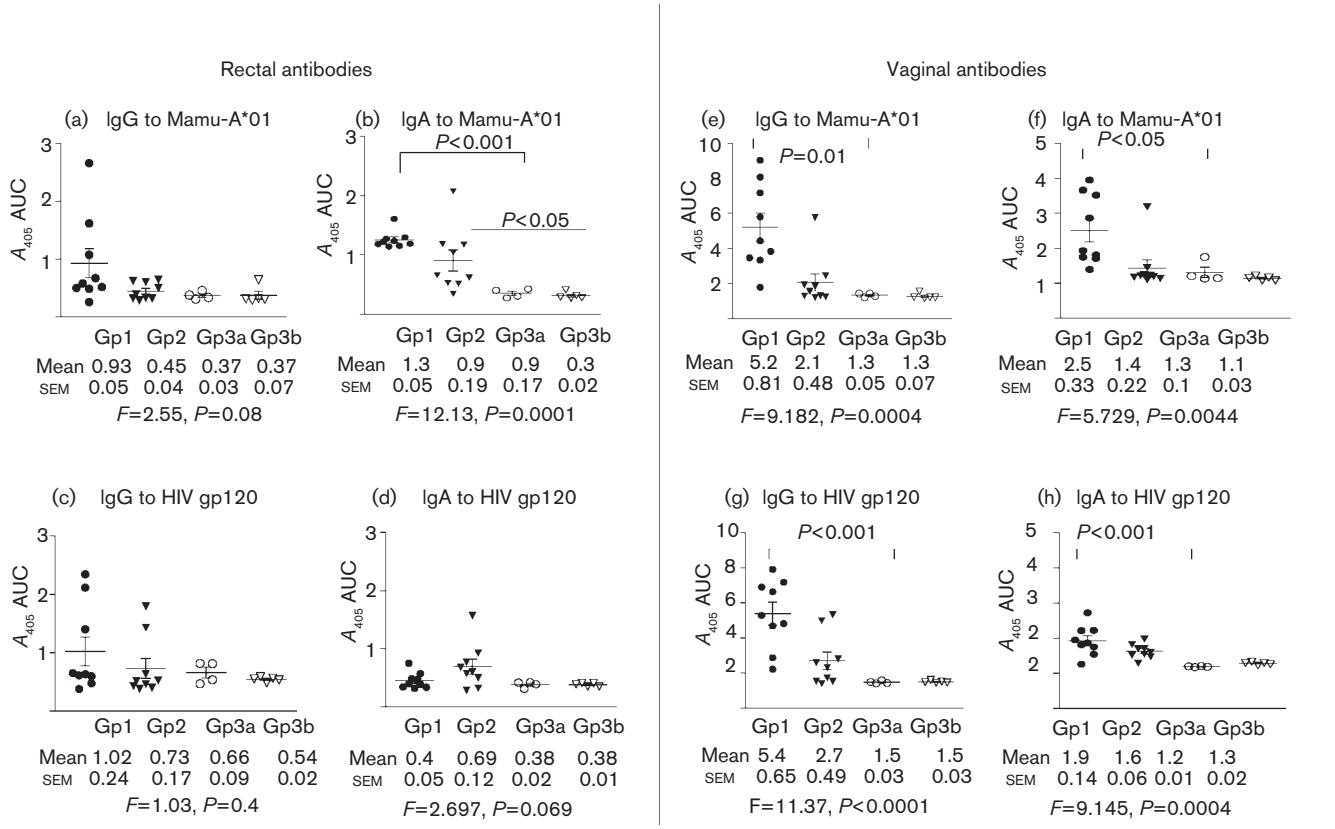

Fig. 5. Comparative rectal (a–d) and vaginal (e–h) IgG and IgA anti-Mamu-A*01 (a, b, e and f) and anti-HIV gp120 (c, d, g and h) antibody levels in macaques SC immunized with the vaccine (group 1) or rectal/SC-immunized (group 2) using CpG-C in the mucosal and TiterMax in the SC immunizations. Control group 3a animals were treated with $HSP_{359–609}$ + dextran + TiterMax and group 3b animals were treated rectally using CpG-C instead of TiterMax and SC boosted with the adjuvant used in group 3a. The results are expressed as means (± SEM) of the AUC of absorbance after the last immunization, 1 or 2 days before challenge with SHIV SF162.P4.

