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Vaccine-induced myeloid cell population dampens protective immunity to SIV

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Vaccines are largely evaluated for their ability to promote adaptive immunity, with little focus on the induction of negative immune regulators. Adjuvants facilitate and enhance vaccine-induced immune responses and have been explored for mediating protection against HIV. Using a regimen of peptide priming followed by a modified vaccinia Ankara (MVA) boost in a nonhuman primate model, we found that an SIV vaccine incorporating molecular adjuvants mediated partial protection against rectal SIVmac251 challenges. Animals treated with vaccine and multiple adjuvants exhibited a reduced viral load (VL) compared with those treated with vaccine only. Surprisingly, animals treated with adjuvant alone had reduced VLs that were comparable to or better than those of the vaccine-treated group. VL reduction was greatest in animals with the MHC class I allele Mamu-A*01 that were treated with adjuvant only and was largely dependent on $CD8^+$ T cells. Early VLs correlated with $Ki67^+CCR5^+CD4^+$ T cell frequency, while set-point VL was associated with expansion of a myeloid cell population that was phenotypically similar to myeloid-derived suppressor cells (MDSCs) and that suppressed T cell responses in vitro. MDSC expansion occurred in animals receiving vaccine and was not observed in the adjuvant-only group. Collectively, these results indicate that vaccine-induced MDSCs inhibit protective cellular immunity and suggest that preventing MDSC induction may be critical for effective AIDS vaccination.

Introduction

Recent studies showed that vaccine-induced T cell responses could control the replication of AIDS viruses and that the magnitude and function of virus-specific T cell responses correlated with protection (1–3). This motivated the development of T cell–based HIV/SIV vaccines. However, vaccine research has focused on improving the induction of desired immune responses, but little is understood of unwanted induction by vaccines of cells that counteract the vaccine's effectiveness. In this study, we sought to use molecular adjuvants to optimize vaccine responses. In the course of this work, we serendipitously discovered a critical factor in vaccine efficacy, namely, the ability of the vaccine to induce myeloid-derived suppressor cells (MDSCs) that suppress the vaccine response.

Our initial working hypothesis was a mucosal "push-pull" model (4). Molecular adjuvants such as TLR2, -3, and -9 agonists, and IL-15 could promote and redirect immune responses for viral protection (5–11). The combination of both induced innate and adaptive immunity for viral reduction (12). Therefore, we used TLR2, -3, and -9 agonists and IL-15 as our base vaccine adjuvant. To improve the quantity and quality of T cell responses (the "push"), we incorporated an NKT cell agonist, α-GalCer, to the base vaccine. α-GalCer has been shown to improve the immunogenicity and effectiveness of cancer (13, 14), malaria (15), and HIV vaccines (16–18). To remove the negative regulators that would allow the immune responses to achieve their full potential (the "pull"), we added the PD-1 modulator B7-DC-Ig, which is a recombinant fusion protein composed of the extracellular domain of B7-DC (also known as PD-L2) fused to the hinge and Fc domain of human IgG1. In mouse tumor studies, B7-DC-Ig enhances antitumor response by modulating $PD-1^{hi}$ Tregs and dysfunctional CD8 T cells. In an SIV chronic infection study, B7-DC-Ig also demonstrated its effect on $PD-1^{hi}$ Tregs (19). SIV-infected T cells expressed high levels of PD-1 to attenuate Ag-specific T cell immunity (20–23), and blockage of this signal with the use of anti–PD-1 antibodies enhanced and restored host immune functions for both prophylactic and therapeutic HIV/SIV vaccines (24–26). We used intrarectal immunization to induce or direct the optimized immune responses to the gastrointestinal mucosa, where mucosal $CD8^+$ T cell responses have been demonstrated to control viral replication and dissemination (27–31).

Our approach was to vaccinate macaques using a peptide-prime/modified vaccinia Ankara (MVA) boost vaccine with different combinations of adjuvants. We included 1 adjuvant-only control group, as it has shown partial protection in our previous study (12). After 3 SIVmac251 challenges, when all the macaques were infected, we observed that the combination of B7-DC-Ig with α-GalCer, TLR agonists, and IL-15 mediated set-point viral load (VL) reduction. In Mamu-A*01 animals, the adjuvant-only group had even lower VLs than the naive group or the group with the same adjuvants plus vaccine. Following the hint of MHC class I restriction, we confirmed that the VL reduction in the adjuvant-only group was mainly $CD8^+$ T cell dependent. To delineate the mechanisms that would explain the lower protection in the vaccinated groups, we hypothesized that our vaccine strategy elicited some regulatory cell populations such as myeloid-derived suppressor

Authorship note: Alison Hogg and Yichuan Wang contributed equally to this work.

Conflict of interest: Linda Liu and Sol Langermann are employees of Amplimmune Inc., which provided B7-DC-Ig and input on study design.

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cells (MDSCs), which suppressed the cellular immune responses induced by vaccine or virus, resulting in loss of protection. Indeed, when we investigated the myeloid cells — $Lin^-DR^{lo}CD33^+CD11b^+$ (Lin^- MDSCs) and $CD14^+DR^{lo}CD33^+CD11b^+$ ($CD14^+$ MDSCs), which phenotypically resemble MDSCs (MDSC-like cells, referred to herein as MDSCs), we found that they were elevated in the animals receiving vaccine, but only slightly so in the animals with adjuvant-only treatment. Moreover, these MDSCs positively correlated with set-point VLs. Recent research showed that the MDSCs could inhibit HIV-specific $CD8^+$ T cell responses in chronically HIV-infected patients (32, 33); yet, the role of MDSCs in HIV/SIV vaccine strategies remains elusive. Our data demonstrate the potential involvement of MDSCs in immune modulation of VLs, probably via vaccine- or virus-induced $CD8^+$ T cell responses, and suggest that counteracting these vaccine-induced regulatory innate cells could be important in designing an effective HIV vaccine.

Results

The adjuvant-only group showed viral reduction during acute and chronic stages of SIVmac251 infection. TLR agonists and IL-15 as a mucosal vaccine adjuvant have been shown to be able to induce protective colorectal $CD8^+$ T cell responses against SIV infection (12). To improve the protective immunity, we added B7-DC-Ig to modulate $PD\text{-}1^{hi}$ Tregs and dysfunctional $CD8^+$ T cells and/or α-GalCer to enhance NKT cell activation as a source of help and of IFN-γ. We immunized 4 groups of rhesus macaques with the peptide-prime/MVA-SIV boost vaccine adjuvanted with IL-15 and TLR agonists, either alone (group 1, basic vaccine) or with B7-DC-Ig (group 2), α-GalCer (group 3), or both (group 4) (Supplemental Table 2; supplemental material available online with this article; doi:10.1172/JCI73518DS1). Two control groups, adjuvant-only (group 5) and naive (group 6), were included (Figure 1 and Supplemental Table 1).

