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TLR agonists and/or IL-15 adjuvanted mucosal SIV-vaccine reduced gut CD4⁺ memory T cell loss in SIVmac251-challenged rhesus macaques

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Abstract

Adjuvant plays an important role in increasing and directing vaccine-induced immune responses. In a previous study, we found that a mucosal SIV vaccine using a combination of IL-15 and TLR agonists as adjuvant mediated partial protection against SIVmac251 rectal challenge, whereas neither IL-15 nor TLR agonists alone as an adjuvant impacted the plasma viral loads. In this study, dissociation of CD4⁺ T cell preservation with viral loads was observed in the animals vaccinated with adjuvants. Significantly higher levels of memory CD4⁺ T cell numbers were preserved after SIVmac251 infection in the colons of the animals vaccinated with vaccine containing any of these adjuvants compared to no adjuvant. When we measured the viral-specific CD8⁺ tetramer responses in the colon lamina propria, we found significantly higher levels of gag, tat, and pol epitope tetramer⁺ T cell responses in these animals compared to ones without adjuvant, even if some of the animals had similarly high viral loads. Furthermore, this CD4⁺ T preservation was positively correlated with increased levels of gag and Tat, but not pol tetramer⁺ T cell responses, and inversely correlated with beta-chemokine expression. The pre-challenged APOBEC3G expression level, which has previously been shown inversely associated with viral loads, was further found positively correlated with CD4⁺ T cell number preservation. Overall, these data highlight one unrecognized role of adjuvant in HIV vaccine development, and show that vaccines can produce a surprising discordance between CD4⁺ T cell levels and SIV viral load.

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1. Introduction

Dramatic loss of resident memory CD4⁺ T cells in the intestine occurs within 2-3 weeks post SIV-infection irrespective of the route of initial viral entry [1-5]. This rapid and profound depletion of CD4⁺ T cells is more severe in the gut mucosa than the other compartments [6], and hard to be reconstituted in patients even on highly active antiretroviral therapy (HAART) [2, 7]. Recently, a long-term benefit of protecting against gut mucosal [8, 9], and systemic CD4⁺ T cell loss [10] has been demonstrated. For example, long-term non-progressors (LTNP) had a higher frequency of mucosal CD4⁺ T cells as compared to progressors [8], and early restoration of mucosal CD4⁺ memory CCR5⁺ T cells in the gut of SIV-infected rhesus predicted LTNP [9]. In a vaccine trial, Letvin et al showed that preserved CD4⁺ central memory T cells several months after infection correlated with long-term protection and predicted the efficacy of an HIV-vaccine better than set-point viral load (VL) [10]. If CD4⁺ T cells were depleted during immunization as demonstrated by Vaccari et al, a decreased protection against SIVmac251 challenge was observed, which further confirmed the important role of CD4⁺ T cells in the course of HIV infection and AIDS development [11]. These studies suggested the significance of protecting mucosal/systemic CD4⁺ T cells from infection and destruction during HIV infection; however, current vaccine strategies hardly achieved this goal. Even if plasma and tissue viral loads were reduced in some of the macaque studies, significant mucosal CD4⁺ T cell preservation was not observed (17, 57). Here we surprisingly observed the opposite discordance, namely that CD4⁺ T cells were preserved even when VL was not reduced. Although vaccine protection of CD4⁺ T cells may be expected to occur if VL is reduced, protection of CD4⁺ T cells without VL reduction is novel and unexpected, and requires further examination, as we have attempted here. Strategies that could protect against both systemic and gut mucosal CD4⁺ T cell loss during HIV/SIV infection would be desirable.

One way to achieve this, which we demonstrated in this study, was to use molecular adjuvants, such as Toll-like receptor (TLR) agonists and/or IL-15, during SIV vaccine immunization. It has been known that adjuvant plays an important role to increase the magnitude, breadth, and the quality of the immune responses, and vaccines with certain adjuvants confer better protection against the subsequent SIV/SHIV challenge. TLR agonists activate and mature dendritic cells to enhance immune responses [12-16], while IL-15 promotes the homeostatic expansion of CD8⁺ memory T cells [17-20], and the induction of higher avidity, longer-lived T cells [21-24]. Both have been shown to be good adjuvants in mouse and macaque models [12, 13, 15, 16, 23, 24]. In our current macaque study, we found that the combination of TLR agonists and IL-15 as an adjuvant in a mucosal SIV vaccine regimen enhanced the quality of vaccine-induced responses, which included long-lived APOBEC3G (A3G) and polyfunctional CD8⁺ T cell responses, and partially protected the SIVmac251 challenged macaques [25]. Though we did not observe any reduction of VLs in the animals vaccinated with the same mucosal SIV vaccine regimen with only TLR agonists or IL-15 alone as an adjuvant [25], we have now surprisingly found a significant preservation of CD4⁺ T cell numbers in the colon mucosa (and to a lesser extent in the ileum and peripheral blood) of these animals 6 months post-infection.