Neutralizing activity

Complement-dependent and -independent neutralizing activity was tested by a T2M-bl-based assay. Neutralizing activity was not detected in control animals after the last immunization. Although a low-titre, complement-independent, neutralizing activity greater than the IC_{50} using SHIV SF162.P4 grown in rhesus PBMCs (passaged in a macaque expressing Mamu-A*01 and DRB*W1/W2), was found in four out of nine group 1 immunized animals after the last immunization, this was also seen in the pre-immunized animals (data not presented) and was not considered to be significant.

Mamu alleles

Mamu class I and II alleles were determined, and the protective Mamu class I lineage Mamu-A*01 or Mamu-A*08 was not found in these Chinese macaques (Goulder & Watkins, 2008). However, seven animals showed Mamu-B*17, of which two were in the immunized group 1, three in the adjuvant-treated group 3a and one in the untreated group 4. Of the three immunized and completely protected animals in group 1, only one expressed Mamu-B*17, whilst another was infected. Surprisingly, three out of four of the adjuvant-treated group 3a animals expressed Mamu-B*17 and, although they were all infected, the viral load was lower than that in the untreated animals, consistent with the Mamu-B*17 allele having some protective effect. Mamu-B*17 was also found in one of the group 2 animals and one in group 3b adjuvant-treated control, but none was protected. All five macaques in the untreated group, one of which expressed Mamu-B*17, were infected and the viral load was not decreased. As the animals expressed neither Mamu-A*01 nor Mamu-A*08, it is unlikely that self-tolerance had been broken by immunization with the Mamu-A*01 and Mamu-A*08 antigens.

DISCUSSION

Following protection or inhibition of SHIV infection elicited by xeno-immunization with a recombinant HLA class I and II HIV–SIV antigens and HSP70 dextramer

Table 1. Analysis of RANTES, MIP-1α and MIP-1β in plasma and in rectal and vaginal fluid in immunized and control macaques

The concentrations of RANTES, MIP-1α and MIP-1β in plasma (a, b) and in rectal (c) and vaginal (d) fluid of SC-immunized group 1, its control (group 3a), rectal/SC-immunized group 2, its control (group 3b) and the untreated control (group 4). Fluids were collected before and after the last immunization. Concentrations were determined before (Pre) and after (Post) immunization. NS, Not significant.

(a) Plasma

	RANTES (ng ml^{-1})		MIP1-α (pg ml^{-1})		MIP-1β (pg ml^{-1})	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**
Group 1	18.5±2	24.5±1.3*	83.5±11.7	159.6±27.6*	8.2±0.5	9.6±1.6
Group 2	16.5±2	21.3±1.1*	89.9±12.5	129.6±7.6†	6.9±0.5	7.1±0.4
Group 3a	18.9±2.1	13.2±3.6	92.3±12.1	80.6±16.9	7.4±0.5	8±2.1
Group 3b	15.8±1.2	21±1.5†	63.8±11.9	123.2±17†	5.3±0.4	7.4±0.3
Group 4	11.9±1.3	12.7±3.2	67.4±10.4	72.4±13.6	6.1±0.8	62±0.7

(b) ANOVA of the post-immunization groups followed by the Bonferroni's multiple comparison test

	RANTES		MIP-1α	
F=	7.254		3.039	
P=	0.0004		0.034	
	t	***P***	***t***	***P***
Group 1 vs 3a	3.954	<0.01	2.545	NS
Group 1 vs 4	4.475	<0.001	3.029	<0.05
Group 2 vs 3b	0.1223	NS	0.2238	NS
Group 2 vs 4	3.258	<0.05	0.2238	NS

