

Adjuvanting a DNA vaccine with a TLR9 ligand plus Flt3 ligand results in enhanced cellular immunity against the simian immunodeficiency virus

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DNA vaccines offer promising strategies for immunization against infections. However, their clinical use requires improvements in immunogenicity. We explored the efficacy of Toll-like receptor (TLR) ligands (TLR-Ls) on augmenting the immunogenicity of a DNA prime-modified vaccinia virus Ankara (MVA) boost vaccine against SIV. Rhesus macaques were injected with Fms-like tyrosine kinase 3 (Flt3)-ligand (FL) to expand dendritic cells (DCs) and were primed with a DNA vaccine encoding immunodeficiency virus antigens mixed with ligands for TLR9 or TLR7/8. Subsequently, the animals were boosted with DNA and twice with recombinant MVA expressing the same antigens. TLR9-L (CpG DNA) mediated activation of DCs in vivo and enhanced the magnitude of antigen-specific CD8⁺ interferon (IFN) γ ⁺ T cells and polyfunctional CD8⁺ T cells producing IFN- γ , tumor necrosis factor α , and interleukin 2. Although this trial was designed primarily as an immunogenicity study, we challenged the animals with pathogenic SIVmac₂₅₁ and observed a reduction in peak viremia and cumulative viral loads in the TLR9-L plus FL-adjuvanted group relative to the unvaccinated group; however, the study design precluded comparisons between the adjuvanted groups and the group vaccinated with DNA/MVA alone. Viral loads were inversely correlated with the magnitude and quality of the immune response. Thus, the immunogenicity of DNA vaccines can be augmented with TLR9-L plus FL.

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Abbreviations used: AUC, area under the curve; CCR, CC chemokine receptor; FL, Fms-like tyrosine kinase 3 (Flt3)-ligand; ICC, intracellular cytokine; i.d., intradermal(ly); MVA, modified vaccinia virus Ankara; NHP, nonhuman primate; pDC, plasmacytoid DC; pDNA, plasmid DNA; TLR, Toll-like receptor, TLR-L, TLR ligand.

The discovery that antigen-encoding DNA plasmids could stimulate humoral and cellular immune responses represents a pivotal milestone in vaccine research (1–3). This discovery enabled the development of one of the most promising vaccination strategies against HIV, the so called “prime-boost” strategy in which the immune response is primed with a plasmid DNA (pDNA) and subsequently boosted with a viral vector such as modified vaccinia virus Ankara (MVA) or adenovirus expressing the relevant antigens (4–7). Studies in nonhuman primates (NHPs) suggest that such prime-boost strategies typically induce antigen-specific CTL responses and result in a lowering of the viral load upon challenge,

although they do not clear the infection (8–10). In humans, although DNA vaccines have been shown to induce immune responses against several pathogens (such as malaria, hepatitis B, and HIV), a major limitation is their poor protective immunogenicity despite the high doses of DNA used (7, 11–13). Therefore, strategies to optimize the immunogenicity of DNA vaccines are clearly needed.

Recent advances in our understanding of the immune system suggest that the innate immune system, particularly DCs and pattern recognition receptors such as Toll-like receptors (TLRs), plays a critical role in initiating adaptive immune responses and in modulating their strength and quality. Thus, there is presently much interest in understanding the

The online version of this article contains supplemental material.

nature of the innate immune parameters that qualitatively govern different types of adaptive immune responses, as well as in exploiting these in the development of novel vaccines against global pandemics and chronic infections such as HIV (14). In this context, given the critical roles played by DCs and TLRs in adaptive immunity, they represent promising targets for boosting vaccine immunogenicity (15, 16). Numerous previous studies have demonstrated the efficacy of using TLR ligands (TLR-Ls) in augmenting both cellular and humoral immune responses to protein antigens or DNA-encoded antigens in mice (17–20). Furthermore, recent studies using NHPs have demonstrated that a TLR7/8-L formulated or covalently linked to recombinant Gag results in an enhancement in the magnitude and “quality” of the T cell response (21, 22). Importantly, TLR-Ls are beginning to be used as vaccine adjuvants in humans. For example, monophosphoryl lipid A, an agonist of TLR4, is being developed by GlaxoSmithKline for use as a vaccine adjuvant and was recently licensed in Europe as a component of an improved vaccine for hepatitis B (Fendrix) (23). The TLR9-L CpG DNA has been administered with the hepatitis B vaccine Engerix-B and was shown to enhance the frequency of seroconversion as well as the magnitude of the antibody response (24, 25). However, in the case of DNA vaccines, despite several experiments demonstrating the induction of strong immunity in mice (1), translation into NHPs or humans has been disappointing.

We performed a preclinical vaccine study in rhesus macaques to test the hypothesis that activated DCs would elicit robust levels of antiviral immunity in prime-boost vaccination against HIV. To this end, we have harnessed the ability of Fms-like tyrosine kinase 3 (Flt3)-ligand (FL) to enhance DC numbers in vivo (26–31) and specific ligands for TLR9 (CpG DNA type B) (19, 32) and TLR7/8 (3M compound 003) to induce DC activation in a rhesus model. Our data suggest that adjuvanting a DNA vaccine with a single injection of CpG DNA at the time of priming results in a pronounced enhancement of the magnitude of the antigen-specific CD8⁺ T cell response and translates into improved control of viral loads after challenge with SIV.

RESULTS

Experimental protocol

The experimental groups and immunization and analyses schedule are shown in Table I and Fig. S1 (available at <http://www.jem.org/cgi/content/full/jem.20071211/DC1>). There were five cohorts with five animals per cohort. Group 1 was vaccinated with the prime-boost approach described previously (i.e., with pDNA expressing the SIV antigens Gag and Pol, as well as HIV89.6 antigens Env, Nef, Vpr, and Vpu) (10) as a multiprotein vaccine without adjuvants. The other three groups of monkeys (groups 2–4) received daily subcutaneous injections of FL for 14 d (from days 1 to 14), and on day 15 were injected intradermally (i.d.) with pDNA alone (group 2), pDNA mixed with TLR9-L (group 3), or pDNA mixed with TLR7/8-L (group 4). In week 11, all animals in groups 1–4 received a secondary immunization with pDNA alone. Then, in weeks 36 and 58, the animals were boosted with rMVA expressing the Gag (SIV), Pol (SIV), and Env (HIV) genes. Group 5 was not vaccinated and did not receive any FL treatment. The time points at which immunological analyses were performed are indicated in Fig. S1.

Administration of FL results in a profound enhancement in the numbers of blood CD11c⁺ DCs and monocytes

DC expansion and activation in the blood was evaluated by flow cytometry (Fig. 1 A). Three subsets of APCs in the blood were identified, as previously described (30, 31, 33, 34). In brief, lineage-positive cells were excluded using a cocktail of antibodies directed against B cells, T cells, and monocytes, and the lineage-negative, HLA-DR⁺ cells were evaluated for the expression of CD11c⁺ and CD123⁺. CD11c⁺ myeloid DCs were defined as CD11c⁺HLA-DR⁺Lin⁻ cells, and CD11c⁻ plasmacytoid DC (pDC) precursors were defined as CD11c⁻CD123⁺HLA-DR⁺Lin⁻ cells. Monocytes were defined as CD14⁺HLA-DR⁺ cells within an entire PBMC population (Fig. 1 A). Administration of FL markedly increased the numbers of HLA-DR⁺Lin⁻ cells from 0.51% at the start of the treatment to 19.9% (39-fold increase) at the completion of FL injection (day 14). This increase was primarily caused by a 51.8-fold expansion of CD11c⁺ DCs (from 0.2 ± 0.12% at day 0 to 9.45 ± 6.99%

Table I. Experimental protocol

Group	n	Days		Weeks/injections				
		1–14 FL treatment ^a	2 (day 15) Vaccine prime	11 Vaccine boost	36 Viral boost I	58 Viral boost II	77 SIV challenge	
1	5	–	pDNA	pDNA	rMVA	rMVA	+	
2	5	+	pDNA	pDNA	rMVA	rMVA	+	
3	5	+	pDNA+TLR9-L	pDNA	rMVA	rMVA	+	
4	5	+	pDNA+TLR7/8-L	pDNA	rMVA	rMVA	+	
5	5	–	–	–	–	–	+	

Each experimental group consisted of five animals. Groups 2, 3, and 4 were treated by daily injections of FL protein for 14 days. At day 15, groups 1–4 were injected with pDNA encoding for HIV/SIV proteins in PBS only (groups 1 and 2), or formulated with TLR9-L (group 3) or TLR7/8-L (group 4). Furthermore, vaccination groups were injected with naked pDNA vaccine without TLR-Ls at week 11 and were boosted with rMVA expressing HIV/SIV antigens at weeks 36 and 58. Group 5 remained untreated until challenge. All animals were challenged intrarectally with a SIVmac₂₅₁ virus at week 77 of the experiment.