In an attempt to explore the possible mechanisms of this CD4⁺ T cell preservation in the SIVmac251 infected macaques, we first examined whether the pre-challenge A3G expression levels and antigen-specific polyfunctional CD8⁺ T cell responses, which we have explored in our previous study, were correlated with CD4⁺ T cell preservation. Interestingly, A3G, which was previously found inversely correlated with plasma VL reduction, demonstrated a positive correlation with CD4⁺ T cell preservation in the SIV-infected colons, indicating the profound effects of APOBEC3G. This was in contrast to prechallenge polyfunctional CD8⁺ T cell responses, which were inversely correlated with postchallenge

plasma viral loads, but surprisingly did not correlate with post-infection CD4⁺ T cell preservation in the colon at all. We further explored cytokine/chemokine expression levels, gp120 binding and neutralizing antibodies, and post-challenge mucosal viral-specific tetramer⁺ CD8⁺ T cell responses, and found that a significantly higher level of SIV-specific tetramer responses in the colons of the adjuvanted animals was induced /maintained even 6 months after SIVmac251 challenge compared to those without adjuvant. These SIV-specific tetramer responses were also positively correlated with the CD4⁺ T cell preservation. It is unusual here to see tetramer responses after 6 months of infection correlating with vaccine efficacy, as such responses are heavily influenced by viral load, which did not correlate.

Taken together, we found that the usage of certain molecular adjuvants in HIV mucosal vaccines was beneficial for improving the gut CD4⁺ memory T cell numbers upon infection, unexpectedly independent of its effect on VL, and thus might be useful for future HIV vaccine design.

2. Material and Methods

2.1. Rhesus macaques, immunization and challenge

25 Indian rhesus macaques (*Macaca mulatta*) were immunized and challenged as previously described [25]. Briefly, the macaques were maintained in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International and with approval of the NCI Animal Care and Use Committee. They were all seronegative for SIV, simian retroviruses 1, 2 and 5, and simian T-cell leukemia/lymphotropic virus type 1 prior to the study. All macaques were Mamu-A*01⁺, Mamu-B*08⁻. Only seven were Mamu-B*17⁺ (Animals A, B, C, D, E, I and P) and were distributed as evenly as possible among the groups. Four groups (5 animals/group) were used for immunization. Group 1: Vac +TLRLs; Group 2: Vac+IL-15; Group 3: Vac+TLRLs+IL-15; and Group 4: Vac only. Each dose of intrarectal peptide vaccine [26, 27] contained 0.5 mg of each peptide mixed with DOTAP (Roche, Palo Alto, CA, 100μl/dose) with or without a combination of 500 μg/dose of D-type CpG oligodeoxynucleotide, 10μg/dose of MALP2, and 1mg/dose of PolyI:C (In Vivogen, San Diego, CA), or 300μg/dose of IL-15 (NCI, NIH). Recombinant MVA-expressing SIVmac239 Gag, pol, env (5×10^8 /immunization) and recombinant MVA-expressing SIV-1 Rev, Tat, Nef (5×10^8 /immunization), previously described [11], were administered intrarectally, again with or without IL-15 or TLR ligands. Intrarectal inoculations were performed as previously described [28]. A fifth group of 5 animals (Group 5) received only TLR agonists and IL-15 without peptide and MVA-SIV to serve as an adjuvant-only control group. Macaques were primed at week 0, 3 and 6 with peptides and/or adjuvants and boosted at week 12 and 15 with MVA-SIV and/or adjuvants [11]. At week 22, all animals received an intrarectal inoculation of 10 ID₅₀ of SIVmac251 (Nancy Miller, NIAID, NIH), shown sufficient to infect 100% of 30 naïve controls in previous studies [11, 29-31]. SIV RNA levels and CD4 counts were measured by Advanced BioScience Laboratories, Inc (Kensington, MD). We collected colon lamina propria as previously described [28].

2.2. Antibodies and flow cytometry

We measured SIV-specific CD4⁺ and CD8⁺ T cell responses by multi-parameter intracellular cytokine staining (ICS) assays as described [32]. Mamu-A*01 tetramers (CM9-Alexa 680, LV10-PE, LA9-Alexa 680, QA9-PE, SL8-Alexa647, and KA9-Alexa 647) were obtained from the MHC tetramer core facility, NIAID. Eight-color ICS assays performed on an LSRII flow cytometer with 4 lasers (BD Biosciences) used SIV peptides and the following monoclonal antibodies from either BD pharmingen: anti-CD3-PE-Cy7 (SP34), anti-CD8-APC-Cy7 (SK1), anti-CD28-FITC (CD28.2), anti-CD95-PE-Cy5 (DX2); or from

eBioscience: anti-CD4- κ 605. Flow cytometric data was analyzed using FlowJo version 8.8 (tree Star, Inc).