(c) Rectal fluid

	RANTES (pg ml^{-1})		MIP-1α (pg ml^{-1})		MIP-1β (pg ml^{-1})	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**
Group 1	38±3.6	56.1±3.1†	12.1±1.5	16.3±2.9	23.4±1.8	33±3.6
Group 2	39.8±8.4	54.7±3.1	9.8±2.7	12±2	14.7±3.2	24.1±2.1
Group 3a	40±5.9	42.5±8.1	9.3±4	13.5±4	22.8±4	21.9±3.1
Group 3b	36.2±10.5	40.4±6.8	5.9±2.5	9.8±3	8.4±3.8	16±4.2
Group 4	51.6±4.1	48.8±3.1	11.2±2.4	8±0.3	21.4±4.1	20.8±1.8

(d) Vaginal fluid

	RANTES (pg ml^{-1})		MIP-1α (pg ml^{-1})		MIP-1β (pg ml^{-1})	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**
Group 1	15.2±3.2	46.1±12.6*	11.6±2.6	21.9±6.8	12.3±3	36.6±12.8
Group 2	15.4±2.9	20.1±3.7	9.5±4.3	6.4±2.6	9.8±1.9	16.4±4.1
Group 3a	21±5.3	15±1.1	9±2.2	10.5±3	17.6±2.7	7.4±2.4
Group 3b	18.3±3.2	26.1±3.3*	17.4±4.4	25.6±6.2*	12.1±2.2	19.1±5.2
Group 4	14.7±4.4	12.9±2	13.3±2.7	21.6±3.8	10.8±3.4	14.2±3.3

*$P<0.05$, compared with pre-immunized samples.
†$P\leqslant 0.01$, compared with pre-immunized samples.

construct (Mörner *et al.*, 2011), the critical question was to ascertain whether allo-immunization was equally effective, as translation from macaque studies to those of humans would necessitate allo-immunization. It was essential to establish whether the recombinant allogeneic constructs were immunogenic, whether they elicited systemic and mucosal antibodies and whether they induced protection against rectal mucosal challenge. Indeed, the recombinant Mamu constructs administered SC (three times) either prevented SHIV infection in three of nine macaques or significantly decreased sequential and peak viral loads in the animals when challenged rectally by up to ten repeated doses of heterologous SHIV SF162.P4. However, rectal mucosal immunization followed by SC immunization failed to prevent SHIV SF162.P4 transmission, although the peak viral load was significantly decreased, and one of