Ten weeks after the second boost (week 26), all macaques were intrarectally challenged with a moderately low dose (1:100) of SIVmac251. Those not infected were rechallenged at 1:100 at week 28 and then at 1:50 at week 30 if necessary, after which all animals were successfully infected (Supplemental Figure 1). One problem in macaque high-dose mucosal challenge studies is that multiple distinct viruses are transmitted (34). To assess the founder virus, we enumerated the viral variants in the infected animals. Of the 12 animals analyzed, 9 had only 1 variant and 3 had 2 variants. Consensus viral sequences from peak viremia confirmed that the transmitted founder viruses from different animals were similar to each other and to the inoculum viruses (Supplemental Figure 2). This study thus mimicked human mucosal HIV-1 transmission, in which typically only one or a few transmitted founder viruses are found (35).

During the acute infection, none of the 4 vaccinated groups showed lower VLs than the naive controls. The only group that showed a significant VL decrease was the adjuvant-only group (P = 0.049), which also demonstrated a trend toward reduced VL by week 2 after infection. This finding was consistent with the maintenance of $CD4^+$ T cell counts in the adjuvant-only group (Figure 1 and Supplemental Figure 3).

For set-point VL, however, a reduction was observed in group 4 (vaccine plus all adjuvants) and an even greater one in the adjuvant-only group (group 5) (Figure 1C). In addition, group 2, which received the base vaccine plus B7-DC-Ig, showed a viral reduction similar to that of the adjuvant-only group (Figure 1C). Both groups 2 and 4 received vaccine with B7-DC-Ig alone or combined with α-GalCer. These reductions suggested that modulating PD-1 by B7-DC-Ig during immunization affected the set-point VLs, consistent with previous studies using an anti–PD-1 antibody as a vaccine adjuvant that reduced the VLs in SIV-infected macaques (25, 26).

The viral reduction in the adjuvant-only group was MHC restricted and mainly $CD8^+$ T cell dependent. As both Mamu-A*01 and -A*02 animals were included in each group, we then investigated whether they behaved differently. In the naive group, both Mamu-A*01 and -A*02 animals showed a similar pattern of viral infection. We observed the same trend in groups 1, 2, and 3, in which Mamu-A*01 and -A*02 animals had comparable VLs (Figure 2A).

However, we saw a different pattern in groups 4 and 5 (Figure 2, A and B). Also, because 37 of 40 animals were female, if we examined only the female animals, group 3 also showed a difference between Mamu-A*01–positive (5.18 logs) and –negative (6.04 logs) animals (P = 0.0085, Supplemental Figure 4). In group 4, the VLs of Mamu-A*01 animals (mean 5.12 ± 0.21) were maintained significantly lower, approximately 1 log (1.04), than those of Mamu-A*02 animals (mean 6.16 ± 0.15). This was in agreement with an earlier report that the Mamu-A*01 allele correlated with slower SIV disease progression (36). In the adjuvant-only group, Mamu-A*01 animals had significantly (up to 2.5 logs) lower VLs compared with those of their Mamu-A*02 counterparts. Among the Mamu-A*01 animals, both group 4 and the adjuvant-only group showed a statistically significant reduction of VLs compared with those seen in naive animals (Figure 2B). Moreover, the adjuvant-only A*01 group had VLs significantly lower than those in group 4 A*01 animals.

We observed this viral reduction in groups 4 and 5 only in Mamu-A*01 animals, suggesting a class I–restricted $CD8^+$ T cell response to the antigen in group 4 and also in the adjuvant-only group, presumably induced by the virus challenge after some innate effect of the adjuvants. To determine whether $CD8^+$ T cells played a role in controlling VLs, CD8 T cells were depleted 6 months after infection. In the Mamu-A*01 animals in group 5, which showed reduced VLs, we found a spike in VL after $CD8^+$ T cell depletion, similar to the spike in the vaccinated animals that showed a greater reduction in set-point VLs (Figure 2C and Supplemental Figure 5). Furthermore, the down-slope of the viral spikes was concomitant with $CD8^+$ T cell recovery. Therefore, although all the groups showed some effect of CD8 depletion, the VL spikes after CD8 depletion correlated with protection, implying that the protective effect was indeed largely $CD8^+$ T cell dependent, as predicted by the Mamu-A*01 restriction, even in adjuvant-only animals that never received vaccine antigens.

Early and peak VLs positively correlated with postvaccination immune activation status. To understand why vaccination reduced the beneficial effect of the adjuvants on VL, we proposed 2 complementary hypotheses: (a) immune activation creates target cells for infection, and (b) vaccine induces regulatory cells. We first measured the vaccine-induced, Gag-specific immune responses in the colonic lamina propria (LP). The Gag-specific Mamu-A*01 tetramer and Mamu-A*02 pentamer responses and Gag-specific cytokine staining of $CD4^+$/$CD8^+$ T cell responses in the 4 vaccinated groups did not significantly differ (Figure 3, A and C).

Virus-specific $CD4^+$ T cells not only provide help for $CD8^+$ T cell responses, but are also preferentially infected upon viral infection (37). The induction of these cells in the colonic LP could potentially exacerbate viral transmission and replication.

Figure 1
The adjuvant-only group had the best viral control upon SIVmac251 infection. (**A**) Schematic illustration of the vaccination and challenge protocols (animals did not receive further viral challenge once they were confirmed infected). (**B**) Effect of SIVmac251 challenge on the plasma SIV RNA VLs of the individual macaques (the days after infection for each animal started from the day of challenge that led to the infection of that animal.). "*" denotes the Mamu-A*01 animals. (**C**) Geometric means of VLs of each group during acute and chronic stages. Repeated-measures ANOVA was used to compare the adjuvant-only group (n = 6) with the naive control group (n = 8). (**D**) Comparison of plasma RNA VLs of groups at 2 weeks or 6 months after infection. The Mann-Whitney U test was used to compare the adjuvant-only group with the naive control group. (**E**) Comparison of absolute CD4+ T cell count ratio (2-week or 6-month after infection versus before infection). The Mann-Whitney U test was used to compare the adjuvant-only group with naive control group. Data represent mean ± SEM. "Vac" refers to Pep/MVA + TLRLs + IL-15.

However, we observed a weak inverse correlation of early VL with Gag-specific IL-2+CD4+ T cell responses (Figure 3B), indicating that the latter might be beneficial for viral control.

We then examined immune activation in postvaccination mucosal tissues and PBMCs. Immune activation, one of the hallmarks of HIV-1 infection, not only promotes viral infection, but also abolishes the benefit of vaccine-induced immune responses by providing more target cells for viral replications. When we measured the Ki67+CCR5+CD4+ T cell frequency in PBMCs, we did not observe any difference among the groups, either after or before vaccination (not shown). In the postvaccination colonic intraepithelial lymphocytes (IELs), however, we found that the adjuvant-only group had a significantly lower frequency of Ki67+CCR5+CD4+ T cells than did the vaccinated groups, and this was positively correlated with 2-week postinfection VLs, but not set-point VLs (Figure 3, D–F). Other activation markers such as CD38, HLA-DR, and CD69 in the CD4+ T cells did not differ significantly among the groups (not shown). One potential mechanism by which the adjuvant-only group had better viral control could be the low frequency of mucosal CCR5+Ki67+CD4+ T cells, which serve as viral targets in colorectal tissues.