2.3. Gp120 binding antibody and SIV neutralization assays

Plasma from each animal was tested 6 months post infection. Binding antibodies to SIV gp120 were assessed by enzyme-linked immunosorbent assay (ELISA). Antibody titer was defined as the reciprocal of the serum dilution at which the optical density (OD) of the test serum was two times greater than that of the negative-control serum diluted 1:50. Neutralizing antibodies were measured in a luciferase reporter gene assay that utilized TZM-bl cells. The samples were tested at 1:10, 1:40 and 1:160. SIVmac251 was used at 200TCID₅₀/well. The percent inhibition was compared to control wells containing only virus and cells.

2.4. Cytokine/chemokine analysis

The following cytokines and chemokines were measured on a Bioplex Array Reader (LUMINEX 100, Bio-Rad Laboratories, Hercules, CA) using Bioplex human cytokine 27-plex panel (Bio-Rad Laboratories, Hercules, CA): IL-2, IL-10, IL-12p70, IL-13, IL-15, IL-17, TNF- α , IFN- γ , MIP-1 β , and RANTES.

2.5. Statistical analyses

We performed statistical analyses with Graph Pad Prism for Mac, version 5 (Graph Pad) and SAS for Windows version 9.1.3. For group-wise comparisons, we used two-tailed Man-Whitney tests, and for correlations, we used Spearman's tests. In all analyses, we used a two-sided significance level of 0.05. Animal M in group 3 and animal O in group 5 were excluded from the analysis because of the lack of SIV-infection.

3. Results

3.1. CD4⁺ memory T cell numbers were preserved after SIV infection in the colons of the macaques immunized with vaccines containing adjuvants

In our recent macaque study, we have intrarectally immunized four groups of macaques with a peptide-prime, MVA-boost SIV vaccine regimen with or without adjuvant: group 1 adjuvanted with a combination of TLR 2, 3, and 9 agonists, group 2 with IL-15, group 3 with both TLR agonists and IL-15, and group 4 with vaccine but without any adjuvant [25]. A group 5 was added with TLR agonists and IL-15 adjuvant only but without vaccine as an adjuvant control group [25]. Upon SIVmac251 challenge, only group 3 animals showed decreased viral loads (VL) and were partially protected, whereas group 1, 2 and 4 had similarly high VL (supplementary Fig. 1) [25]. In the current study, we measured the CD4⁺ T cell count/ratio in the peripheral and gut tissues six months post SIV infection in the same animals. Since animal M in group 3 and animal O in group 5 never showed any signs of infection, and thus had relatively normal CD4⁺ T cell count and ratio, we excluded these two animals from the analyses. Interestingly, though groups 1 and 2 had a similarly high VL as group 4, the post/pre CD4⁺ T cell ratios were higher in the colons of macaques vaccinated with adjuvants (groups 1-3) than in those without adjuvants (group 4) (Figure 1A, Supplementary Fig 2A-E). Even with only five animals per group, significantly greater CD4⁺ T cell preservation in group 1 was observed (even without counting one high outlier in group 1, $p=0.016$). Also, the combination of all three adjuvanted groups showed significantly higher CD4⁺ T cell levels than the group without adjuvants ($p = 0.019$, Fig. 1A, without the outlier in group 1, $p=0.03$). Furthermore, higher levels of CD4⁺ T cell numbers were also observed in the PBMCs and ileum of the adjuvanted animals, and the ratios of post/pre challenged CD4 absolute counts in the PBMCs and ileum of the adjuvanted animals

showed a trend toward higher values than those of group 4, but none of these comparisons in the PBMC and ileum among individual groups was statistically significant (Supplementary Figure 3 A-D).

Since memory CD4⁺ T cells play important roles in SIV/HIV infections, we measured the CD4⁺ T cell subsets using CD95 and CD28 markers. CD28⁺CD95⁺CD4⁺ T cell percentage within the total lymphocytes was defined as T central and transitional memory CD4⁺ T cells (TCM & Trm), whereas CD28⁻CD95⁺CD4⁺ T cell percentage within the total lymphocytes was defined as T effector memory (TEM). Consistent with the fact that intestinal tissues such as colon are different from other mucosal tissues such as lung, behaving as an immune inductive site (similar to lymph nodes) as well as an immune effector site, these cell subsets were observed in the pre-challenge colon lamina propria (LP) (Supplementary Fig 2F). As shown in Figure 1B&C, both group 1 and 2 had significantly greater CD4⁺ TEM, TCM & Trm subpopulations compared to the ones without adjuvant (group 4), and the adjuvanted groups as a whole had higher frequencies in the colon LP during the chronic infection stage. Furthermore, CD4⁺ TCM & Trm and TEM subpopulations in the SIV chronically-infected colon LP were closely associated with each other ($r=0.98$, and $P<0.0001$). However, if we compared the infected colon LP samples of the Vac+adjuvanted groups to those of uninfected animals (median 6% for TCM&TRM and 2% for TEM, respectively), CD4⁺TCM & Trm were all decreased >50%, while TEM were near normal except in the group without adjuvant where the CD4 reduction was almost twice as great. In the infected ileum samples, the adjuvanted groups had higher CD4⁺ TCM & Trm frequencies, but not TEM (supplementary Fig 3E-F).