^aDaily injections for 14 consecutive days.

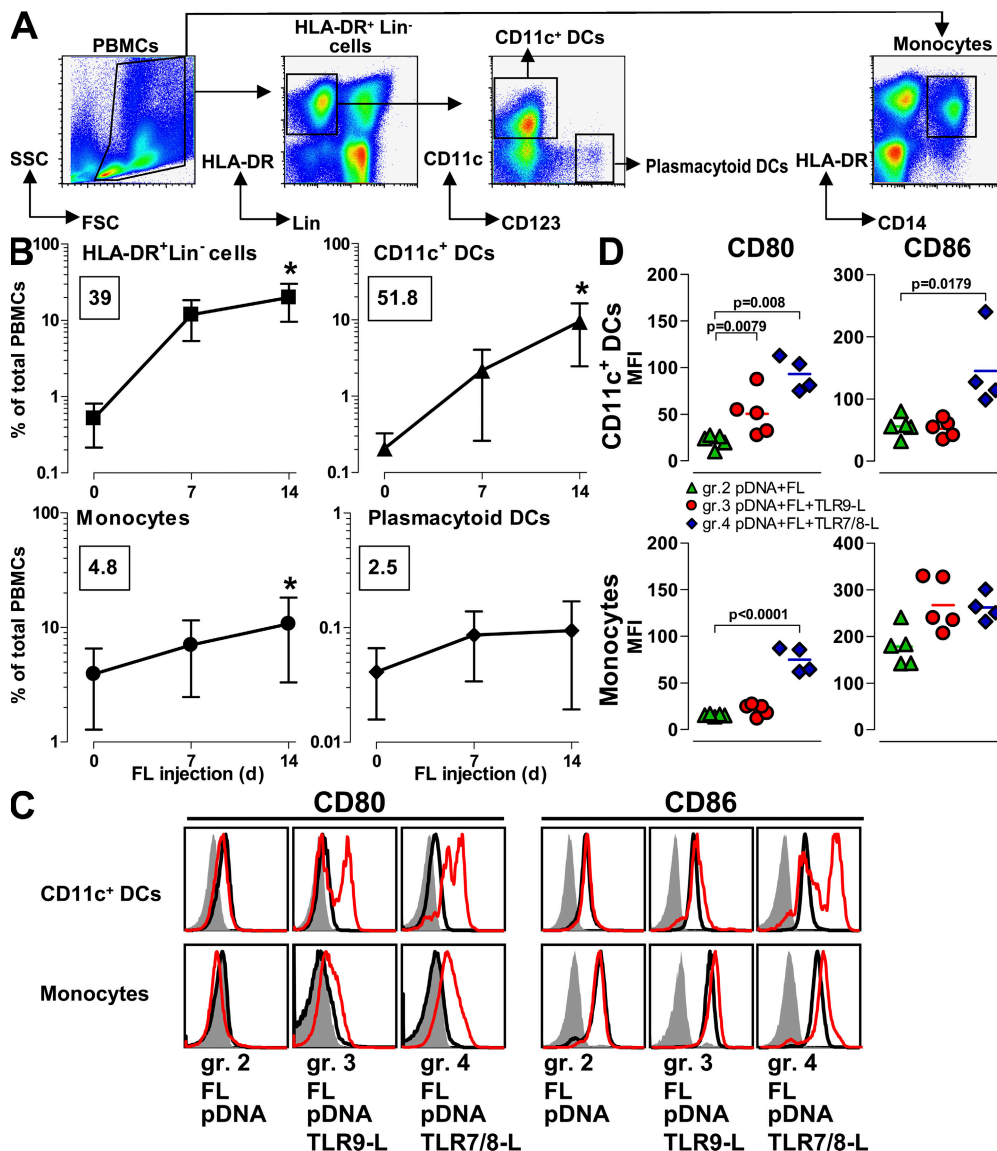


Figure 1. Expansion and activation of APCs in vivo. (A) Gating strategy for APC populations in FL-treated animals. Total PBMCs were isolated using standard procedures, and the mononuclear fractions were analyzed by flow cytometry to assess the frequencies of various APCs in the blood. Total DC population subsets were defined by a lack of lineage marker expression ($CD3^-CD14^-CD20^-$) and expression of HLA-DR. $CD11c^+$ DCs were defined as $CD11c^+HLA-DR^+Lin^-$, and pDCs were defined as $CD123^{++}HLA-DR^+CD11c^-Lin^-$. Monocytes were defined as $HLA-DR^+CD14^+$ within the entire PBMC population. (B) Animals were injected subcutaneously with 100 μ g/kg FL daily from days 0 to 14. The frequencies of specific APC subsets in the blood at the indicated time points are shown. Numbers in squares represent the mean fold increase between days 0 and 14. Error bars represent SD. Mean frequencies of specific APC subsets at days 0 and 14 were compared by a nonparametric Mann-Whitney test ($n = 5$). $P < 0.05$ was considered statistically significant (*). (C) Activation of $CD11c^+$ DCs and monocytes was assessed by staining for CD80 and CD86 surface markers at days 14 (1 d before vaccination; black line) and 16 (1 d after vaccination; red line). Shading represents the isotype. (D) Mean fluorescent intensity (MFI) of CD80 and CD86 expression was measured on activated $CD11c^+$ DCs and monocytes (four out of five animals are presented in group 4 [blue diamonds]) caused by problems with PBMC isolation. Error bars represent the mean frequencies \pm SEM. Mean MFIs were compared by a nonparametric Mann-Whitney test ($n = 4$ or 5). $P < 0.05$ was considered statistically significant. FSC, forward scatter; SSC, side scatter.

at day 14; Fig. 1 B). A 4.8-fold increase in the frequency of monocytes ($HLA-DR^+CD14^+$) was also noted (from $3.9 \pm 2.63\%$ at day 0 to $10.74 \pm 7.43\%$ at day 14). In contrast, FL increased the numbers of pDC precursors only 2.5-fold (from $0.041 \pm 0.025\%$ to $0.094 \pm 0.075\%$). These observations are consistent with previous studies (30, 31, 33) and demonstrate that FL is a very potent DC growth factor in vivo.

TLR9-L and TLR7/8-L induce activation of FL-expanded DCs and monocytes in vivo

We next evaluated whether injection of TLR9-L or TLR7/8-L resulted in the activation of APCs in vivo. Animals were injected i.d. with TLR9-L or TLR7/8-L mixed with pDNA on day 15 (Table I) and 1 d later (on day 16; Fig. 1 C, red line), and the activation of DCs was evaluated by the up-regulation

of the co-stimulatory molecules CD80 and CD86 relative to the peak of APC expansion at day 14 (Fig. 1 C, black line). The expression of CD80 was substantially up-regulated on both CD11c⁺ DCs and monocytes in animals that received the pDNA plus TLR7/8-L or TLR9-L (red line) in comparison with the group that received the pDNA alone (Fig. 1, C and D). CD86 expression on CD11c⁺ DCs was considerably enhanced by TLR7/8-L, but not by TLR9-L, and was not up-regulated on monocytes by either (Fig. 1, C and D). The reasons for the differential induction of co-stimulatory molecules on the distinct APC subsets by TLR9-L versus TLR7/8 are at present unclear. Furthermore, because TLR9 is reported not to be expressed by CD11c⁺ DCs or monocytes (35), their observed activation *in vivo* is likely to have been caused indirectly by proinflammatory cytokines rather than by direct TLR9-L-mediated stimulation of these cells. In this context, pDCs that express TLR9 and TLR7 (35) were diminished in numbers 1 d after the injection of TLR9-L or TLR7/8-L (unpublished data), likely reflecting the rapid migration of these cells into secondary lymphoid organs in response to stimulation with the TLR-Ls. Furthermore, induction of CD80 and CD86 on the remaining blood pDCs was not enhanced by injection of TLR9-L or TLR7/8-L.

A single injection of TLR9-L into FL-treated animals at the time of initial DNA priming markedly enhances the magnitude of polyfunctional, antigen-specific CD8⁺ T cells after the MVA boost

Antigen-specific CD8⁺ and CD4⁺ T cell responses were evaluated using intracellular cytokine (ICC) staining and ELISPOT 2 wk after each immunization with the DNA vaccine (i.e., weeks 4 and 13) or 1 wk after each boost with MVA (i.e., weeks 37 and 59; Fig. S1). For SIV Gag stimulation, one pool of overlapping peptides spanning the entire Gag protein was used to assess the frequencies of Gag-specific CD8⁺ T cells. To measure Env-specific T cell responses, the pool of peptides encompassing the full-length HIV Env protein was used in both ICC and ELISPOT assays. After the first or second DNA vaccination, the magnitudes of the antigen-specific CD8⁺ or CD4⁺ T cell responses were below the threshold of detection in all groups (Fig. 2 C; and not depicted).