Myeloid cells, which resembled MDSCs, were upregulated during vaccination and early viral infection and positively correlated with set-point VLs. The above CD8 depletion study clearly demonstrated the importance of CD8+ T cells in controlling viral replication, including in the adjuvant-only group. However, this cannot explain the paradoxical observation that Mamu-A*01 animals in the adjuvant-only group showed better CD8+ T cell–dependent VL control than did those in group 4 that received the same adjuvants plus vaccine. Besides the immune activation examined above, regulatory cells may be critical. Accumulating data demonstrate that MDSCs have a remarkable ability to suppress T cell responses in cancer patients and HIV-1–infected individuals (32, 33, 38–40). We hypothesized

Figure 2

The viral control in the adjuvant-only control group was MHC class I restricted and mainly dependent on CD8+ T cells. (**A**) Comparison of geometric means of plasma SIV RNA VLs of Mamu-A*01+ (red) and Mamu-A*01– (black) macaques in each of the 6 groups. *P* values of the differences between Mamu-A*01+ and 01– animals in groups 4 and 5 were calculated by repeated-measures ANOVA. (**B**) Comparison of geometric means of plasma SIV RNA VLs of the animals from the 6 groups in Mamu-A*01+ and Mamu-A*01– macaques. *P* values of the differences between groups in Mamu-A*01+ animals were calculated by repeated-measures ANOVA. (**C**) Effect of CD8 depletion on the plasma SIV RNA VLs of macaques in vaccinated, naive, and adjuvant-only groups. Data represent mean ± SEM.

that MDSCs are altered in the SIV vaccine–immunized animals. To assess the roles of MDSCs, we investigated 2 subsets of myeloid cells, which phenotypically resemble the MDSCs described originally in cancer patients: Lin–DRloCD33+CD11b+ (Lin– MDSCs) and CD14+DRloCD33+CD11b+ (CD14+ MDSCs) (Supplemental Figure 6).

Similarly to HIV-infected humans (32, 33), MDSCs maintained high frequencies in the acutely and chronically infected macaques (Figure 4). During vaccination, we observed different kinetics in these 2 subsets (Figure 4). The frequency of Lin– MDSCs did not change after the first MVA-SIV boost, but showed a significant increase after the second boost in the 4 vaccinated groups ($P < 0.0001$), whereas the adjuvant-only group did not change after either boost. In contrast, we found that the frequency of CD14+ MDSCs was increased after both boosts in the 4 vaccinated groups as well as in the adjuvant-only group, though the latter showed a much smaller increase than the former. The naive animals showed no change in MDSC frequency at the 2 time points measured before challenge, weeks 17 and 20. Overall, the vaccinated groups significantly upregulated their MDSC frequencies after the second boost. Most importantly, after the second boost, we saw significantly lower frequencies of MDSCs in the adjuvant-only group compared with those in the pool of all vaccinated groups (Figure 4, B and D). Thus it was the vaccine, but not the adjuvant, that induced the expansion of MDSC frequencies during immunization. More MDSCs in vaccinated groups might inhibit the induction and function of protective CD8+ T cell responses, which would indirectly affect the set-point VLs.

To test whether the MDSCs were suppressive, we used 2 approaches: addition of sorted MDSCs to T cells plus APCs or depletion of MDSCs from PBMCs. First, we performed in vitro T cell proliferation assays using FACS-sorted MDSCs from SIV vaccine–immunized, preinfected or postinfected PBMCs. CFSE-labeled purified T cells were either cultured alone or cocultured with autologous MDSCs at the ratios indicated in Figure 5A for 3 to 4 days, with the stimulation of SIV/HIV-1–specific peptide pool included in the vaccine (in the presence of APCs). Reduction of T cell proliferation showed that the sorted MDSCs (CD3–DR–CD33+CD11b+) significantly inhibited the SIV antigen–

Figure 3

Early and peak VLs inversely correlated with Gag-specific CD4+ T cell responses and positively correlated with immune activation status of the postvaccinated colonic tissue. (**A**) Comparison of vaccine-induced IL-2+CD3+CD4+ T cell frequency in the colonic LP 3 weeks after the last boost (n = 26). (**B**) IL-2+CD3+CD4+ T cell frequency in the colonic LP 3 weeks after the last boost was inversely correlated with acute infection VLs (n = 26). Spearman's analysis of correlation was used to calculate the R and P values. The lines represent least-squares, best-fit linear regression. (**C**) Comparison of Gag-specific CD8+ T cell responses in the colonic LP 3 weeks after the last boost (n = 26). There were no detectable levels of Gag-specific IL-2+CD4+ T cell or tetramer+ CD8+ T cell responses in the adjuvant or naive groups before challenge. (**D**) Analysis of Ki67+CCR5+CD4+ T cell frequency in colonic IELs 3 weeks after the second boost (n = 32). The Mann-Whitney U test was used to compare the pool of the vaccinated groups with the adjuvant-only group. (**E** and **F**) The frequency of Ki67+CCR5+CD4+ T cells in colonic IELs 3 weeks after the second boost (n = 32) was positively correlated with week-2 postinfection plasma VLs, but not with the set-point VLs. Spearman's analysis of correlation was used to calculate the R and P values. The lines represent least-squares, best-fit linear regression. Data represent mean ± SEM.

specific CD8+ T cell responses (P = 0.0005 to reject the null hypothesis that the slopes were zero [i.e., no effect of MDSCs], using a least-squares regression of the arcsine-transformed percentages as a function of MDSC/T cell ratio) (Figure 5A). We obtained consistent results (all negative slopes) in all 6 animals tested (Figure 5A) and obtained similar results using anti-CD3/CD28 stimulation of the responder T cells without added APCs (data not shown). Second, since depleting the CD33+ cells from PBMCs resulted in greater than 90% depletion of MDSCs, we further compared the T cell responses to anti-CD3/CD28 stimulus in the PBMCs, with or without CD33 depletion. The results showing increased responses in PBMCs from all 6 tested animals after MDSC depletion (P = 0.03 by nonparametric Wilcoxon test) confirmed the suppressive effect of MDSCs on CD8+ T cells (Figure 5B).