In accord with group 1 and 2 animals' having high set-point/tissue VLs, but high post/pre challenged CD4⁺ ratios and memory T cell numbers in the colons, we observed an unexpected dissociation or discordance of CD4⁺ memory T cell preservation with both colon tissue and set-point VLs (colon tissue VL and plasma set-point VL were positively correlated with each other, with $r=0.74$, $p<0.0001$. Fig. 1D-F, supplemental Fig. 4A-D), indicating that colon CD4⁺ T cell numbers in the adjuvant animals were independent of VLs. The mechanisms behind this surprising dissociation were therefore pursued.

3.2. Pre-challenge A3G expression level, but not viral-specific polyfunctional CD8⁺ T cells, correlated with CD4⁺ T memory cell preservation

In our previous study, we found that the vaccine-induced pre-challenge mesenteric LN APOBEC3G (A3G), and viral-specific polyfunctional CD8⁺ T cells inversely correlated with reduced set-point plasma VL. In this study we first investigated whether these two factors contributed to the CD4⁺ T cell number preservation. None of these factors correlated with CD4⁺ T cell numbers in the PBMC (data not shown). In the colon, however, A3G, but not viral-specific polyfunctional T cells (data not shown), positively correlated with CD4⁺ T cell number preservation, suggesting the possible involvement of A3G in maintaining high CD4⁺ memory T cell numbers in the SIV-infected colons (Figure 2). However, preservation of CD4⁺ T cells in the colon reservoir may lead secondarily to greater CD4⁺ T cell numbers in the blood and other tissues.

As there is no single defined mechanism, and multiple factors such as β -chemokines, cytokine storm, antigen-specific humoral and cellular responses have been reported to influence CD4⁺ T cell depletion during HIV infection, we therefore evaluated these factors in this study for their potential involvement in mediating CD4⁺ T cell preservation.

3.3. Plasma β -chemokines positively correlated with plasma VL, and showed inverse correlation trends with CD4⁺ T memory cell preservation

Cytokines and chemokines played important roles in modulating CD4⁺ T cell depletion during HIV infection via multiple mechanisms. For example, overproduction of IL-4, IL-10 increases susceptibility to activation induced cell death; high levels of IL-2, IL-15 cause immune activation, which facilitates the viral infection; in contrast, β -chemokines protect the CD4⁺ T cell from viral infections via co-receptor blockage. To further explore the mechanisms by which the disassociation of VL and CD4⁺ T cell counts/ratio occurred in the adjuvanted animals, we further measured the expression levels of cytokines and chemokines in the plasma of the animals 6 months post-infection. The expression levels of IL-2, IL-12, IFN γ , IL-15, TNF α , IL-17, IL-13, and IL-10 were low and not significantly different from twice the assay background (horizontal lines in the figures), and most importantly, there was no difference between the adjuvanted and un-adjuvanted animals for all the cytokines measured (Supplementary Fig. 5). β -chemokines, MIP-1 β and RANTES, had relatively high expression levels, but there was no difference among the groups (Figure 3A&B). β -chemokines could prevent HIV/SIV infection via the blockage of the co-receptor for viral entry, but contrarily could also attract CCR5⁺ target cells, including CD4⁺ T cells, to the foci of the HIV/SIV infection and thus fuel the viral infection. In this cohort of monkeys, we documented that both MIP-1 β and RANTES were positively correlated with colon tissue VL (Fig. 3E-F) and set-point VL, and showed inverse correlation trends relative to CD4⁺ T memory cell preservation in the colon (Fig. 3C-D), implying that high production of β -chemokines in the SIV-infected plasma might play a deleterious role in viral replication or may be induced by higher VL, and thus either way was not responsible for the CD4⁺ T cell number preservation.

3.4. Neither gp120-binding antibody nor SIV neutralizing antibody showed correlation with CD4⁺ memory T cell preservation

Recently, vaccine-induced non-neutralizing anti-envelope antibody activities were reported to be associated with control of both acute and chronic viremia in rhesus macaques [33]. We therefore evaluated the SIV-gp120 binding antibody activities. After immunization but before SIVmac251 challenge, only three animals, F (group 1), L (group 2), V (group 2), developed significant antibody titers to gp120 [25]. Six months after SIV infection, most of the animals had measurable binding antibody titers against gp120; however, no significant differences were detected between adjuvanted and un-adjuvanted animals (Supplementary Fig. 6). Furthermore, SIVgp120 binding antibody activities did not correlate with set-point ($p=0.39$) or colonic tissue ($p=0.88$) VLs either.