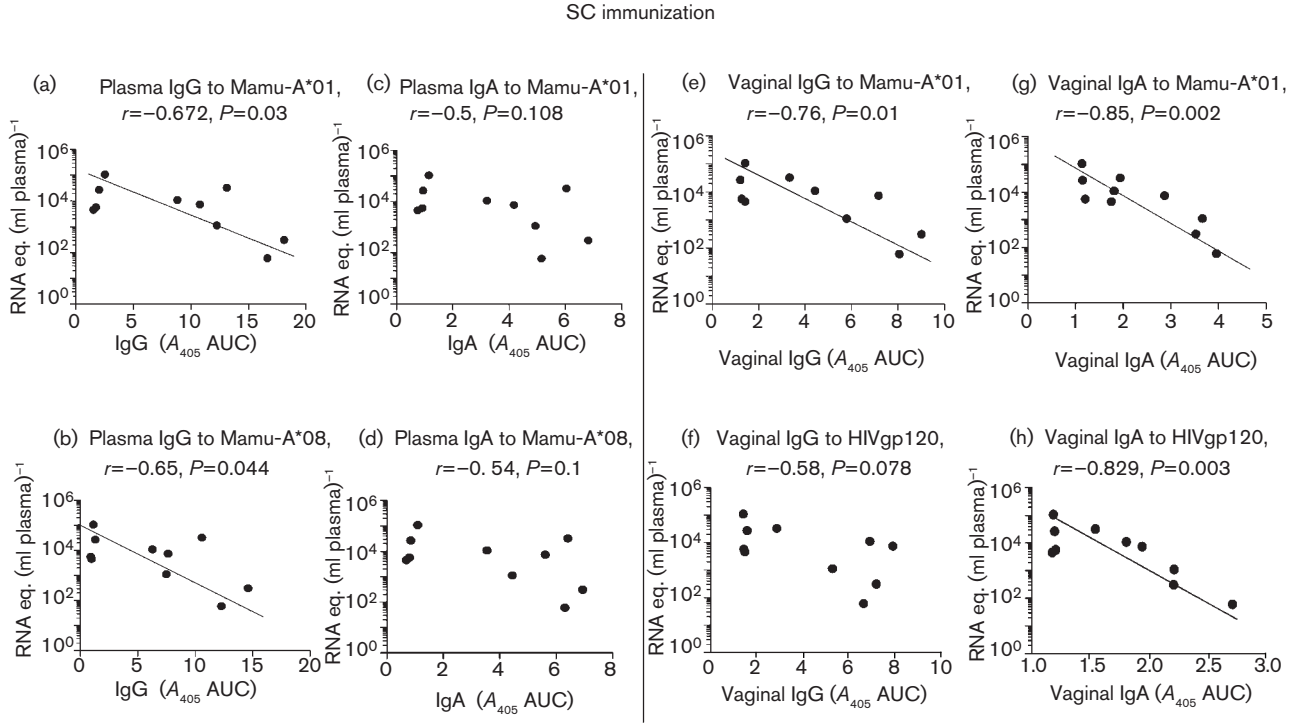

Fig. 6. Correlation between peak viral load [RNA eq. (ml plasma)$^{-1}$] at week 3 after challenge and plasma (a–d) or vaginal (e–h) IgG and IgA antibody levels against Mamu-A*01, Mamu-A*08 or HIV gp120 antigen, after the last immunization and before challenge, expressed as AUC of absorbance in SC-infected animals (groups 1 and 3a).

the five adjuvant-treated controls remained uninfected. The lack of complete protection might be ascribed to the significantly lower serum IgG antibody levels to the Mamu class I and HIV gp120 antigens compared with systemic immunization alone. This was less evident with serum IgA and was not evident with rectal IgG or IgA antibodies. Mucosal allo-immunization has been attempted previously only in one macaque study, applying unmatched mononuclear cells to rectal or vaginal mucosa (Bergmeier *et al.*, 2005). A significant decrease in SIV infectivity was elicited with CD4$^+$ T-cells *ex vivo*, but the animals were not challenged *in vivo*.

It is unlikely that protection and/or a decrease in viral load would have been achieved without either HLA class I and II constructs or HIV and SIV antigens, as HLA xeno-immunization has clearly established that both HLA and HIV/SIV constructs are critical in eliciting protection against SHIV SF162.P4 infection (Mörner *et al.*, 2011). Indeed, no difference from the controls was observed when a group of eight macaques was immunized with the vaccine without the HLA constructs or when another group of eight macaques was immunized without HIV and SIV antigens (Mörner *et al.*, 2011). It is also unlikely that the recombinant HLA constructs exerted significant adjuvant activity, as another control group of six macaques with all vaccine constituents (as in the protected group 1) but without the TiterMax adjuvant showed no protection and poor immune responses. In the early experiments, most macaques were protected from SIV infection if they were immunized with inactivated SIV grown in a human CD4$^+$ T-cell line and challenged with SIV grown in the same human CD4$^+$ T-cell line (Arthur *et al.*, 1995; Carlson *et al.*, 1990; Desrosiers *et al.*, 1989; Dormont *et al.*, 1995; Murphey-Corb *et al.*, 1989; Stott *et al.*, 1994). However, in the more limited studies, protection was greatly decreased if the animals were immunized with human CD4$^+$ T-cells alone (Langlois *et al.*, 1992; Stott, 1991). It is noteworthy that, although the control animals (group 3a) treated with $HSP_{359–609}$ + dextran + TiterMax showed a peak viral load significantly higher than that in the immunized animals, the sequential viral load was less significant than that recorded in the untreated group 4 animals. This is likely to be a non-specific adjuvant effect exerted by the three agents, which has often been observed with other adjuvants. Importantly, none of the systemically treated controls showed complete prevention of SHIV infection, unlike three of the nine immunized animals.