To investigate whether MDSCs were associated with viral control, we performed a Spearman's correlation analysis between VLs and the 2 subsets of MDSCs (Figure 6). There was no correlation in the prevaccination samples. After the first boost, both subsets of MDSCs showed positive correlations with set-point (Figure 6, A and B, and Supplemental Figure 7), but not early (not shown), VLs. After the second boost, with the upregulation of Lin- MDSCs, we found no correlation between MDSCs and VLs when all the animals were pooled. However, when we focused on the groups that received all the adjuvants (groups 4 and 5) and on the naive group (group 6), we found positive correlations (or trends) between set-point VLs and Lin- MDSCs or CD14+ MDSCs (Figure 6, D and E, and Supplemental Figure 7). Upon infection, both Lin- and CD14+ MDSCs were upregulated compared with prevaccination levels. However, only the Lin- MDSCs in the acute infection positively correlated with set-point VLs. Because of the interest in developing a mucosal vaccine, we examined the MDSCs in colorectal tissues after the second boost. We observed a positive correlation between

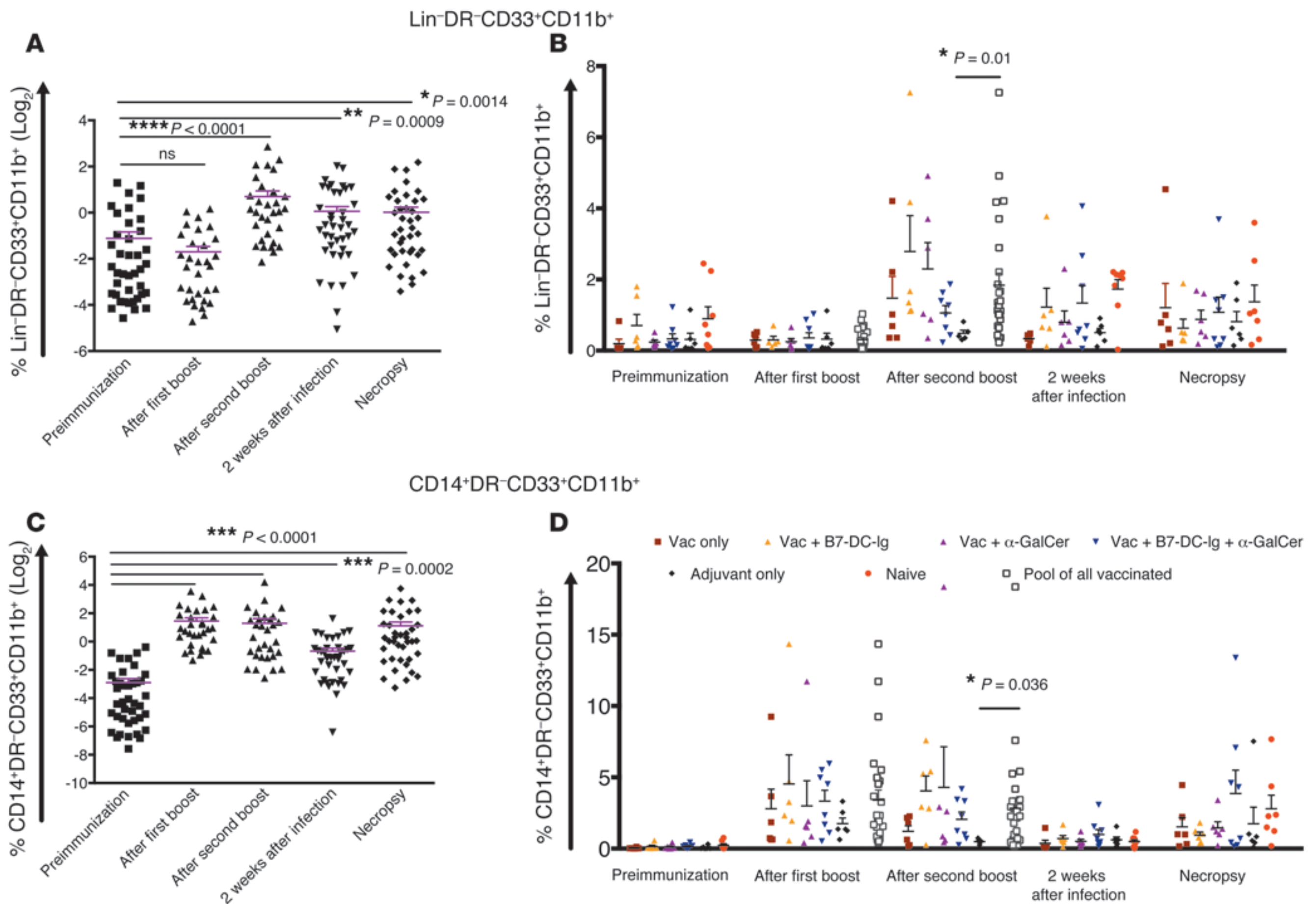

Figure 4

Myeloid cells, which resembled MDSCs, were upregulated during vaccination and SIVmac251 infection. (**A** and **C**) Overall frequency of Lin⁻ (**A**) and CD14⁺ (**C**) MDSCs in the PBMCs of vaccinated (n = 32) and SIV-infected animals (n =40). Kruskal-Wallis tests were used to compare the levels of Lin⁻ and CD14⁺ MDSCs in the postvaccination and postinfection PBMCs with the preimmunization PBMCs (n = 40). Dunn's multiple comparison tests were used for multiple comparison corrections. (**B** and **D**) Group comparison of Lin⁻ (**B**) and CD14⁺ (**D**) MDSCs in the PBMCs during the course of immunization and infection. The naive animals were received shortly before challenge and tested at weeks 17 and 20, with no difference found between these dates. The week-20 data for the naive animals are shown in **B** and **D** as preimmunization values for these animals, but these are also the immediately-before-challenge values for the naive animals. In the post-second-boost samples, the Mann-Whitney U test was used to compare the adjuvant-only group with the pool of the 4 vaccinated groups (groups 1–4). Data represent mean ± SEM.

CD14⁺ MDSCs and set-point VLs in the Mamu-A*01 animals (Figure 6G), but not in the Lin⁻ MDSCs (not shown), suggesting different roles played by different subsets of MDSCs in the systemic and mucosal immune systems.

FOXP3⁺CD4⁺ Tregs, another important immune regulator, have been shown to be involved in HIV infection. In contrast to MDSCs, we did not find significant alterations in Tregs during the immunization. In fact, CD4⁺ Tregs demonstrated significant inverse correlations with set-point VLs after the boosts, which was not consistent with any deleterious effect, but the correlations became nonsignificant upon infection (Figure 6, C, F, and I). Taken together, these data highlight the important adverse effect of MDSCs, but not CD4⁺ Tregs, in modulating set-point VLs.

Latent TGF-β, which was upregulated in the vaccinated animals, played an important role in the induction of MDSCs during vaccination. Various factors including IL-6, IL-13, GM-CSF, IL-1β, TGF-β, and prostaglandins have been shown to have important implications in the in vitro and in vivo differentiation and induction of MDSCs (41, 42). To investigate the possible factors that regulate the expansion, and maybe the function, of MDSCs during SIV vaccination, we quantified these factors in the plasma of the vaccinated and naive animals before viral challenge. Consistent with the lack of obvious side effects of the current vaccine regimen, no significant amount of pro- or antiinflammatory cytokines were detected in the plasma samples that were collected 3 weeks after the last boost (Figure 7, A and B). None of the animals had a detectable level of GM-CSF, only 1 animal showed a detectable level of IL-1β, and the majority of the animals did not have detectable levels of IL-6 or IL-13 (Figure 7, A and B). The expression level of PGE2 in the vaccinated animals was comparable to that of the naive control animals and within the range of the level seen in healthy human plasma samples (Figure 7C). Additionally, none of these cytokines or factors correlated with MDSCs (data not shown). Thus, none of these cytokines were significantly increased, and the data did not suggest any important role played by these cytokines in the induction of MDSCs using the current vaccine regimen.