The monkeys were boosted with 10⁸ PFU rMVA 25 wk after the second DNA vaccination (week 36) and a second time on week 58 (Table I). Immune responses were measured 1 wk after each MVA boost by ICC and ELISPOT. CD3⁺CD8⁺ and CD3⁺CD4⁺ T cells were analyzed for IFN- γ , TNF- α , and IL-2 intracellular staining, as shown in Fig. S2 (available at <http://www.jem.org/cgi/content/full/jem.20071211/DC1>). Robust responses could be detected, particularly after the second MVA boost (Fig. 2). The magnitude of the Gag-specific CD8⁺ T cell response in the TLR9-L-injected group was considerably enhanced after the second MVA boost (with a mean frequency of 1.5% Gag-specific CD8⁺ IFN- γ ⁺ T cells) relative to group 1 (Fig. 2 A). The frequency of Env-specific CD8⁺ IFN- γ ⁺ T cells was also

significantly enhanced (Fig. 2 A). Interestingly, FL + TLR7/8-L did not enhance the magnitude of the response despite potent activation of DCs and monocytes (Fig. 2 A). Consistent with these results, there was also a significant enhancement of the Gag and Env-specific IFN- γ -secreting cells, as measured by ELISPOT, in the animals that received FL + TLR9-L relative to group 1 (for the Gag-specific response, a mean of 1,500 spots per 10⁶ PBMCs, with one animal showing 3,700 spots per 10⁶ PBMCs; Fig. 2 B). Furthermore, even after the first MVA boost, there was a nearly eightfold enhancement in the frequency of IFN- γ ⁺ CD8⁺ T cells per total CD8⁺ T cells in the group that received FL + TLR9-L (0.8%) relative to group 1 (0.1%; Fig. 2 C), and there was a large augmentation in the Env-specific CD8⁺ T cell response in group 3 (FL + TLR9-L) relative to group 1 or 2 (Fig. 2 C). The FL + TLR7/8-L group showed no major increase in the magnitude of the response (Fig. 2 C).

In addition to the magnitude, the quality of a T cell response is thought to correlate with protection (36, 37). One measure of T cell quality is based on the frequency of polyfunctional antigen-specific T cells that simultaneously produce IFN- γ , TNF- α , and IL-2 (38, 39). We thus evaluated the frequencies of such cells by ICC staining at weeks 1 or 10 after the second MVA boost. There was a very marked increase in the frequency of polyfunctional CD8⁺ T cells that simultaneously produced IFN- γ , TNF- α , and IL-2 in the FL + TLR9-L group relative to group 1 (Fig. 2 D). At the peak of the response (1 wk after second MVA), the frequencies of polyfunctional CD8⁺ T cells in the FL + TLR9-L group (group 3) were higher than in groups 1 and 2, and this was also apparent 9 wk later (Fig. 2 D). However, relative to the total antigen-specific T cell responses, even the unadjuvanted control group 1 monkeys exhibited a strikingly high representation of polyfunctional cells (up to 36% of the antigen-specific cytokine-producing T cells; Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20071211/DC1>). Furthermore, the majority of IFN- γ -producing cells produced at least one other cytokine (Fig. S3). However, no enhancement of the relative representation of polyfunctional CD4⁺ or CD8⁺ T cells was noted in group 2, 3, or 4 compared with group 1 (Fig. S3).

An important caveat to this experiment was the unequal distribution of Mamu-A*01 animals in each group. Animals in groups 2, 3, 4, and 5 contained, respectively, 3, 4, 3, and 2 animals typed positive for the Mamu-A*01 allele with PCR analyses. Group 1 did not contain Mamu-A*01-positive animals. Animals that express Mamu-A*01 have been shown to be particularly efficient at controlling SIV replication (40, 41). However, recent experiments (42) and our own results (unpublished data) suggest that this may not be the case. Nevertheless, it was important to determine the extent to which the differences in the immune responses could be attributable to Mamu-A*01. Therefore, we initially analyzed the magnitude of the Gag-specific IFN- γ ⁺ CD8⁺ T cell response only in Mamu-A*01⁺ animals. As indicated in Fig. 2 E, the Gag-specific IFN- γ ⁺ CD8⁺ T cell responses were enhanced in