The possibility that serum IgG and mucosal IgA antibodies to the viral construct were correlates of protection is greatly enhanced by the significant inverse correlation between peak viral load and serum or mucosal (vaginal) IgG and to a lesser extent by IgA antibodies. However,

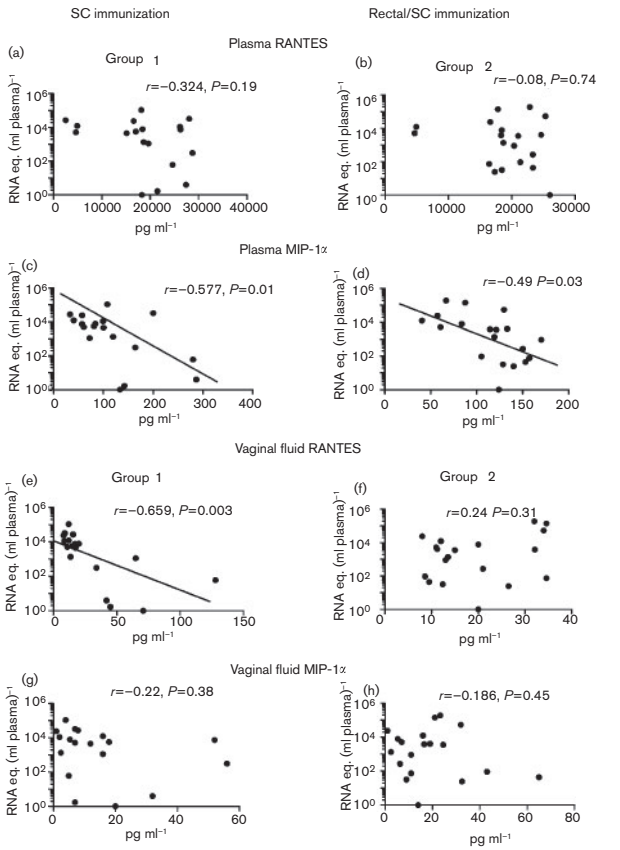

Fig. 7. Correlation between plasma and vaginal fluid RANTES and MIP-1α, collected after the last immunization, and the peak viral load [RNA eq. (ml plasma)$^{-1}$] in the SC-immunized (group 1) and controls (a, c, e, g) or rectal/SC-immunized and controls (b, d, f, h).

complement-dependent or -independent neutralizing activity was not demonstrated, unlike in xeno-immunization in which the former was inversely correlated with viral load (Mörner *et al.*, 2011). The alternative function of antibodies in antibody-dependent cell-mediated cytotoxicity cannot be excluded, although expert advice suggested this to be unlikely. None the less, the protective function of serum antibodies was confirmed in xeno-immunization by preventing SHIV SF162.P4 infection in passive transfer of immune serum from protected to naïve macaques, unlike serum from unimmunized animals (Mörner *et al.*, 2011). Examination of RANTES in the vaginal fluid of group 1 showed a significant inverse correlation with peak viral load ($P<0.01$), as with MIP-1α and peak viral load in the plasma ($P<0.05$). These results suggest that the blocking function by the CC chemokines of CCR5 co-receptors may have inhibited HIV-1 entry into CD4$^+$ T-cells and dendritic cells in the mucosal tissues or in blood. An attempt to differentiate the antibody levels to Mamu antigens or HIV gp120 in the three protected macaques from the six infected macaques in group 1 failed with both serum and mucosal antibodies, as well as with CC chemokines, suggesting that additional immune factors may have been involved, such as innate antiviral factors or cellular immunity.