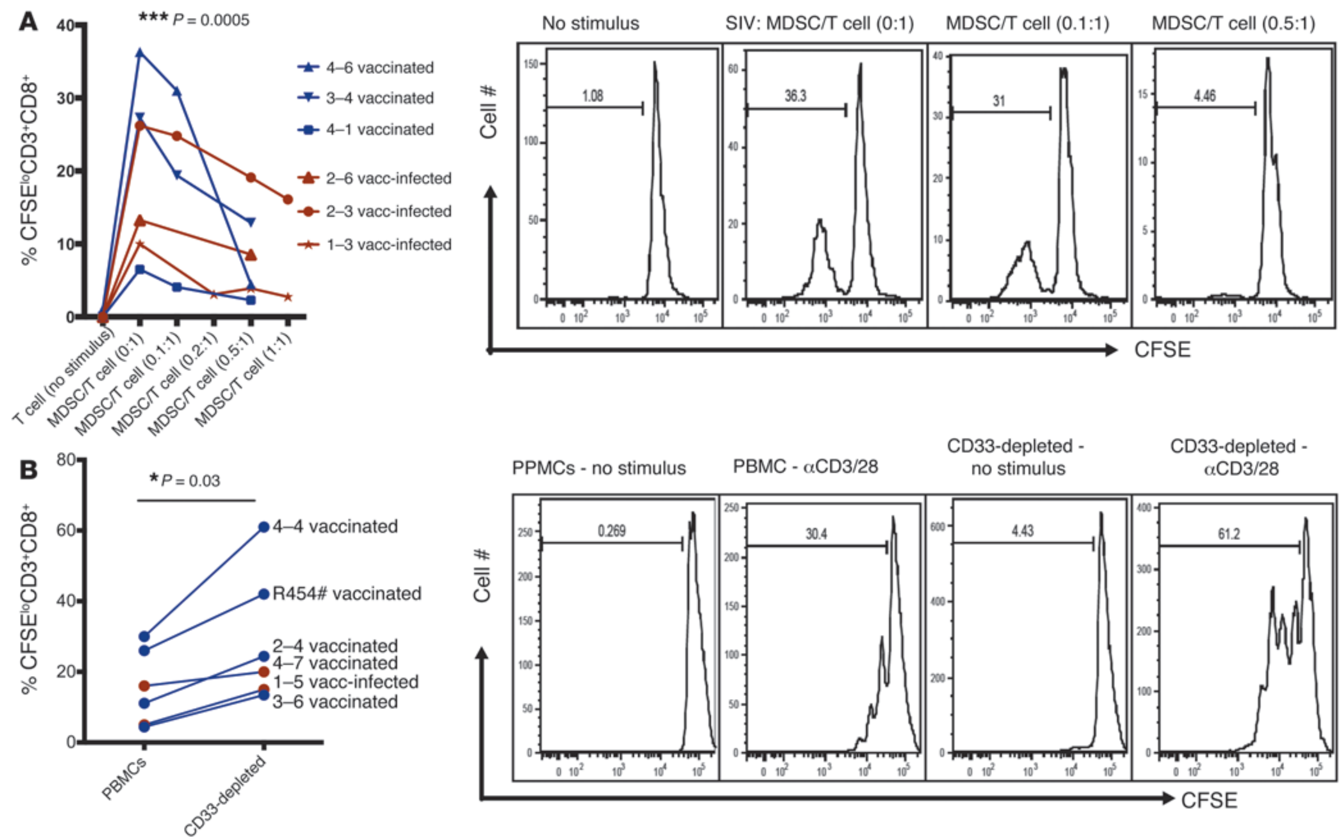

Figure 5
MDSCs from PBMCs of the SIV vaccine–immunized (prior to infection) or vaccinated animals after SIVmac251 infection suppressed $CD8^+$ T cell responses. (**A**) Suppression effects of MDSCs ($DR^-CD11b^+CD33^+$) on antigen-specific $CD8^+$ T cell function in a coculture experiment. $CD3^+$ T cells from PBMCs of SIV vaccine–immunized (Vaccinated) or vaccinated-then-SIVmac251-infected (Vacc-infected) macaques were stimulated with the SIV peptide pool (same as that used in the vaccination) in the presence of APCs. MDSCs from the same animal were added to the coculture at different ratios. $CD8^+$ T cell proliferation was evaluated by CFSE dilution after 3 to 4 days of culture. Unstimulated T cells were used as a negative control. Right: Representative flow cytometry plot from 1 animal. Left: Results from 6 animals from 5 independent experiments. (**B**) Depletion of $CD33^+$ cells enhanced $CD8^+$ T cell responses. PBMCs or PBMCs with CD33 depletion (CD33 depleted) were stimulated with anti-CD3/28 antibodies for 3 to 4 days. $CD8^+$ T cell proliferation was evaluated by CFSE dilution. Unstimulated PBMCs or CD33-depleted PBMCs were used as negative controls. Right: Representative flow cytometry plot from 1 animal. Left: Results from 6 animals from 5 independent experiments, R454# was an animal from another cohort with similar immunization. For the purified MDSC suppression assay (**A**), a least-squares regression of the slopes of the arcsine-transformed percentages over the ordinal MDSC/T cell ratios was used, rejecting the null hypothesis that the slopes were zero. The Wilcoxon signed-rank test was used for in vitro MDSC depletion assays (**B**).

Latent TGF-β, however, was significantly elevated in the plasma of the vaccinated animals compared with that in naive controls ($P < 0.001$), though it was still within the range of healthy human donors (Figure 7D). Interestingly, the level of latent TGF-β in the plasma also positively correlated with the frequency of Lin^- MDSCs ($P = 0.02$) and showed a positive correlation trend ($P = 0.062$) with the frequency of $CD14^+$ MDSCs (Figure 7, E and F). TGF-β is a pleiotropic cytokine critical for many physiological and immunological processes. TGF-β is synthesized in an inactive (latent) form, and additional events are required to cleave the precursor to produce the active form and thus exert its function to regulate adaptive immunity components, such as T cells (43). In an ongoing SIV vaccine study that we are conducting, we found that the neutralization of TGF-β using an anti–TGF-β1 antibody intrarectally during intrarectal vaccination greatly reduced the frequency of Lin^- MDSCs, but not $CD14^+$ MDSCs, in PBMCs and did so to an even greater degree in the bone marrow (Y. Sui et al., unpublished observations). Collectively, all these data showed that TGF-β played an important role in the differentiation of MDSCs.