Neutralizing antibodies against SIVmac251 in the plasma of the 6-month post-challenge samples were also measured. No difference among the groups, and no correlations with either the plasma set-point or colon VLs or CD4⁺ T cell preservation in the blood or colon were observed (data not shown).

3.5. Increased tetramer responses in the colons of the adjuvanted macaques were positively associated with CD4⁺ memory T cell preservation

To evaluate the role that local mucosal cellular immunity played in CD4⁺ T cell preservation of the SIV-infected colons, we examined six Mamu A*01 tetramer responses targeting gag, pol, tat, and vif regions of SIV in the colonic mucosa. After three peptide priming immunizations and two MVA-SIV boosts, the macaques generated as high as about 20% tetramer positive cells in the colon LP [25]. However, the adjuvanted and the un-adjuvanted groups had similar levels of tetramer responses, implicating that adjuvants did not further enhance the magnitudes of the responses. Nevertheless, upon intrarectal SIVmac251 challenge, 19 out of the total 20 vaccinated animals got infected [25]. Thus, the high levels

of tetramer responses did not prevent viral transmission. In fact, the pre-challenge tetramer responses in the colons correlated with neither set-point nor tissue VLs, nor CD4⁺ T cell preservation.

We then measured the same six-tetramer responses in the colon LP six months post-infection. To evaluate the augmenting effect of adjuvant as a whole, the tetramer responses from animals of group 1, 2, and 3 were pooled together as the total adjuvanted group. As shown in Figure 4, the tetramer responses specific to LA9, Tat2, and pol were significantly increased in the colonic LP of the adjuvanted groups compared to those of group 4, the unadjuvanted group. Gag-specific tetramer CM9, and Tat-specific tetramer SL8 showed increasing trends, but Vif did not differ between adjuvanted and un-adjuvanted groups. The overall tetramer responses were 2-fold higher in the animals with adjuvant compared to the ones without. Even with five animals per group, we still observed statistically significantly greater tetramer responses in group 1, and 2 vs. those of group 4. This is unexpected, as the tetramer responses 6 months after infection are usually more dependent on infection and viral load levels (which were comparable in groups 1, 2 and 4) than on vaccine priming.

Though antigen-specific individual or total tetramer responses did not correlate with either set-point or colon tissue VLs (data not shown), total tetramer responses did positively correlate with both total and CD4⁺ T memory cell preservation in the colons (Figure 5). Among the individual tetramers, Gag-specific tetramers CM9 and LA9 were strongly associated with CD4⁺ T cell preservation, followed by tat2 and vif, but not SL8 or pol at all (Figure 5), whereas for CD4⁺ TCM & Trm or TEM in the infected colon LP, tetramers CM9, LA9 and Tat2 were correlated, but not SL8, pol or vif (Supplemental Fig. 7&8), indicating that different tetramer responses might play different roles in term of association with different subsets of CD4⁺ memory cells. Overall, the data implied that vaccines co-administrated with molecular adjuvants improved viral-specific immune responses, and reduced CD4⁺ T memory cell loss after viral infection. However, the causative mechanism remains to be determined.

In PBMC, there were no such correlations observed, though CD4⁺ T cells in the PBMC of the adjuvanted animals were also preserved. This agreed with the compartmentalization phenomena observed in the SHIV models [34].

4. Discussion

In our recent SIV-macaque study, we have evaluated the protective efficacy of using TLR agonists, IL-15 and the combination of these two as adjuvants in a peptide-primed/MVA-boosted mucosal SIV vaccine against intrarectal SIVmac251 challenge [25]. As shown before, mucosal administration of the combination of both adjuvants in the macaques induced high levels of antigen-specific CD4⁺ and polyfunctional CD8⁺ T cell responses after immunization, and conferred partial protection against the subsequent SIVmac251 challenge [25]. In contrast to the HIV-envelope-expressing poxvirus challenge in mice [16], neither adjuvant alone conferred protection against SIV infection or reduced the set point blood or colon tissue VLs. In this study, we further examined the CD4⁺ memory T cell numbers in the colons of the same animals 6 months post SIVmac251-infection. One interesting observation was that using adjuvant during immunization, irrespective of whether it was the combination of TLR agonists and IL-15, or either one alone, reduced CD4⁺ memory T cell loss in the intestine of these SIV chronically infected macaques. Thus, there was a discordance between effects of vaccine adjuvant on viral load and effects on preservation of CD4⁺ T cells. As CD4⁺ T cell count is usually inversely correlated with VLs in most of the HIV-1/SIV infected individuals/animals, the finding of greater mucosal CD4⁺ T cell preservation in the presence of high VLs suggests that mucosal CD4⁺ T cell count in

some of the cases could be independent of VLs. This is in agreement with the lack or delay of mucosal immune reconstitution during prolonged treatment of HIV infection (37). Thus, the current results that it is possible to preserve CD4⁺ T cells even in the face of high viral load might point toward strategies to protect CD4⁺ T cell besides reducing VLs.