The difference in antibodies to Mamu and HIV gp120 between the protected SC-immunized group 1 and the rectal/SC-immunized group 2 animals with limited protection is of interest, as it was expected that the SC-boosted rectal immunization might be more effective against rectal challenge with SHIV than immunization by the SC route alone. However, the latter had significantly higher serum and to a lesser extent vaginal IgG and IgA antibodies than the former. Furthermore, whereas the SC-immunized group showed a significant inverse correlation between peak viral load and serum IgG and to a lesser extent IgA antibodies to Mamu antigens, the converse was found in the rectal/SC-immunized animals. Vaginal IgG and IgA antibodies to both Mamu and HIV gp120 antibodies showed a significant inverse correlation (or a trend) with peak viral load only in the SC-immunized animals. These differences in antibody titres and correlation with viral load may account for the prevention of infection in three macaques and the greater decrease in the viral load in the SC-immunized compared with the rectal/SC-immunized animals. The possibility of rectal mucosal tolerance elicited by rectal/SC immunization cannot be excluded. An intriguing observation was that rectal washings showed only modest increases in IgG and IgA antibodies when compared with vaginal washings. The difference between vaginal and rectal antibodies might be accounted for by the iliac lymph nodes functioning as an inductive immunological site from which B- and T-cells home preferentially to the vaginal and cervical mucosa, compared with rectal, colonic or control ileal tissue (Mitchell *et al.*, 1998).

Among the CC chemokines, only RANTES (CCL-5) was significantly increased in plasma and in rectal and vaginal fluid in the SC-immunized but not in the rectal/SC-immunized animals. Furthermore, a significant inverse correlation between the peak viral load and RANTES was found only in the vaginal fluid of the SC-immunized animals. MIP-1α showed a significant increase only in plasma and significant inverse correlations with the peak viral load in both group 1 and 2 animals. It is therefore likely that only RANTES enhanced the protection elicited by IgG and IgA specific antibodies.

In conclusion, we have presented evidence that only systemic allo-immunization with recombinant Mamu class I and II constructs with HIV/SIV antigens elicits significant protection when challenged with heterologous SHIV SF162.P4, either preventing infection or decreasing the viral load by up to 3 logs. The potential disadvantage of allo-immunization is that it induces allo-antibodies, which might cause allograft rejection, if one were required. However, in HIV-1-endemic regions, the risk-to-benefit ratio of potential allograft failure argues overwhelmingly in favour of allo-immunization. Systemic allo-immunization may prove to be an alternative vaccine strategy, especially

as it is independent of HIV mutation and cytotoxic T-lymphocyte or antibody escape. This is the first demonstration that systemic allo-immunization with a recombinant MHC class I and II, HIV gp140 and SIV p27 complex elicits significant inhibition of heterologous SHIV infection in macaques.

METHODS

Selection and preparation of recombinant Mamu alleles. Three Mamu class I and one class II alleles were selected on the basis of the frequency of these alleles found in Chinese rhesus macaques and the appropriate Mamu allele/peptide combinations for efficient folding: (i) Mamu-A*01 (CTPYDINQM), (ii) Mamu-A*08 (KPCVKLTP) and (iii) Mamu-B*17 (IRFPKTFGW). Preparation of these Mamu class I heavy chains and β_2-microglobulin has been described previously (Schøller *et al.*, 2010). The Mamu class II protein (DRB*W602) was purchased from Dr W. Kwok (Benaroya Research Institute, Seattle, WA, USA).

Preparation of trimeric HIV gp140, SIV p27 and $HSP_{359–609}$. Monobiotinylated HIV strain YU2 gp140 trimer and SIV Gag p27 were prepared as described previously (Mörner *et al.*, 2011). The 28 kDa C-terminal fragment of *Mycobacterium tuberculosis* HSP70 ($HSP_{359–609}$) (Babaahmady *et al.*, 2007) was subcloned to enable *in vitro* biotinylation, as described elsewhere (Mörner *et al.*, 2011). All biotinylated vaccine components were linked to streptavidin-coated divinyl sulfone-activated dextran (Scholler *et al.*, 2010) using concentrations described previously (Mörner *et al.*, 2011).