Discussion

With an increasing number of HIV/SIV vaccine strategies focusing on the induction and maintenance of virus-specific $CD8^+$ T cell responses (1, 2), the role of immune-regulatory cells has become more important. Here, we demonstrated for the first time to our knowledge that set-point VLs, which were mostly determined by protective $CD8^+$ T cells, were positively associated with vaccine- and/or SIV-induced expansion of MDSCs. Although correlation analysis does not delineate cause and effect, the data, taken together, lead most logically to a model in which the induced expansion of MDSCs during vaccination and early SIV infection contributed to the suppression of the protective $CD8^+$ T cellular responses and thus resulted in a loss of protection against SIV

Figure 6

Chronic set-point VLs positively correlated with myeloid cells, but inversely correlated with FOXP3+CD4+ T cells during vaccination or infection. Spearman's analysis of correlations between set-point VLs and the frequencies of Lin− (**A** and **D**) or CD14+ (**B** and **E**) MDSCs or FOXP3+CD4+ T cells (**C** and **F**) in PBMCs after the first (n = 31) and second boosts (n = 20) and between set-point VLs and CD14+ MDSC frequency after the last boost in the colon of Mamu-A*01 (A*01+) animals (**G**) (n = 23); or CD14+ MDSCs (**H**) and FOXP3+CD4+ T cell (**I**) frequencies in PBMCs 2 weeks after infection (n = 40). The lines represent least-squares, best-fit linear regression. Maroon squares indicate group 1, yellow triangles indicate group 2, purple triangles indicate group 3, blue triangles indicate group 4, black diamonds indicate group 5, and red circles indicate group 6.

replication. These data suggest that the current SIV/HIV vaccine regimens induce a strong cellular response, but that the subsequently triggered induction of MDSCs attenuates T cell–mediated protection. Therefore, prevention of this MDSC expansion may be critical to achieving vaccine efficacy.

We observed, to our surprise, that the Mamu-A*01 animals in the adjuvants-plus-vaccine group had higher set-point VLs than those in the adjuvant-only group. Furthermore, the CD8 depletion study and class I MHC restriction revealed that CD8+ T cell responses in the adjuvant-only group, which were probably induced by viral infection but influenced by the adjuvant, were essential for viral control. Though we found that anti-CD8 could also deplete a subpopulation of NK cells in rhesus macaques, the partial protection in the adjuvant-only group was restricted by the MHC class I molecule Mamu-A*01, not Mamu-A*02, and only CD8 T cells, but not NK cells, would discriminate between Mamu-A*01 and Mamu-A*02. Therefore, it is likely that the protection in the Mamu-A*01 animals in the adjuvant-only group was mainly due to CD8+ T cells, although we cannot rule out some contribution by CD8+ NK cells. To explore the possible mechanisms by which the adjuvant-only group manifested better protection than did the vaccinated groups, we hypothesized that immune regulators such as Tregs and MDSCs are involved. Downregulation of negative regulators such as Tregs during acute infection has been shown to be essential for optimal T cell responses and host resistance to pathogens such as *Toxoplasma gondii*, *Listeria monocytogenes,* and vaccinia virus, and restoration of Tregs led to enhanced susceptibility to these pathogens (44). In this study, however, we found positive associations of set-point VLs only with MDSCs, but not with Tregs. MDSCs represent a het-

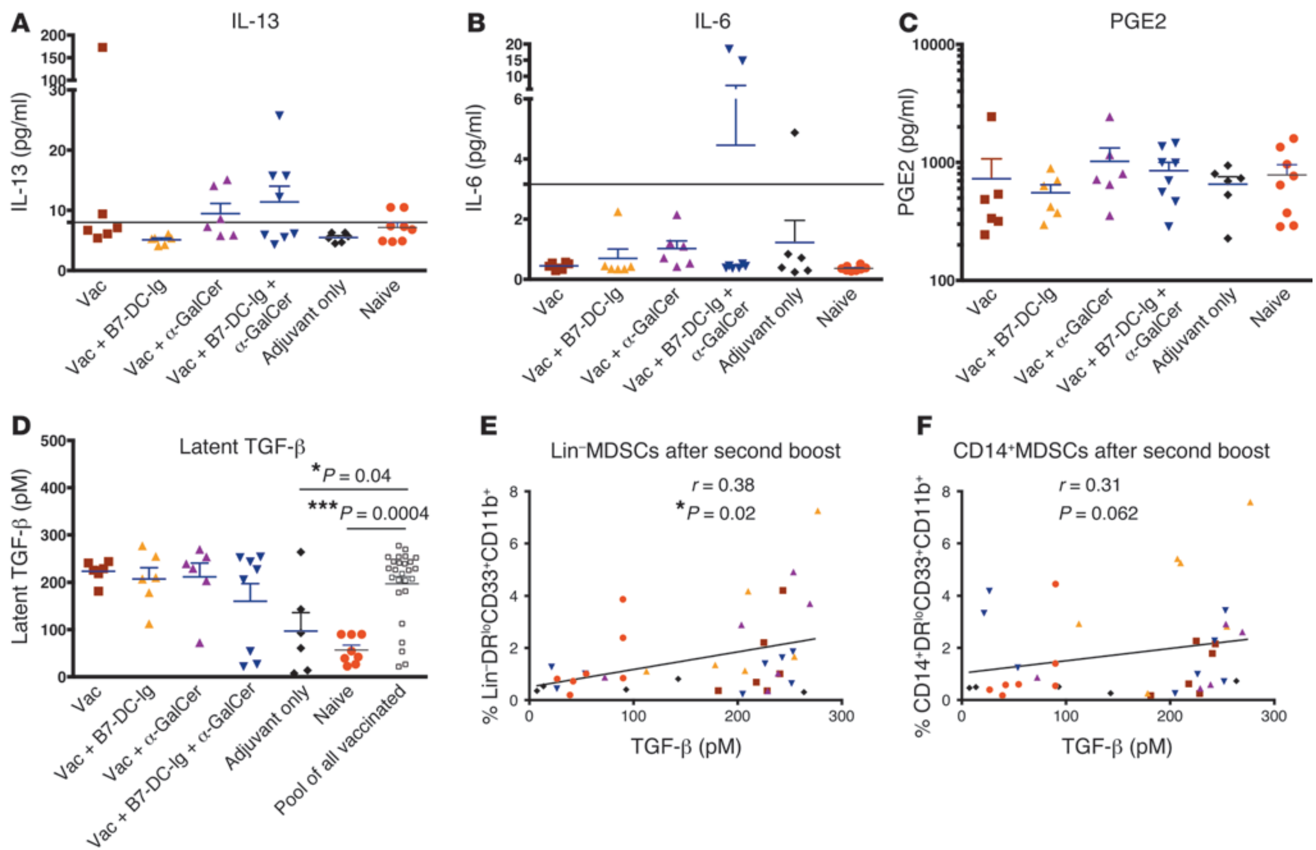

Figure 7

Expression levels of cytokines and factors in the plasma of vaccinated animals 3 weeks after the last vaccination. (**A**–**D**) Expression levels of IL-13, IL-6, PGE2, and latent TGF-β (n = 40). Data represent mean ± SEM. The black lines in IL-13 and IL-6 plots indicate the detection levels of the ELISA kits. The Mann-Whitney U test was used to compare the pool of the 4 vaccinated groups (groups 1–4) with the naive and adjuvant-only groups for latent TGF-β. (**E** and **F**) Spearman's analysis of correlations between expression levels of latent TGF-β and MDSCs. The lines represent least-squares, best-fit linear regression.

erogeneous population of cells that consist of myeloid progenitor cells and immature myeloid cells, which have the ability to suppress the effector immune response (45). In contrast to murine MDSCs, which are defined by the expression of Gr-1 and CD11b, the corresponding cells in humans are inadequately characterized due to the lack of uniform markers. MDSCs have been intensively studied in cancer and were found to have important implications in disease progression (38–40). To investigate the MDSCs, we chose 2 subsets of markers that have shown statistical association with human cancer disease stages (38–40). These subsets have been shown to have suppressive activity in HIV-infected humans (32, 33). We found that both subsets correlated with VLs, though the Lin⁻ MDSCs seemed to correlate better.