Mucosal CD4⁺ T cells are central players in the pathogenesis of HIV infection. Upon HIV-1/SIV natural infection, memory CD4⁺ T cells are first depleted from the gut, and never totally recovered even under HAART therapy [2, 35]. As preexisting CD4⁺ T memory cells are critical in generating secondary immune responses, the loss of these during acute SIV infection sets the stage for immunodeficiency. Furthermore, intestinal mucosal tissues are not only the reservoir for HIV and the site where CD4⁺ T cells are depleted early during HIV infection, but also a major T cell organ. Protection of gut CD4⁺ T cells from depletion will affect both mucosal and systemic immunity. Thus achieving mucosal memory CD4⁺ T cell preservation is one of the major goals of HIV vaccine development. In this study, we found that TLR agonists, IL-15, either one alone or as a combination, used as an adjuvant could impact the CD4⁺ T cell number in the blood and colon, especially the CD4⁺ memory T cell numbers in the SIV-infected colons.

In an attempt to identify the factors that contributed to the CD4⁺ T cell preservation independent of VL, we found that the expression levels of cytokines and β -chemokines, SIV gp120 binding and neutralizing antibodies against SIVmac215 did not differ among the groups, and most importantly, did not positively correlate with CD4⁺ T cell preservation at all. The two strongest correlations with the CD4⁺ T cell preservation in this study were found to be 1) the expression level of A3G in pre-challenge mesenteric LN samples; and 2) antigen-specific tetramer⁺ CD8⁺ T cell responses in the colons of animals 6 months post-infection (in contrast to tetramer⁺ response after immunization but prior to challenge, which are usually better predictors of protective vaccine effects).

A3G is a DNA deaminase (cytidine deaminase) that mediates innate resistance to retroviral infections such as HIV-1 infection [36, 37]. Elevated A3G mRNA levels have been reported in both HIV-1 infected LTNP and HIV-exposed seronegative individuals, and also correlated with a slow progression in LTNP [38, 39]. In the previous study with the same animals, we found that pre-challenge A3G levels correlated inversely with VL reduction [25]. In this study, we further discovered that the pre-challenge A3G level also correlated with the CD4⁺ T cell preservation after infection. Since both VL and CD4⁺ T cell preservation correlated with A3G, it was possible that the observed CD4⁺ T cell preservation in this study was indirectly related to viral load reduction, but the lack of a direct correlation between VL and CD4⁺ T cell preservation argues against that possibility. It is more likely that A3G might directly preserve CD4⁺ T cells. As we found adjuvant/vaccine-induced A3G expression was widely distributed in monocyte/macrophage, DC, and CD4⁺ memory T cell subsets in the pre-challenge mesenteric LNs and colons, the increased A3G may provide several advantages to contribute to CD4⁺ memory T cell preservation directly or indirectly: 1) A3G expressed in the CD4⁺ CD95⁺ T cell may render the cells intrinsically resistant to viral-induced T cell depletion; 2) A3G expressed in monocyte/macrophages and DCs may provide further barriers for viral transmission in the peripheral tissues; 3) A3G-mediated G-to-A hypermutations may generate stop codons in the viral genes, and thus dampen the virus's ability to deplete CD4⁺ T cells. Overall, our data suggest that the role of innate factors in CD4⁺ T cell preservation, and maybe in the LTNP as well, may have been underappreciated.

The CD4⁺ memory T cell preservation is likely to be multifactorial, for we also found that CD4⁺ T memory cell preservation in this cohort correlated with antigen-specific tetramer⁺ CD8⁺ T cell responses in the colons 6 months post-infection. There is accumulating

evidence that CD8⁺ T cells could control viral replication, and protect CD4⁺ T cells from depletion [40-45]. For example, depleting CD8⁺ T lymphocytes using anti-CD8 antibody strongly supported the notion that CD8⁺ T cell played an important role to control viral replication [46, 47]. Likewise, genes associated with elite control of virus and preservation of CD4⁺ T cells mapped to the HLA class I locus, strongly implicating CD8⁺ T cells in this elite control [45]. While CD8⁺ T cells might protect CD4⁺ T cells from virus, the reverse is also possible that CD4⁺ T cells help preserve CD8⁺ T cell responses [Rosenberg, 1997 #3801]. Studies from mice showed that memory CD8⁺ T cell functionality depends on help from CD4⁺ T cells. Signals from CD4⁺ T cells, both intrinsic and extrinsic, help to mold the quality of the memory T cells, and in particular their proliferation potential [48-50]. In the absence of CD4⁺ T cell help, the memory CD8⁺ T cells are more susceptible to TRAIL-mediated apoptosis, and the induced primary CD8⁺ T cell responses lack high-avidity and longevity [22, 51, 52]. It was therefore impossible to distinguish cause and effect in the correlation between CD4⁺ T memory cell preservation and antigen-specific tetramer⁺ CD8⁺ T cell responses in this study. However, the adjuvanted animals had higher antigen-specific tetramer responses and CD4⁺ memory T cell numbers than those of the un-adjuvanted ones even in the presence of similar VL, indicating the pronounced and beneficial effects of the adjuvant. Interestingly, we also observed that the adjuvant only group appeared to have a higher level of CD4⁺ memory T cells than the vaccine only group, and similarly, the adjuvant only group had a higher level of CD8⁺ tetramer responses, which were higher than the vaccine only group. These data seem to suggest that adjuvant alone can mediate a certain level of memory CD4⁺ T cell preservation in the colon. This might be associated with lower viral loads in the adjuvant only group than the vaccine only group, or other un-identified factors.