Animals and immunization and SHIV challenge. Thirty-two rhesus macaques of Chinese origin, serologically negative for SIV, simian retrovirus and simian T-cell leukemia virus, were treated according to the guidelines set out by the Institutional Animal Care and Use Committee at the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences. Immunized groups of nine macaques each were given the vaccine candidate formulated as an emulsion with TiterMax Gold (Sigma-Aldrich) or CpG-C (Invivogen) as shown in Fig. 1(e). The control macaques in group 4 (n=5) were untreated, those in group 3a (n=4) were given only $HSP_{359–609}$ + dextran + TiterMax Gold (SC three times) and those in group 3b (n=5) were given the same formulation but using CpG-C (twice rectally), followed by SC (twice) with the same controls but using TiterMax Gold (twice).

SHIV SF162.P4 was expanded in macaque PBMCs expressing the Mamu-A*01 and DRB*W1/W2 alleles (provided by Dr Nancy Miller, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA). Rectal challenges were carried out 4 weeks after the last immunization with 25 $TCID_{50}$ SHIV SF162.P4 twice weekly until the animal was infected [>200 copies (ml plasma)$^{-1}$] and this was carried out up to ten times, after which the three uninfected animals were considered to be protected. The plasma viral load was monitored by real-time reverse transcriptase activity, and translated into RNA equivalents ml^{-1}; the detection limit was 200 copies ml^{-1}.

MHC genotypes of class I and II alleles. MHC genotypes relevant to this study were examined by PCR with sequence-specific primers or by cloning and sequencing methods (Otting *et al.*, 2011; Qiu *et al.*, 2008) of Mamu-A*01, Mamu-A*08, Mamu-B*17, DQB1*18, DRB1*w101 and DRB1*w201. DNA or RNA was isolated from the PBMCs of these monkeys, and locus-specific or allele-specific primers were used to amplify the alleles. The genotypes were determined by gel electrophoresis, direct sequencing or cloning into vectors for sequencing (Table S2).

Collection of blood and rectal and vaginal fluids. Blood and rectal and vaginal fluids were collected before and after each immunization and before virus inoculation, as described previously (Lehner *et al.*, 1994) (Fig. 1e). The blood plasma and fluids were stored at −80 °C until use.

Antibody assays of serum, rectal and vaginal fluid IgG and IgA antibodies to Mamu-A*01, Mamu-A*08 and HIV gp140 by ELISA. IgG and IgA antibodies to Mamu-A*01, Mamu-A*08 and HIV gp140 were assayed using ELISA, as described previously (Bogers *et al.*, 2004). IgG and IgA antibody titres are presented as absorbance values by calculating the AUC.

Neutralizing activity. Macaque sera were tested for their neutralization activity against SHIV SF162.P4, propagated in rhesus PBMCs. A TZM-bl cell-based assay was used, i.e. HeLa-cell derivatives that express high levels of CD4 and both CCR5 and CXCR4 co-receptors. Neutralization activity in serum was analysed in the presence of complement, using serum from a healthy AB^+ blood donor as a source of complement, and in the absence of complement, using heat-inactivated AB^+ serum (Mörner *et al.*, 2011).

Assay of RANTES, MIP-1α and MIP-1β in plasma and mucosal fluids. Quantification of RANTES, MIP-1α and MIP-1β was carried out using a Luminex bead assay using Fluorokine Multianalyte Profiling kits (R&D). Twenty microlitres of 1 : 50-diluted plasma and undiluted rectal and vaginal fluid samples was added to a mix of analytes for RANTES, MIP-1α and MIP-1β and incubated for 3 h, followed by strepavidin-labelled antibodies to the chemokines and phycoerythrin–biotin. The beads were then analysed on a Bio-Plex 200 System (Bio-Rad).

Statistical analysis. ANOVA with a Bonferroni multiple comparison test was used when three or more comparisons were made. Spearman's rank correlation coefficient was applied for analysis of correlations between the viral load and immunological parameters. Statistical analysis of the sequential viral load was carried out by aligning the starting week of infection for each macaque in a group, to account for different times of infection following repeated doses of virus challenge. The AUC was used for the absorbance of all serially diluted serum antibody titrations (Gilbert *et al.*, 2010).

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