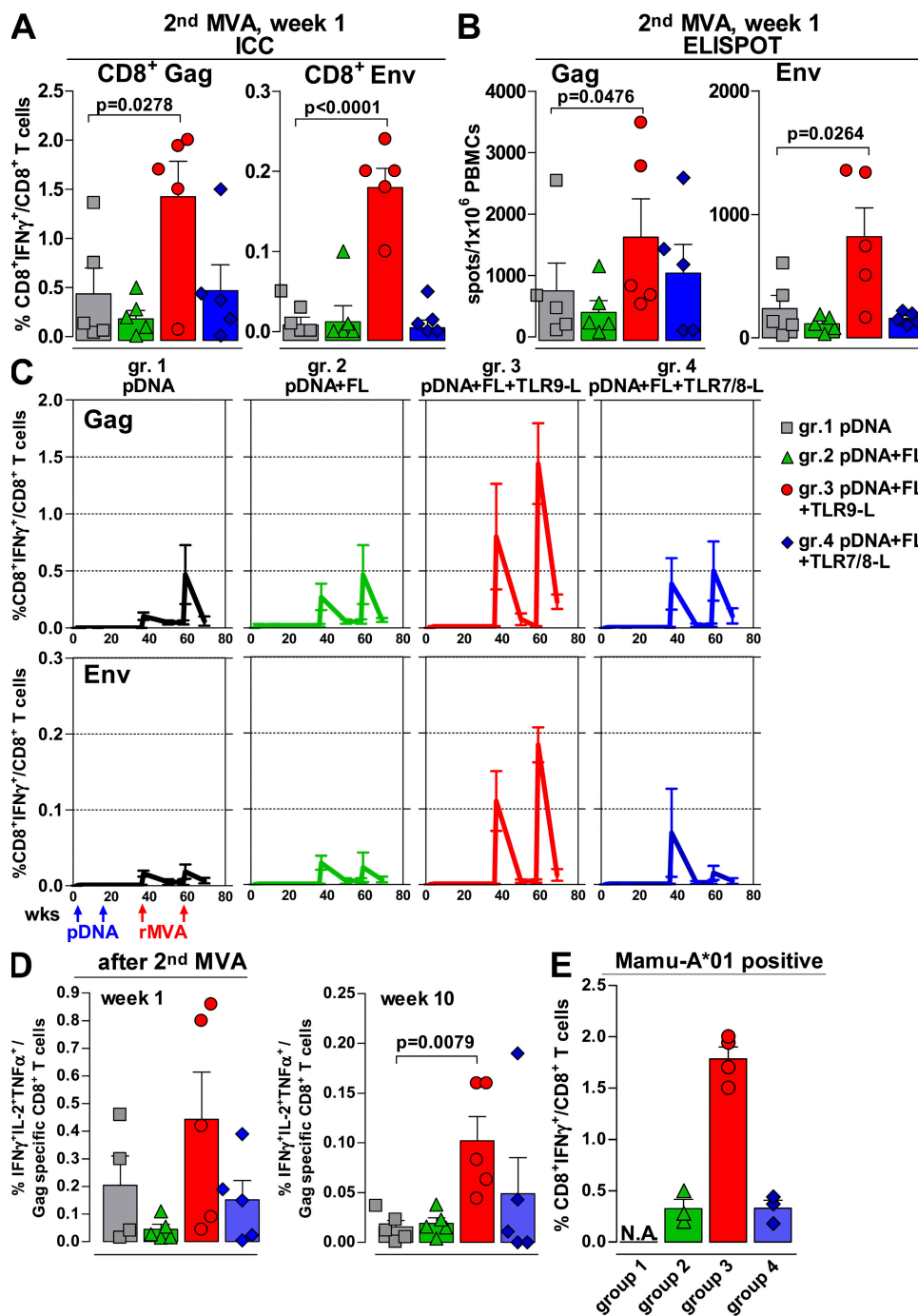


Figure 2. A single injection of TLR9-L into FL-treated animals at the time of initial DNA priming markedly enhances the magnitude of polyfunctional, antigen-specific CD8⁺ T cells after the MVA boost. Total PMBCs were stimulated with a pool of overlapping peptides spanning SIV Gag or HIV Env proteins at week 1 after a second rMVA boost vaccination. IFN- γ -producing T cells were detected by intracellular staining (A) or ELISPOT (B). Each symbol represents an individual animal in the experimental group; bars represent the mean frequencies \pm SEM ($n = 5$). (C) Kinetics of Gag- (top) and Env- (bottom) specific IFN- γ -producing CD8⁺ T cells. Animals in each group were vaccinated as described in Table I and Fig. S1, and PMBCs were stimulated as described. Each line represents the mean of all individuals in the group \pm SD ($n = 5$). (D) Frequencies of multicytokine IFN- γ , TNF- α , and IL-2-producing Gag-specific T cells after the second rMVA boost vaccination. Total PMBCs at weeks 1 (left) and 10 (right) after the second rMVA boost vaccination were stimulated with a pool of overlapping Gag peptides and stained for intracellular IFN- γ , TNF- α , and IL-2. Percentages of cells simultaneously producing all three cytokines of total Gag-specific CD8⁺ T cells are shown. Error bars represent the mean frequencies \pm SEM. All vaccinated groups were compared with group 1 by a nonparametric Mann-Whitney test. $P < 0.05$ was considered statistically significant ($n = 5$). (E) Frequencies of IFN- γ -producing Gag-specific CD8⁺ T cells at week 1 after a second rMVA boost vaccination in Mamu-A*01-positive animals from groups 2, 3, and 4. Each symbol represents an individual animal in the experimental group. Error bars represent the mean frequencies \pm SEM ($n = 3$ or 4). N.A., not available.

group 3, relative to group 2 or 4, suggesting that the enhanced immune response induced by FL + TLR9-L was not simply caused by the unequal distribution of Mamu-A*01 animals. Also, specific CD8⁺ T cell responses against HIV Env protein, which does not demonstrate Mamu-A*01-specific epitope dependence, were enhanced only in group 3, treated with FL and TLR9-L (Fig. 2 C). Collectively, these data suggest that a single injection of TLR9-L at the time of DNA priming results in profound increases in the magnitude of polyfunctional antigen-specific T cells after the MVA boost.

Studies of specific cellular responses and infection control in the vaccinated groups after SIV challenge

The robust immune responses stimulated by CpG DNA raised the question of whether there was an enhanced control of virus upon challenge with SIV. Although the present experiment was not designed as a challenge study (in terms of the limited numbers of animals per group and the unequal distribution of Mamu-A*01⁺ animals), given the robust immune responses observed by adjuvanting with CpG DNA, we attempted to test the protective efficacy of the adjuvanted vaccine against a high dose mucosal challenge with SIV. The animals were thus challenged with a high dose (10 animal infectious doses per animal) of the highly pathogenic SIV_{mac251}, delivered atraumatically via the rectal route, 19 wk after the final MVA boost. Importantly, this protocol relied solely on protective responses directed to Gag and Pol antigens, because given the relative lack of cross-reactivity between SIV and the HIV Env or Nef used in the present vaccine (10), no contribution from these antigens was expected. 2 wk after the challenge and coincident with the peak viremia, there was a significant enhancement in the frequency of Gag-specific IFN- γ ⁺ CD8⁺ T cells (mean of 7.8%, with two animals \geq 10%) in the group that received FL + TLR9-L relative to group 1 (Fig. 3 A). In addition, there was a noticeable but statistically insignificant increase in such cells in the FL-alone group 2 or the FL + TLR7/8-L group 4 (Fig. 3 A). At week 10 or 24 after challenge, the magnitude of the Gag-specific IFN- γ ⁺ CD8⁺ T cell response had reached a plateau at \sim 1.7% for groups 2, 3, and 4 compared with a mean of 0.7% for group 1 (Fig. 3 A). There was also a noticeable, although statistically insignificant, enhancement in the frequencies of Gag-specific CD4⁺ T cells, particularly at week 24 (Fig. 3 A). The responses after challenge at both early (week 2) and late (week 24) time points correlated well with the magnitude of the Gag-specific CD8⁺ T cell responses noted after the second MVA boost (Fig. 3 B, left and right, respectively). This suggested a direct link between the magnitudes of the vaccine-induced T cell response and expansion of antigen-specific T cells after viral challenge.

Furthermore, at weeks 2, 10, and 24 after challenge, there was an increase in the frequencies of polyfunctional, Gag-specific CD8⁺ (not depicted) and CD4⁺ T cells (Fig. 3 C). The relative representation of polyfunctional cells within

the Gag-specific CD8⁺ T cell compartment was not altered by adjuvants (unpublished data). However, the relative representation of polyfunctional cells within the Gag-specific CD4⁺ T cell compartment was enhanced at weeks 10 and 24 in groups 2, 3, and 4 relative to group 1 (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20071211/DC1>). Thus, administration of TLR9-L at the time of the primary DNA vaccination resulted in an enhanced magnitude of polyfunctional antigen-specific CD4⁺ and CD8⁺ T cells after challenge with SIV.

Although immune measurements often correlate with efficacy, the ultimate test for any vaccine is the control of infection. Thus, we followed the viral titers in the SIV_{mac251}-infected animals. Our vaccine contained only two antigens that were specific to SIV (Gag and Pol), and it is the CD8⁺ T cells (and not antibody) stimulated by these antigens that are known to confer protection. The immunogen Env (the primary target for antibody-mediated neutralization) was encoded by an HIV env gene, and thus, neutralizing antibodies stimulated by HIV Env are not protective against SIV (43). Thus, we were in effect evaluating the protective capacity of a CD8⁺ T cell response that was specific to Gag and Pol.

One animal in group 1 and another in group 3 did not appear to have taken the challenge, as viral loads remained undetectable throughout the study and other parameters of immune activation (such as the frequency of CD4⁺ T cells in the gut) remained normal. These two animals were excluded from the statistical analyses of viral loads. Challenge of unvaccinated animals with SIV resulted in a geometric mean peak viral load of 23 million copies per milliliter at week 2 (Fig. 4, A and B). Animals that received the DNA vaccine alone had a peak mean viral load of 7 million copies per milliliter. However, in the groups that received FL + TLR9-L or FL + TLR7/8-L, there was a 6.3–8.5-fold reduction in the peak viral loads (3.6 million and 2.7 million copies per milliliter, respectively; Fig. 4, A and B). Viral loads were followed for a longer term (up to 24 wk; Fig. 4 A). The unvaccinated animals reached a set-point load geometric mean of 2.5 million copies per milliliter by weeks 10–24 (Fig. 4 A). The animals in group 1 reached a geometric mean set-point value of \sim 0.36 million copies per milliliter. In contrast, with the exception of one animal, there was a profound diminution of viral loads in the animals in group 3 (Fig. 4 A). Three of the animals had a geometric mean of viral loads of 448 copies per milliliter, which was barely above the threshold of detection of 125 copies per milliliter (Fig. 4 A). The animals that received FL or FL + TLR7/8-L also had noticeably enhanced control of viral titers, although the effects were not as pronounced as in the FL + TLR9-L group (Fig. 4 A). In particular, there was a statistically significant decrease in the cumulative viral loads, as measured by the area under the curve (AUC) for the entire 24-wk period after challenge, in the FL + TLR9-L and FL + TLR7/8-L groups relative to the unvaccinated group (Fig. 4 C). There was a noticeable but statistically insignificant difference in the viremia reduction measured by the AUC between group 1 and groups 3 and 4.