Using TLR agonists and/or IL-15 as adjuvant during immunization may affect many aspects of the host. For example, the activation of TLRs by their cognate ligands leads to production of inflammatory cytokines, upregulation of MHC, type I IFN, IL-15R α , A3G, and costimulatory factors in the antigen presenting cells, in addition to enhancing T/B cell priming and activity [53], and IL-15 is able to induce long-lived antigen-specific T cell responses with high avidity [21, 22, 24]. Furthermore, molecular adjuvants are known to augment both primary and anamnestic immune responses. Though we do not know the exact mechanism(s) of the CD4⁺ T cell preservation in these animals vaccinated with adjuvant, we envision that the adjuvant used during immunization somehow modulated the microenvironment of the host, including inducing innate immunity factors, such as A3G expression in HIV susceptible cells, and controlling the quality and memory of the T and B cell immunity. As a result, the CD4⁺ T cells in the adjuvanted animals might be more resistant to SIV-induced apoptosis. Moreover, under the pressure of antigen-specific CD8⁺ T cells, it is also possible that the viruses in the chronically infected animals were mutated to a less virulent form with reduced ability to induce CD4⁺ T cell depletion. Studies to analyze the effect of the adjuvant on the survival and apoptotic properties of the CD4⁺ T cells after viral challenge, and the biological characteristics of viruses during chronic infections are underway. Another interesting finding was that T cell responses to different SIV epitopes correlated differently with CD4⁺ T cell preservation. Gag tetramer⁺ T cells clearly associated with CD4⁺ T cell preservation better than the others, which was consistent with other studies showing that CD8⁺ T cells from elite controllers were more likely targeting Gag than CD8⁺ T cells from progressors, and depletion of Gag-specific (but not nef-specific) CD8⁺ T cells abrogated the suppressive activity against viral replication [54-56]. Besides gag, our data also suggested that CD8⁺ T cells specific for tat, but not pol, correlated with CD4⁺ T cell preservation. The association of preserved CD4⁺ T cells in the colons with gag and tat tetramer responses in the SIV-infected macaques might be useful for future HIV vaccine design. However, we also realize that correlations by definition cannot prove any causation.

A different type of dissociation between control of viral replication and mucosal CD4⁺ T cell preservation was observed recently by several SIV vaccine studies using MVA vectors [57, 58]. Engram et al reported that macaques vaccinated with MVA that expressed SIVmac239 gag and tat showed no protection from systemic or mucosal CD4⁺ T cell depletion and no improved survival, despite a one log reduction of the peak and early set-point VLs [58]. In another study in which macaques were vaccinated with a DNA/MVA-prime/boost regimen, the loss of CCR5⁺CD4⁺ T cells was found equivalent in vaccinated and control macaques at 2 or 3 weeks post infection, despite a three log reduction at mucosal sites of SIV RNA in the vaccinated group [57]. However, an apparently better preservation of the CCR5⁺ CD4⁺ T cells that repopulate this site in the vaccinated animals was also observed later [57]. In our current study with peptide/MVA-prime/boost regimen, animals vaccinated with either TLRs or IL-15-alone did not show a reduction of the VLs while the combination of both types of adjuvant significantly reduced VLs. Nevertheless, colon CD4⁺ T cell numbers were maintained in all the adjuvanted groups, compared to the vaccine-only group. In this regard, our study differed somehow from both of these other studies, in that the discordance between VL and CD4⁺ T cell numbers was in the opposite direction (preserving CD4⁺ T cells without reducing VL rather than reducing VL without preserving CD4⁺ T cells), possibly due to the usage of the mucosal molecular adjuvants. As is known, IL-15 plays a key role in the generation and maintenance of memory CD8⁺ [59] and CD4⁺ T cells [60]. TLR 3 and 9 agonists, which have been included in the current regimens, have been shown to induce efficient cross-presentation from mature DC [61], and, if combined with cationic liposomes, could produce uniquely effective vaccine adjuvants capable of eliciting strong T cell responses against protein and peptide Ags via a cross-priming mechanism [62]. CpG was demonstrated to exert its cross-priming effect on B cells as well [63], in addition to its ability to stimulate B cell proliferation, differentiation, and antibody production [64]. Furthermore, TLR ligands like Poly I: C and CpG directly enhance the survival of activated CD4⁺ T cells without augmenting proliferation both *in vitro* and *in vivo* via the mechanism of up-regulation of Bcl-x_L, but not Bcl-2 and Bcl-3 [65]. It was also found that poly I: C could serve as an adjuvant to induce durable and protective CD4⁺ T cell responses at mucosal surfaces, which were multifunctional [66]. Thus, adjuvants enhance the induction of humoral, T-helper, cytotoxic T-lymphocyte immune responses in the vaccine models by utilizing multiple mechanisms.