Although MDSCs have been intensively investigated in the pathogenesis of cancer and some viral infections (46), their emerging roles in the effectiveness of HIV/SIV vaccines are less appreciated. Elevated levels of MDSCs were found to be associated with progressive HIV-1 infection (32), and the expansion of MDSCs dampened T cell function in HIV-1–seropositive individuals (33), but MDSCs have not been studied in the context of HIV vaccines. In this study, we speculated that the expanded MDSCs play an important deleterious role in promoting viral replication via the inhibition of virus-specific CD8⁺ T cells that can otherwise effectively control viral replication. As it remains elusive which qualities of CD8⁺ T cell responses mediate HIV/SIV control, we cannot further delineate the mechanisms by establishing a direct correlation between effective virus-specific CD8⁺ T cell activity and MDSCs. Nevertheless, a lower level of MDSCs during vaccination or upon acute SIV infection, even without prior vaccination, was possibly beneficial in enhancing the induction of protective antiviral immunity.

The current paradigm suggests that MDSCs acquire suppressive function only after exposure to factors present in inflammatory or tumor microenvironments. Inflammatory and/or activated T cells are necessary for activation of suppression in MDSCs (47). In addition, recent studies also demonstrated that vaccinia virus (48, 49), as well as HIV tat and gp120 proteins, induced expansion of MDSCs that suppressed immunity (33, 50). As these components were all included in the vaccine regimen, but not in the adjuvant-only group, it was possible that combination of the activated T cells, vaccinia virus, and vaccinia vector–expressed SIV proteins drove the expansion and function of MDSCs. Consistent with this possibility, our data demonstrated that MDSCs, which were able to suppress T cell responses, were elevated in the vaccinated groups, but not in the adjuvant-only group.

Adjuvant can alter the suppressive function of MDSCs. It had been demonstrated that CpG and polyI:C induced differentiation of MDSCs (or M2 macrophages) into M1 macrophages to aid in tumor elimination, and MDSCs responding to CpG lost their ability to suppress T cell function and produced more Th1 cytokines (51–53). However, these beneficial effects of the TLR agonists alone cannot explain the different outcomes of VL control in the groups that received adjuvant plus vaccine or adjuvant alone, as all these groups received the same TLR agonists. To understand the apparent paradox that the VL reduction was greater in the adjuvant-only group than in group 4, which received the same adjuvants plus vaccine, we examined both the immune activation of $CD4^+$ T cells and the expansion of MDSCs. Both mechanisms appeared complementary to each other in determining the outcome, acting primarily at different stages of the infection. The immune activation of $CD4^+$ T cells (creating more targets for virus), which was not increased in the adjuvant-only group, was positively correlated with early, but not set-point, VLs, implying greater effect on acute viral control. Based on the association of mucosal $CD4^+Ki67^+CCR5^+$ T cells with acute VLs, one possible mechanism is the diminished immune activation in the rectal mucosa of the adjuvant-only animals before viral challenge, which led to the lower peak VLs at the acute phase. Another possible innate mechanism could be the upregulation of APOBEC3G by the adjuvant, which we observed previously (12). Indeed, all the animals receiving at least the base adjuvant of TLR ligands and IL-15 had increased levels of APOBEC3G in the colonic LP cells compared with those in the naive controls, although the levels of APOBEC3G did not correlate with VL (data not shown). Conversely, the MDSCs, which were also maintained at a lower level in the adjuvant-only group, were positively correlated with set-point, but not early, VLs, suggesting a greater role of MDSCs in modulating the chronic infection. It is possible that the lower frequency of MDSCs in the adjuvant-only group was more permissive for the induction and function of $CD8^+$ T cells to decrease the set-point VLs. The inclusion of an adjuvant-only group in this study revealed the possible deleterious role of vaccine-induced MDSC expansion. Overall, our data demonstrate that, compared with adjuvant only, the addition of the vaccine induced events that promoted viral replication, such as the proliferation of mucosal viral target $CD4^+$ T cells, and the expansion of suppressive cell subsets, such as MDSCs. In terms of MDSC expansion, one of the factors involved could be TGF-β. We demonstrated that vaccination increased plasma latent TGF-β levels, which were positively correlated with Lin^- MDSCs. Most importantly, in another similar SIV vaccine study, we observed that the frequencies of Lin^- MDSCs in macaque PBMCs, and more prominently in the bone marrow, were significantly decreased in the animals treated with anti–TGF-β antibody (Y. Sui et al., unpublished observations), suggesting the important role of TGF-β in the differentiation, and maybe the function, of MDSCs.

Our findings may have important applications in the development of other HIV/SIV vaccine platforms as well. Our date indicate that it may be worth evaluating possible vaccine-induced MDSC expansion in other HIV/SIV vaccine regimens. For example, many HIV clinical trials, such as the HVTN 503/Phambili trial (54) and the recently prematurely halted HVTN 505 trial, focused on developing strong cellular immune responses; however, our data suggest that poor vaccine efficacy may be explained by an alternative mechanism involving the induction of negative regulatory cells such as MDSCs. If such a mechanism indeed exists, it might help explain why some vaccine platforms have appeared less effective than others, despite the ability to induce similar or higher cellular immune responses.

In summary, our findings demonstrate a positive association of chronic VL with MDSCs and suggest that vaccine-induced expansion of MDSCs plays a role in abrogation of vaccine-induced protective immune responses. Thus, targeting MDSCs to prevent such adverse effects, thereby allowing vaccines to be protective, might be a promising strategy for HIV vaccine design.

Methods

Animals. A total of 40 adult Indian rhesus macaques (*Macaca mulatta*) were used in this study. They were maintained in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International and with approval of the Animal Care and Use Committee of the National Cancer Institute (NCI). All macaques were free of SIV, simian retroviruses 1, 2, and 5, and simian T cell leukemia/lymphotropic virus type 1 before the study. The MHC complex alleles of the animals were tested and are shown in Supplemental Table 1.