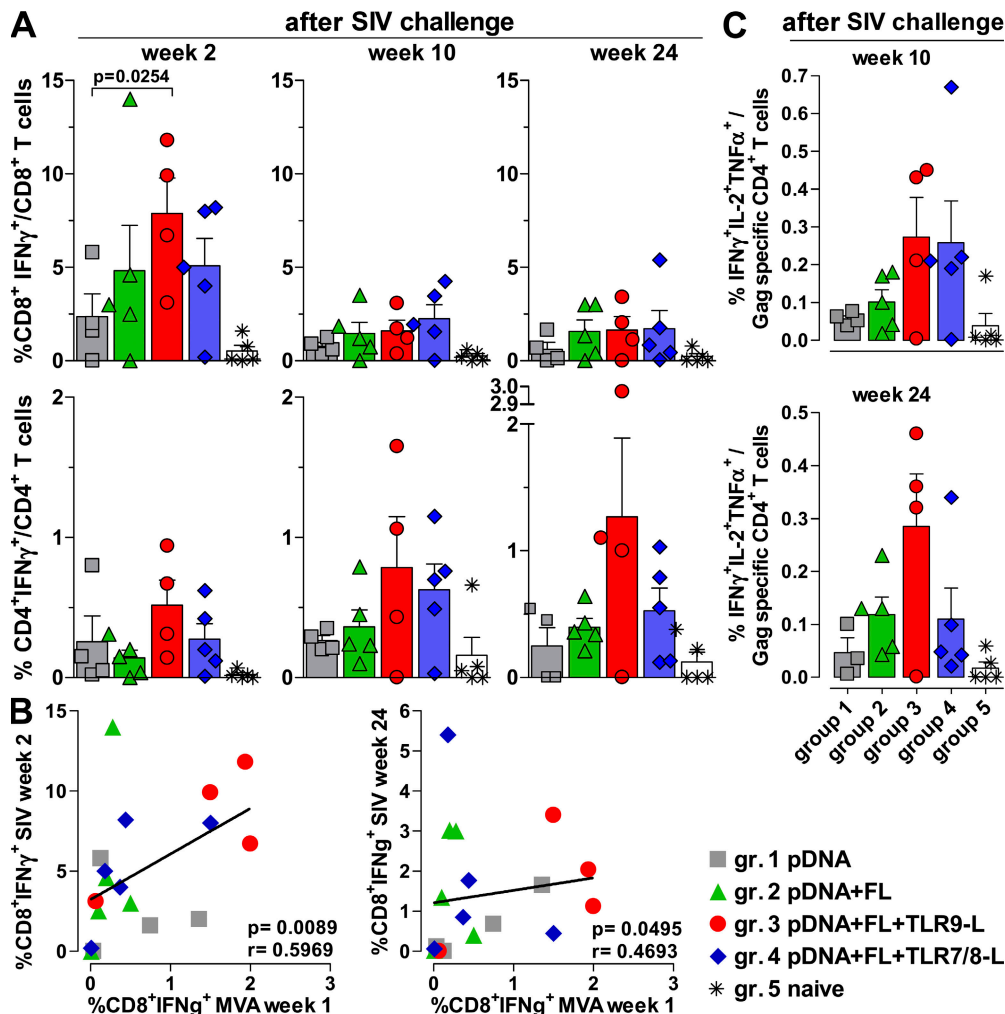


Figure 3. Co-injection of TLR9-L and pDNA at priming markedly enhances the magnitude of polyfunctional antigen-specific CD8⁺ T cell response after SIV challenge. All animals in vaccinated groups 1–4, as well as naive animals in group 5, were challenged intrarectally with SIVmac₂₅₁ virus at week 77 of the experiment. Total PMBCs were stimulated with a pool of overlapping Gag peptides at weeks 2, 10, and 24 after SIV challenge. (A) Frequencies of IFN- γ -producing CD8⁺ (top) and CD4⁺ (bottom) T cells were detected by ICC staining. Each symbol represents an individual animal in the experimental group; bars represent the means \pm SEM ($n = 4$ or 5). (B) Correlates of Gag-specific CD8⁺ T cell responses after SIV challenge. Magnitude of expansion of Gag-specific CD8⁺ T cells after the second rMVA vaccination correlates with specific T cell responses at week 2 (left) or 24 (right) after SIV challenge. The p -values were calculated for correlation efficiency and were considered significant at $P < 0.05$. Spearman's rank correlation coefficient values are represented (r). Diagonal lines represent linear regression. (C) Frequencies of multicytokine IFN- γ -, TNF- α -, and IL-2-producing Gag-specific T cells after SIV infection. Total PMBCs at weeks 10 (top) and 24 (bottom) after the second vaccination were stimulated with a pool of overlapping Gag peptides and stained for intracellular IFN- γ , TNF- α , and IL-2. Percentages of all three cytokine-producing cells of total Gag-specific CD4⁺ T cells are shown. Each symbol represents an individual from a vaccinated group (represented by different colors). Error bars represent the mean frequencies \pm SEM ($n = 4$ or 5). All vaccinated groups were compared with group 1 by a nonparametric Mann-Whitney test.

Given the caveats noted, we addressed the potential effects of Mamu-A*01 by analyzing the viral loads within the Mamu-A*01⁺ animals. As shown in Fig. 4 D, there was a significant reduction in viral loads in groups 3 and 4 relative to group 5. Furthermore, there was a reduction in the loads in groups 3 and 4 relative to group 2, although this did not achieve statistical significance. Furthermore, we analyzed whether the observed lack of viral control in response to vaccination without any adjuvants (group 1) was caused by the absence of Mamu-A*01 in group 1. We thus evaluated data

from an independent study, in which eight monkeys (four Mamu-A*01⁺ and four Mamu-A*01⁻) were vaccinated twice with a DNA vaccine encoding the SIV antigens gag, pol, env, nef, vpr, and vpu, followed by two boosts with MVA expressing the same antigens (unpublished data). The vaccination regimen was similar to the present study, but the critical difference was that all the antigens encoded by this vaccine were SIV specific. The viral loads were compared with viral loads in the unvaccinated group, which also consisted of eight animals (four Mamu-A*01⁺ and four Mamu-A*01⁻).