Thus, our data suggest that adjuvant had profound effects on the host to impact mucosal CD4⁺ T cell numbers so that a higher frequency of memory CD4⁺ T cells was maintained in the SIV-infected colons of the adjuvanted animals despite high viral loads. As a consequence of greater CD4⁺ T cell, especially gut mucosal CD4⁺ T cell, preservation, one would expect to see improved immune reconstitution and prolonged survival time of the host, if anti-retroviral therapy were used to control the VLs. Further confirmation of these would support the usage of molecular adjuvants in future human HIV vaccine clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. CD4⁺ memory T cell numbers were preserved after SIV infection in the colons of the macaques immunized with vaccines containing adjuvants

(A). Post/pre CD4⁺ T cell ratios were measured in the colon lamina propria after and before SIV challenge. (B, C). CD4⁺ central and transitional memory T lymphocyte (TCM & Trm: CD28⁺CD95⁺CD4⁺ T cell percentage within the total lymphocyte gate) and effector memory T lymphocyte (TEM: CD28⁻CD95⁺ CD4⁺ T cell percentage within the total lymphocyte gate) responses were measured in the colon lamina propria 6 months post SIV infection. Comparisons among the groups were performed by two-tailed Mann Whitney tests. (D-F). Correlations between colon VL and post/pre CD4⁺ T cell ratios (6 months post infection and before infection), TCM & Trm and TEM (both 6 months post infection) were evaluated by a two-tailed Spearman's rank test. The animals in different groups were color-coded: Vac+TLRLs in red, Vac+IL-15 in blue, Vac+TLRLs+IL-15 in green, Vac-only in orange, and TLRLs+IL-15-adjuvant-only in purple. Data show mean and S.E.M.

Figure 2. Pre-challenge A3G expression level correlated with CD4⁺ T memory cell number preservation

Correlations between pre-challenge A3G expression level in MLN and post/pre CD4⁺ T cell ratios (A, 6 months post infection and before infection); TCM & Trm and TEM (B, C, both 6 months post infection) in the colons were evaluated by a two-tailed Spearman's rank test.

The animals in different groups were color-coded: Vac+TLRLs in red, Vac+IL-15 in blue, Vac+TLRLs+IL-15 in green, Vac-only in orange, and TLRLs+IL-15-adjuvant-only in purple.

Figure 3. Serum β -chemokines did not contribute to CD4⁺ T memory cell preservation
 Serum MIP-1 β (A) and RANTES (B) expression levels of the macaques 6 months post-infection were measured. Correlations between serum MIP-1 β / RANTES expression levels and TCM & Trm in the colon (C, D), and colon VL (E, F) were evaluated by a two-tailed Spearman's rank test. The animals in different groups were color-coded: Vac+TLRLs in red, Vac+IL-15 in blue, Vac+TLRLs+IL-15 in green, Vac-only in orange, and TLRLs+IL-15-adjuvant-only in purple. Data show mean and S.E.M.

Figure 4. Virus specific tetramer⁺CD8⁺ T cell responses in the colon lamina propria of the macaques 6 months post-infection

(A). Gating strategies of virus specific tetramer⁺CD8⁺ T cells. (B). 6 virus specific Mamu A*01 tetramer responses were measured in the colon lamina propria of the macaques 6 months post-infection. The sum of these 6 tetramer responses was also shown. Comparisons among the groups were performed by two-tailed Mann Whitney tests. Data show mean and S.E.M.

Figure 5. Correlations between CD4⁺ memory T cell ratios / numbers and virus specific tetramer⁺CD8⁺ T cell responses in the colons of the macaques post SIVmac251 infection
Correlations between CD4⁺ memory T cell ratios / numbers and virus specific tetramer⁺CD8⁺ T cell responses in the colons of the macaques post SIVmac251 infection were evaluated by a two-tailed Spearman's rank test. The animals in different groups were color-coded: Vac+TLRLs in red, Vac+IL-15 in blue, Vac+TLRLs+IL-15 in green, Vac-only in orange, and TLRLs+IL-15-adjuvant-only in purple.