Immunization and viral challenge. The 40 macaques were divided into the following 4 immunization groups (see Supplemental Table 1): group 1 (Vac): Pep/MVA + TLRLs + IL-15; group 2: Pep/MVA + TLRLs + IL-15 + B7-DC-Ig; group 3: Pep/MVA + TLRLs + IL-15 + α-GalCer; and group 4: Pep/MVA + TLRLs + IL-15 + α-GalCer + B7-DC-Ig. The adjuvant-only and naive groups were designated as groups 5 and 6. PD-1 modulator (B7-DC-Ig) was used at 10 mg/kg per dose and α-GalCer at 300 μg per dose. For immunization, each peptide vaccine (Supplemental Table 2 and refs. 5, 21) contained 0.5 mg of each peptide mixed with DOTAP (100 μl per dose; Roche) with a combination of 500 μg per dose of D-type CpG oligodeoxynucleotide, 10 μg per dose of MALP2, 1 mg per dose of PolyI:C (InvivoGen), and 300 μg per dose of recombinant human IL-15 (Peprotech), with or without B7-DC-Ig/α-GalCer. Two recombinant MVA viruses expressing (a) SIVmac239 Gag, Pol and Env, and (b) SIVmac239 Rev, Tat, and Nef (5×10^8/immunization for each) (55) were administered intracolorectally, also with different adjuvants. Intracolorectal inoculations were performed as previously described (12). The adjuvant-only group received only TLR agonists, IL-15, α-GalCer, and B7-DC-Ig without peptides or MVA-SIV. Macaques were primed at weeks 0, 3, and 6 with peptides and adjuvants and boosted at weeks 13 and 16 with MVA-SIV and adjuvants (22). At weeks 26, 28, and 30, all animals received an intrarectal inoculation of 1:100, 1:100, and 1:50 diluted SIVmac251 stock, which was provided by Nancy Miller of the National Institute of Allergy and Infectious Diseases (NIAID), NIH, until they were infected. Once the animals were confirmed infected with the day-11 postinfection samples, they were not challenged further. Eight macaques were included to serve as naive controls. After challenge, SIV RNA levels and absolute $CD4^+$ T cell counts were monitored for 6 to 14 months by Advanced BioScience Laboratories Inc. Set-point plasma VLs were calculated as the average of the geometric means of plasma VLs from days 42 to 147 after challenge.

CD8 depletion. Six months after SIVmac251 infection, 16 macaques from vaccinated, adjuvant, and naive groups were administered anti-CD8 antibody, M-T807R1, or control mAb by intravenous injection. For optimal depletion of $CD8^+$ lymphocytes, 3–5 mg/kg of M-T807R1 or control antibody was administered 3 times (days 0, 4, and 7). Blood was collected at different time points to verify the depletion of $CD8^+$ T cells. The changes in VLs and $CD4^+$ T cell counts were monitored before and after the depletion.

Flow cytometric analysis of T cell assays and MDSCs. SIV-specific T cells were measured in mononuclear cells isolated from colonic LP and IELs by flow cytometric intracellular cytokine analysis, as previously described in

detail (12, 56). For tetramer staining, we used Mamu-A*01-CM9 tetramer obtained from the NIAID's MHC tetramer core facility. The following antibodies were purchased: CD3-PE-Cy7, CD8-APC-Cy7, Ki67-APC, HLA-DR-PE-Cy5, and CCR5-PE (from BD Pharmingen); CD28-FITC, CD95-PE-Cy5, IFN-γ-Alexa Fluor 700, IL-2-Alexa Fluor 647, TNF-α-PE, and CD69-Alexa Fluor 700 (from Biolegend); CD38-FITC (from STEMCELL Technologies); and CD4-qdot 605 (from eBioscience). MDSCs were measured in the PBMCs and colonic LP. Cells were first incubated with Fc Receptor (FcR) blocking reagent (Miltenyi Biotec) to saturate FcR and were then labeled with mAbs. CD33-PE was purchased from Miltenyi Biotec, and CD14-V450, HLA-DR-APC-Cy7, Lin-FITC, and CD11b-PE-Cy5 were purchased from BD. Violet or yellow viability dye was used to distinguish the live/dead cells (Invitrogen). Data acquisition was performed with an LSRII flow cytometer, and data were analyzed with FlowJo software (Tree Star Inc.).

MDSC sorting and T cell proliferation assays. A BD FACSAria (BD Biosciences) was used for flow cytometric sorting. The strategy used for MDSC sorting involved sorting $CD3^-HLA\text{-}DR^-CD11b^+CD33^+$ cells from live PBMCs. The purity of the sorted MDSCs was greater than 95%. T cell proliferation was evaluated by CFSE dilution. Purified T cells were labeled with CFSE (3 μM; Invitrogen), stimulated with SIV/HIV-1–specific peptides included in the vaccine (1 μg/ml each, Supplemental Table 2) in the presence of APCs ($CD3^-DR^+$), and cultured alone or cocultured with autologous MDSCs at the ratios indicated in Figure 5A for 3 to 4 days (or stimulated with anti-CD3/CD28 without added APCs; not shown). Depletion of MDSCs was performed by incubating the PBMCs with PE-conjugated CD33 antibody and then depleting the $CD33^+$ cells using anti-PE magnetic Microbeads (Miltenyi Biotec). Greater than 90% of the MDSCs were depleted from the PBMCs. PBMCs or CD33-depleted PBMCs were labeled with CFSE followed by stimulation with anti-CD3/CD28 antibodies (5 μg/ml CD3, 2 μg/ml for CD28; obtained from NIH Nonhuman Primate Reagent Resource) for 3 to 4 days. In both assays, the cells were then stained for surface marker expression with CD3/CD4/CD8 antibodies, and T cell proliferation was analyzed on a flow cytometer (BD LSR II; BD Biosciences).

ELISA. ELISA kits that cross-react with macaque samples were purchased from Mabtech (for GM-CSF, latent TGF-β, IL-6, and IL-13) and R&D Systems (for PGE-2 and IL-1β). Plasma samples were collected 3 weeks after the last boost.

Statistics. We performed statistical analyses with Prism for Mac, version 6 (GraphPad Software) and SAS/STAT software, version 9.3 of the SAS System for Windows (SAS Institute Inc.). We used a 2-sided significance level of 0.05 for all analyses. The Mann-Whitney U test was used to compare the 2-week postinfection VLs and CD4 counts of the adjuvant-only group with naive controls; the immune activation of the post-boost colonic tissue of the adjuvant-only group with the pool of the 4 vaccinated groups; and the MDSCs in the post-second-boost PBMCs of the adjuvant-only group with the pool of the 4 vaccinated groups. A repeated measures ANOVA was used to compare the acute VLs of the adjuvant-only group with those of the naive controls; the VLs of Mamu-A*01+ animals of each group with the those of the Mamu-A*01– animals in the same group; as well as to compare groups within the Mamu-A*01 subset. For the purified MDSC suppression assay (Figure 5A), a least-squares regression of the arcsine-transformed percentages was used to determine whether the slopes over the ordinal MDSC/T cell ratios were significantly different from zero (i.e., whether the response was changed by the addition of MDSCs). A Wilcoxon test was used for in vitro MDSC depletion assays (Figure 5B). Kruskal-Wallis tests were used to compare the postvaccinated and postinfected levels of MDSCs with the preimmunized levels, with Dunn's multiple comparison tests to correct for multiple comparisons. Spearman's analysis was used for all the correlations.

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