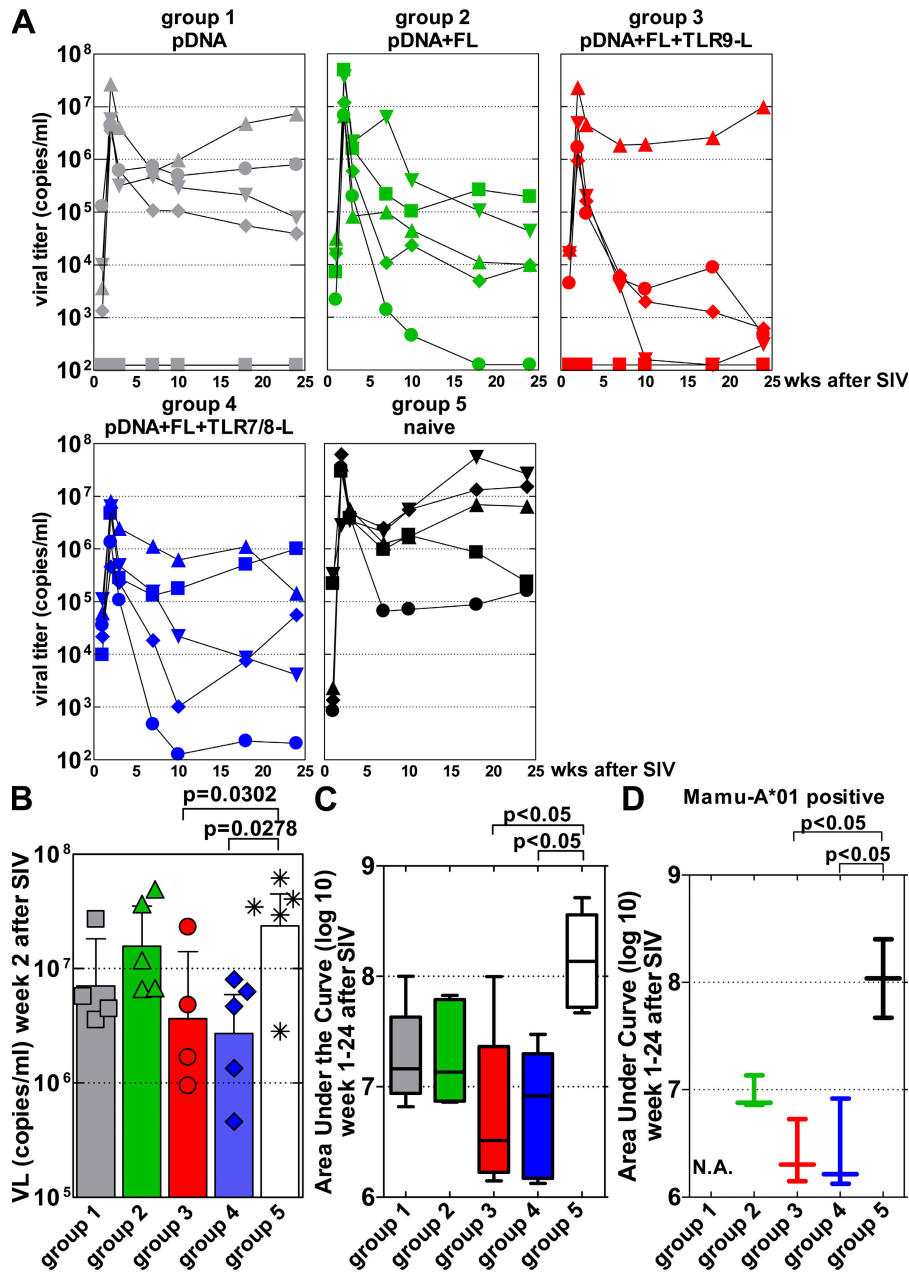


Figure 4. Control of the viremia in the vaccinated groups after SIV challenge. All animals in vaccinated groups 1–4, as well as naive animals in group 5, were challenged intrarectally with SIV_{mac251} virus at week 77 of the experiment. (A) Plasma was collected at weeks 1, 2, 3, 7, 10, 18, and 24 after challenge and tested for SIV viral loads (SIV RNA copies/ml). Symbols represent the dynamics of viremia for each animal in the group. In groups 1 and 3, single animals remained uninfected over the entire challenge phase and were not included in the statistical analyses. (B) Viral titers at the peak of viremia at week 2 after SIV challenge. Each symbol represents an individual animal in the experimental group; bars represent the geometric mean frequencies \pm SEM. All vaccinated groups were compared by a nonparametric Mann-Whitney test. $P < 0.05$ was considered statistically significant ($n = 4$ or 5). (C and D) AUC from weeks 1 through 24 was calculated for each animal in all experimental groups (C), or Mamu-A*01-positive animals only in groups 2, 3, 4, and 5 (D). Log-transformed areas (AUC [\log_{10}]) were used in the analysis. Error bars represent the median with range (C, $n = 5$; D, $n = 2$ or 3). Analysis of variance was used for the comparisons among the groups and adjusted for multiple comparisons with the Bonferroni method. $P < 0.05$ was considered significant ($n = 4$ or 5). N.A., not available.

There was no statistical difference in the viral loads between the Mamu-A*⁻⁰¹ versus Mamu-A*⁻⁰¹ animals when analyzed for the viral loads at the peak (week 2) or set point (week 24) of viremia, and kinetics of viral load control were

similar for Mamu-A*⁻⁰¹ and Mamu-A*⁻⁰¹ animals (unpublished data). In addition, recent work suggests that two other haplotypes, Mamu-B*08 and Mamu-B*17, also exert an influence on the immune control of SIV infection (44, 45).

We thus tested the animals for these haplotypes, and there were two Mamu-B*17 animals and one Mamu-B*08 animal within the entire cohort. One of the Mamu-B*17 animals was in group 1, and the other was in group 5. The Mamu-B*08 animal was in group 2. Thus, group 3 which displayed the best control did not contain any Mamu-B*08 or Mamu-B*17 animals. Collectively, these data suggest that the observed enhancement in immune responses and viral control in the FL + TLR9-L group are unlikely to be caused by Mamu-A*01 effects alone.

Correlates of protective immunity

We evaluated potential correlations between the immune responses and viral loads (Fig. 5). Importantly, there was a strong inverse correlation between the viral loads at the set point at week 24 and the magnitude of the Gag-specific IFN- γ ⁺ CD8⁺ T cell response after a second MVA at primary phase at week 1 (Fig. 5 A), memory phase at week 10 (Fig. 5 B), or 2 wk after SIV challenge (Fig. 5 C). Also, the frequencies of polyfunctional CD4⁺ (Fig. 5 D) or CD8⁺ (Fig. 5 E) T cells correlated with the control of viremia at week 24 after SIV challenge, suggesting the identification of associates of protection after immunization. Additionally, we determined if preservation of central memory CD4⁺ T cells correlated with the control of the viremia after the challenge, as previously reported (46, 47) (Fig. 5 F). Percentages of CD95⁺ CD28⁺CCR chemokine receptor (CCR) 7⁺-expressing, central memory CD4⁺ T cells measured in the blood 24 wk after challenge were well maintained in vaccinated monkeys and correlated inversely with viral loads at the viremia set point (Fig. 5 F).

Finally, we evaluated the frequency of CD4⁺ T cells in the gut, which have been shown to be destroyed rapidly during SIV infection. Vaccination has been shown to preserve these cells, especially the CD4⁺ central memory T cells (48). Our data suggest a strong correlation between vaccine-induced immunity and the frequency of CD4⁺ T cells in the gut (Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20071211/DC1>). In particular, animals in groups 1 and 5 exhibited a rapid diminution in the frequency of intestinal CD4⁺ T cells (Fig. S5 A). In contrast, in animals vaccinated with FL or FL + TLR9-L or FL + TLR7/8-L, there was a pronounced reconstitution of the CD4⁺ T cells at week 18 after challenge (Fig. S5, A and B). Furthermore, there was a striking inverse correlation between the frequency of CD4⁺ T cells in the gut and the viral loads in plasma (Fig. 5 G), signifying an association of viral control and loss of intestinal CD4⁺ T cells, as well as a direct correlation between the magnitude of the Gag-specific CD8⁺ T cell response at 2 wk after SIV challenge (Fig. 5 H).

DISCUSSION

The results presented in this paper suggest that activating DCs with TLR9-L at the time of the initial immunization with a DNA vaccine results in an enhanced antigen-specific CD8⁺ T cell response and improved control of viral loads

after challenge with SIV. Three critical issues deserve discussion: (a) the mechanism by which TLR9-L acts, (b) the correlates of protective immunity, and (c) the influence of Mamu-A*01 haplotypes on the observed results.

Mechanism of action of adjuvants

An important mechanistic question is whether the observed increases in immune responses were caused by enhanced DC numbers, activation of DCs, or both. Our data suggest that it is the TLR9-L, rather than FL, that mediated the enhanced immunogenicity, because animals that received FL alone showed no augmentation of immune responses. This is consistent with previous studies suggesting that, although FL alone is known to enhance immune responses to soluble antigens and DNA vaccines in mice (28, 29), in humans it failed to enhance immune responses to protein antigens despite documented increases in DC numbers (33). However, it is formally possible that TLR9-L and FL acted in a synergistic manner to enhance the immune response. FL is known to expand DCs in most tissues of the body, including dermal DCs in the dermal layer of the skin (49). Thus, it is possible that the increased numbers of dermal DCs at the local site of vaccination (i.d.) might have facilitated enhanced uptake of the DNA vaccine directly or by phagocytosis of apoptotic cells that had acquired the DNA vaccine *in situ*. In addition, increased DC numbers in the draining lymph nodes may have enhanced the likelihood of cross-presentation of antigens derived from apoptotic bodies of activated DCs migrating from the skin (50). However, our data suggest that enhanced DC numbers alone do not lead to amplification of cellular immune responses. It is thus likely that the major effect was mediated via TLR9-L stimulation.

Another mechanistic question is how the innate immune activation by TLR9-L versus TLR7/8-L translates into the observed effects on adaptive immune responses. Our data suggest that both TLR9-L and TLR7/8-L induce potent activation of myeloid DCs and monocytes in the blood. Such systemic effects on DCs were used as surrogate measures of innate immune activation caused by TLR-Ls *in vivo*. However, it must be stressed that such systemic effects are likely to have been secondary effects, mediated perhaps by the release of proinflammatory cytokines released systemically by the direct TLR-mediated activation of DCs and other cells at the site of vaccination (51). In humans and macaques, TLR9 and TLR7 are preferentially expressed on pDCs, whereas TLR8 is expressed preferentially on myeloid DCs and monocytes (22, 32, 35). Thus, TLR9-L and TLR7-L can directly activate only pDCs, whereas TLR7/8-L can directly activate both myeloid DCs and pDCs (22, 32, 35). We observed a diminution in pDC numbers in the blood, which was likely caused by the rapid activation and translocation of such cells into the secondary lymphoid organs. In contrast, both TLR9-L and TLR7/8-L appear to potently activate myeloid DC and monocytes *in vivo* (Fig. 1). This is likely to have been caused indirectly, as a result of proinflammatory cytokines released by other cells, presumably pDCs (51). In any case, it

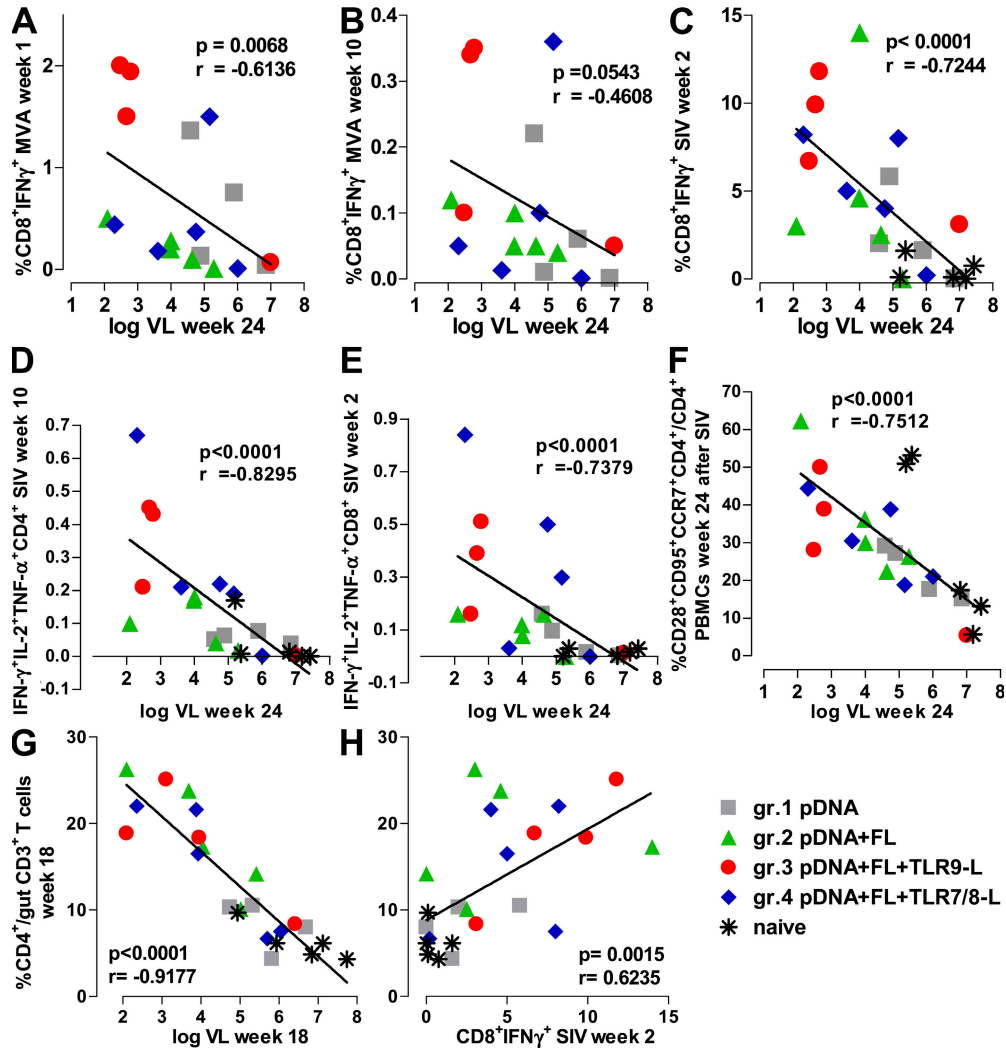


Figure 5. Correlates of immune responses and protection. Control of viral loads at the set point of challenge at week 24 was tested for correlation with Gag-specific T cell responses after the last rMVA boost (A and B) or after SIV infection (C–F). Magnitudes of expansion of Gag-specific CD8⁺IFN- γ ⁺ T cells after the second rMVA vaccination at weeks 1 (A) and 10 (B), and at week 2 after SIV challenge (C) inversely correlated with the viremia level of experimental animals. Magnitudes of multicytokine IFN- γ , TNF- α , and IL-2-producing Gag-specific CD4⁺ (D) and CD8⁺ (E) T cells after SIV challenge also showed strong inverse correlation with the control of the viral load at week 24. (F) Frequencies of central memory CD4⁺ T cells were measured in PBMCs at week 24 after challenge. Population of central memory CD4⁺ T cells were defined as CD95^{high} CD28^{high} CCR7^{high}, and their frequency correlated inversely with the viral loads at week 24 after SIV, showing preservation of memory cells in vaccinated animals. (G and H) Rectal biopsies were collected at week 18 after challenge. Lamina propria lymphocyte CD4⁺ T cells are represented as the percentage of total CD3⁺ T cells extracted from the gut tissue. (G) Proportions of CD4⁺ T cells in mucosal tissue inversely correlated with the viral loads at week 18 after challenge. (H) Direct correlation between the magnitude of expansion of Gag-specific CD8⁺ T cells at week 2 after SIV challenge and control of mucosal CD4⁺ T cell by trial animals at week 18. Each symbol represents an individual animal from a vaccinated group (signified by different colors) or naive animals (asterisks). The p-values were calculated for correlation efficiency and were considered significant at $P < 0.05$. Spearman's rank correlation coefficient values are represented (r). Diagonal lines represent linear regression.

is striking that the TLR7/8-L failed to enhance the magnitude of T cell response despite potent innate immune stimulation, as judged by DC activation in the blood. One possibility is that the TLR7/8-L, which is a small molecule, administered in a soluble form rapidly diffuses from the site of vaccination. In fact, recent experiments show that direct conjugation of TLR7/8-L to a protein antigen results in an enhanced immune response (22). In addition, we observed differences in the expression patterns of CD86 and CD80 in response to

TLR9-L versus TLR7/8-L (Fig. 1). Whether this influenced the differences in the magnitude of the adaptive immune responses induced by TLR9-L versus TLR7/8-L remains to be determined.

Correlates of protective immunity

The magnitude of the antigen-specific IFN- γ ⁺ CD8⁺ T cell response after MVA challenge and after SIV-infection was inversely correlated with the viral loads (Fig. 4, D–F) and

with CD4⁺ T cells levels in gut tissue (Fig. 5 D), which was consistent with numerous previous studies (52–54). However, the frequency of IFN- γ ⁺ IL-2⁺ TNF- α ⁺ polyfunctional CD8⁺ and CD4⁺ T cells after challenge showed an even stronger inverse correlation with the viral loads (Fig. 5, D and E), suggesting that not only the magnitude but also the quality of the T cell response were critical correlates of protection (36, 38, 39). In the case of TLR7/8-L, there was a statistically significant lowering of viral loads despite no increase in the frequency of antigen-specific CD8⁺ T cells (Fig. 4, B and C). This implies that TLR7/8-L might be inducing a different quality of T cells, not measured in this study, that results in enhanced control of infection.

Influence of Mamu-A*01 and other haplotypes

Previous studies have suggested that animals expressing the MHC class I allele Mamu-A*01 are particularly efficient at controlling SIV replication (40, 41), whereas recent experiments suggest that this may not be so (42). Our analysis of the magnitude of the Gag-specific IFN- γ ⁺ CD8⁺ T cell response only in Mamu-A*01⁺ animals indicated (Fig. 2 E) that the Gag-specific IFN- γ ⁺ CD8⁺ T cell responses were enhanced significantly in group 3 relative to group 2 or 4, suggesting that the adjuvant effects of TLR9-L were not simply caused by the unequal distribution of Mamu-A*01 animals. Furthermore, the CD8⁺ T cell responses against HIV Env protein, which does not demonstrate Mamu-A*01-specific epitope dependence, were enhanced only in group 3, which was treated with FL and TLR9-L (Fig. 2 C). Furthermore, our analysis of the viral loads within the Mamu-A*01⁺ animals (Fig. 4 D) showed a significant reduction in viral loads in groups 3 and 4 relative to group 5. There was also a reduction in the loads in groups 3 and 4 relative to group 2, although this did not achieve statistical significance. Finally, our analysis of data from an independent study, in which eight monkeys (four Mamu-A*01⁺ and four Mamu-A*01⁻) were vaccinated twice with a DNA vaccine encoding the SIV antigens gag, pol, env, nef, vpr, and vpu, followed by two boosts with MVA expressing the same antigens, failed to reveal an effect for the Mamu-A*01 haplotype on viral loads (unpublished data). There was no statistical difference in the viral loads between the Mamu-A*01⁺ versus Mamu-A*01⁻ animals when analyzed for the viral loads at the peak (week 2) or set point (week 24) of viremia, and kinetics of viral load control were also similar for Mamu-A*01⁺ and Mamu-A*01⁻ animals (unpublished data). As discussed, neither group 3 nor group 4 contained any Mamu-B*08 or Mamu-B*17 animals, and, thus, these haplotypes cannot have contributed to the observed enhancements in viral controls. Collectively, these data suggest that the observed enhancements in immune responses and viral control in the FL + TLR9-L group are unlikely to be caused by Mamu-A*01 effects alone.

Perspectives

In summary, these data indicate that strategies that enhance the activation of DCs may be beneficial in enhancing the

magnitude and quality of immune responses induced by DNA vaccines. This may provide a solution to the problems associated with the suboptimal immunogenicity of DNA vaccines in humans. In particular, our data suggest that TLR9-L may serve as a useful adjuvant in enhancing specific immunity in an HIV infection model. It is important to stress that in the vaccine used in the present study, only Gag and Pol were SIV specific, whereas the other antigens, including Env, were HIV specific. Therefore, the elicitation of robust, high quality CD8⁺ T cell responses alone, in the absence of a neutralizing antibody response, can exert a major effect on viral control. The additional benefits in protection that are likely to accrue from using a vaccine encoding all antigens that are SIV specific remain to be determined. Finally, the potential clinical utility of such a study is critically dependent on strategies that circumvent the onerous FL administration schedule. One such strategy might involve formulating FL into nanoparticles that slowly release the cytokine over the course of a few days.

MATERIALS AND METHODS

Animals. A cohort of 25 adult Indian rhesus macaques (*Macaca mulatta*) was maintained in the Yerkes National Primate Research Center and Field Station facility. Animals were cared for under guidelines established by the Animal Welfare Act and the NIH “Guide for the Care and Use of Laboratory Animals,” with protocols approved by the Emory University Institutional Animal Care and Use Committee. NHPs were sex and weight matched and divided into five groups of five animals each (Table I). Animals in groups 2, 3, 4, and 5 contained, respectively, 3, 4, 3, and 2 animals typed positive for the Mamu-A*01 allele with PCR analyses. Group 1 did not contain Mamu-A*01-positive animals.

Injections and reagents. Animals were injected subcutaneously for 14 consecutive days (day 1–14) with 100 μ g/kg/d of human FL protein (PBS diluted) provided by Amgen. Cloning of pGA1-SHIV 89.6 VLP pDNA has been previously described (10). pDNA was purified by QIAGEN and injected i.d. at 2 mg per animal in PBS at weeks 2 (day 15) and 11 of the study. In group 3, pDNA was mixed and co-injected i.d. with 2 mg per animal of TLR9-L CpG 2006 type B (Coleypharma). Animals in group 4 were injected i.d. with a combination of pDNA and 0.3 mg/liter/animal of TLR7/8-binding 3M-003 synthetic compound provided by 3M Pharmaceuticals. The MVA double-recombinant virus expressing the HIV 89.6 Env and the SIV 239 Gag-Pol (55) was administered subcutaneously at 10⁸ PFU per animal at weeks 36 and 58 of the experiment.

Challenge and viral loads. All monkeys were challenged by a rectal inoculation of 10 animal infectious doses per animal of SIVmac₂₅₁ (provided by N. Miller, National Institute of Allergy and Infectious Diseases, Bethesda, MD) at week 77 of the trial. Plasma samples were collected at the time points indicated in the figures (weeks 1, 2, 3, 7, 10, 18, and 24 after challenge) and tested for viral RNA using the SIVmac RNA bDNA assay (Bayer Diagnostics). The detection threshold of the bDNA assay was 125 copies per milliliter.

Cell isolation and staining. PBMCs were isolated with a standard method from heparinized or citrated blood samples. Intestinal mucosal cells were isolated from rectal biopsy samples. Rectal biopsy pellets were washed with HBSS medium twice and resuspended in RPMI 1640 containing antibiotics. Tissues were digested for 1.5 h in the presence of collagenase (type 4; Worthington) and DNase (type 1; Roche). Digested pellets were disrupted by pipetting and were filtered twice through a cell strainer. Both PBMCs and mucosal cells were resuspended in PBS containing 10% FBS and were

stained for flow cytometry. All cells were analyzed on FACSCalibur or three-laser LSRII instruments (both from BD Biosciences). The following antibodies were used for flow cytometric analysis: CD3 (SP34-2 or FN-18), CD4 (L200), CD8 (SK-1), CD14 (M5E2), CD20 (2H7), and HLA-DR (L243); CD123 (7G3); CD80 (L307.4); CD86 (2331-FUN-1), CD11c (S-HCL-3), CD28 (CD28.2), CD95 (DX2), IL-2 (MQ1-17H1), TNF- α (MAb11), and IFN- γ (B27; BD Biosciences); and CCR7 (150503; R&D Systems). All cytometry data were analyzed using FlowJo software (Tree Star, Inc.).

ICC staining. Approximately 2×10^6 PBMCs were stimulated with total pools of SIV-Gag or HIV-Env proteins at the final concentration of 2 $\mu\text{g}/\text{ml}$ (15-mers overlapping by 11; courtesy of the NIH AIDS Reagent Program) in 5-ml polypropylene tubes in RPMI 1640 medium containing 10% FBS, and anti-human CD28 and anti-human CD49d (1 $\mu\text{g}/\text{ml}$ each; BD Biosciences) in a final volume of 500 μl . Ovalbumin peptide (SIINFEKL)-stimulated PBMCs were used as negative controls. After 2 h of incubation at 37°C, 20 μl of medium containing 10 $\mu\text{g}/\text{ml}$ Brefeldin A was added, and cells were cultured for an additional 4 h at 37°C at an angle of 5 degrees. Cells were surface stained with fluorochrome-conjugated antibodies to CD8 and CD4. Cells were incubated with fluorochrome-conjugated antibodies to CD3, IFN- γ , IL-2, and TNF- α in perm/wash solution (BD Biosciences) for 20 min at 4°C. Cells were washed twice with Perm/wash, washed once with PBS/10% FBS, and resuspended in 2% PFA in PBS. Approximately 500,000 lymphocytes were acquired for analysis.

ELISPOT. MULTISCREEN 96-well filtration plates (Millipore) were coated with the anti-IFN- γ capture antibody at a concentration of 5 $\mu\text{g}/\text{ml}$ in PBS and refrigerated overnight. Plates were washed two times with RPMI 1640 medium and blocked with RPMI 1640 containing 10% FBS for 1 h at 37°C. Plates were washed five more times with RPMI 1640/10% FBS medium, and cells were seeded in duplicates in 100 μl of complete medium at concentrations of 2×10^5 cells per well. SIV-Gag or HIV-Env total peptide pools were added in a volume of 100 μl in RPMI 1640/10% FBS medium resulting at the end concentration of 1 $\mu\text{g}/\text{ml}$ of each peptide in reaction. Cells were cultured at 37°C for ~20–36 h under a 5% CO₂ atmosphere. Plates were washed six times with wash buffer (PBS with 0.05% Tween 20) and incubated with 1–2 μg of respective biotinylated anticytokine antibody diluted in wash buffer containing 2% FBS. Plates were incubated for 2 h at 37°C and washed six times with wash buffer. Avidin-horseradish peroxidase (Vector Laboratories) was added to each well and incubated for 60 min at 37°C. Plates were washed five times with wash buffer, and spots were developed with stable DAB used as substrate (Research Genetics). Spots were counted by using an automated ELISPOT reader (CTL). Data were normalized for the background by control samples stimulated with an ovalbumin peptide (SIINFEKL) and are represented as several spot-forming cells per 10^6 PBMCs. The following capture and detection antibody pairs were used: anti-human IFN- γ capture antibody (clone B27; BD Biosciences) and anti-human IFN- γ detection antibody (clone 7-B6-1; Diapharma Group, Inc.).

Statistical analysis. All data were analyzed using Prism software (GraphPad Software, Inc.). Cellular responses, mucosal CD4⁺ T cell numbers, and viral loads data were compared using the nonparametric Mann-Whitney test. $P < 0.05$ was considered statistically significant. For viremia control, AUC from weeks 1 to 24 was calculated for each infected animal. Log-transformed areas (AUC [\log_{10}]) were used in the analysis. Analysis of variance was used for the comparisons among the groups and adjusted for multiple comparisons with the Bonferroni method; $P < 0.05$ was considered significant. For correlation analysis, all datasets were calculated for correlation efficiency and were considered significant at $P < 0.05$. Spearman's rank correlation coefficient values were evaluated and represented (r).

Online supplemental material. Fig. S1 represents the experimental design of the trial. Fig. S2 shows the gating strategy for specific, cytokine-producing CD4⁺ and CD8⁺ T cells. Fig. S3 shows the quality of Gag-specific

IFN- γ - and/or TNF- α - and/or IL-2-producing T cells after the last MVA boost. Fig. S4 demonstrates the quality of Gag-specific, IFN- γ - and/or TNF- α - and/or IL-2-producing CD4⁺ T cells after SIV challenge. Fig. S5 shows the frequency of CD4⁺ T cells in the intestine after SIV challenge. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20071211/DC1>.

We warmly acknowledge the outstanding assistance of the following people: Sissy Chikazawa, Stephanie Ehner, Elizabeth Strobert, and all Yerkes personnel, both at the field and main station, for their excellent care of the animals and sample collections; the NIH AIDS Reagent Program for the provision of peptide for cellular assays; and Amgen for providing FL. We are very grateful to David Watkins for his assistance in genotyping the Mamu-B*08 and Mamu-B*17 haplotypes. In addition, we are grateful to Chris Ibegbu for technical support, Lakshmi Chennareddi for assistance with the statistical analysis, and the Emory Center for AIDS Research.

This work was supported by NIH grant R21 AI056947 and by the Bill and Melinda Gates Foundation (subcontract from the Fred Hutchinson Cancer Institute). The authors have no conflicting financial interest.

Submitted: 14 June 2007

Accepted: 28 September 2007

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