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MAdCAM costimulation through Integrin- $\alpha_4\beta_7$ promotes HIV replication

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Human gut-associated lymphoid tissues (GALT) play a key role in the acute phase of HIV infection. The propensity of HIV to replicate in these tissues, however, is not fully understood. Access and migration of naive and memory CD4⁺ T cells to these sites is mediated by interactions between integrin $\alpha_4\beta_7$, expressed on CD4⁺ T cells, and MAdCAM, expressed on high endothelial venules. We report here that MAdCAM delivers a potent costimulatory signal to naive and memory CD4⁺ T cells following ligation with $\alpha_4\beta_7$. Such costimulation promotes high levels of HIV replication. An anti- $\alpha_4\beta_7$ mAb that prevents mucosal transmission of SIV blocks MAdCAM signaling through $\alpha_4\beta_7$ and MAdCAM-dependent viral replication. MAdCAM costimulation of memory CD4⁺ T cells is sufficient to drive cellular proliferation and the upregulation of CCR5, while naive CD4⁺ T cells require both MAdCAM and retinoic acid to achieve the same response. The pairing of MAdCAM and retinoic acid is unique to the GALT, leading us to propose that HIV replication in these sites is facilitated by MAdCAM- $\alpha_4\beta_7$ interactions. Moreover, complete inhibition of MAdCAM signaling by an anti- $\alpha_4\beta_7$ mAb, an analog of the clinically approved therapeutic vedolizumab, highlights the potential of such agents to control acute HIV infection.

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INTRODUCTION

Most HIV infections throughout the world occur following the exposure of the host mucosal surfaces to the virus. The subsequent events that allow irreversible establishment of HIV infection remain poorly defined. Studies of mucosal transmission in the *SIV/Rhesus macaque* (RM) non-human primate model indicate that suboptimally activated CD4⁺ T cells are the initial targets of infection.^{1,2} Various lines of evidence suggest that because the frequency of these cells and the amount of virus that they produce are low, infection of these cells may fail to establish irreversible infection in the host.^{2,3} The establishment of an irreversible infection is instead believed to involve the passing of the virus from suboptimally activated cells in the genital and rectal mucosa to fully activated CD4⁺ T cells, some of which migrate into draining lymph nodes.^{2,3} A key determinative step then occurs as these cells traffic to inductive sites in the gut tissues, most notably Peyer's Patches (PPs) and mesenteric lymph nodes (MLNs).⁴ There appears to be an intrinsic relationship between HIV/SIV replication during acute infection (AI) and the trafficking/homing of the target cell in the GALT.^{5–7} The high level of virus replication in PPs and MLNs is a central event and a primary source of viremia in AI. It is this aspect of AI that has led to the concept that both HIV and SIV are predominantly gut-tropic viruses.^{8,9}

Proviral DNA is also found in the lamina propria (LP), the major effector site within the gut-associated lymphoid tissues (GALT).¹⁰ Importantly, during AI, massive loss of memory CD4⁺ T cells occur along with the degradation of LP ultrastructure.^{11–13} Damage to the LP is considered a major factor in the development of advanced HIV disease.⁸ It is generally assumed that the burst of viral replication in GALT occurs because of the high frequency of activated CD4⁺/CCR5⁺ T cells that appear within these sites. Lymphocytes trafficking through PPs and MLNs, however, are subject to unique regulatory stimuli, raising the possibility that these tissues possess additional features rendering them particularly permissive to infection.

Migration of CD4⁺ T cells from the genital and rectal mucosa to PPs and MLNs is a regulated process that requires those cells to extravasate through the high endothelial venules (HEVs) that service the GALT (Supplementary Figure 1).^{5,7} Extravasation is achieved by a series of receptor-counter receptor interactions involving proteins expressed on both the surfaces of circulating lymphocytes and HEVs.¹⁴ These interactions have been described as a multi-step adhesion cascade.¹⁵ A number of components of this adhesion cascade are common to extravasation of lymphocytes into many tissues, yet trafficking of lymphocytes into PPs and MLNs is somewhat unique, in that it is mediated

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predominantly by the interaction of integrin $\alpha_4\beta_7$ ($\alpha_4\beta_7$) and L-selectin (CD62L) on the surface of the lymphocytes, with MAdCAM and L-selectin-specific ligands on the endothelial cells.^{15–17} These interactions are regulated by the dynamic changes in the expression levels of L-selectin and in the expression levels, aggregated state, and conformation of $\alpha_4\beta_7$. Importantly, $\alpha_4\beta_7$ is the only integrin capable of binding to MAdCAM.¹⁶ It is the tissue-specific expression of MAdCAM on the surface of the gut HEVs that defines $\alpha_4\beta_7$ as the gut-homing integrin. Thus, MAdCAM is central to the trafficking of CD4⁺ T cells to PPs and MLNs, and is therefore linked in an inexorable way to the gut-tropic nature of HIV.

A subset of integrins, most notably LFA-1, but also $\alpha_4\beta_7$, in addition to functioning as homing receptors, deliver costimulatory signals to CD4⁺ T cells.^{18–21} The natural ligand of LFA-1 is ICAM, and through its interaction with LFA-1, ICAM can synergize with CD28 in promoting T cell activation.^{22,23} Central to this process is the role of LFA-1 in stabilizing immunological synapses (IS). Similar to the LFA-1/ICAM interaction, $\alpha_4\beta_7$ mediates costimulatory signals to T cells through its interaction with MAdCAM, and it also synergizes with CD28. Increased adhesion does not adequately account for this synergy. Signaling through $\alpha_4\beta_7$ and CD28 can be separated in space and time such that MAdCAM and CD80/86 (B7-1 and B7-2, respectively,) can appear on separate cells (remote costimulation), and MAdCAM signaling can also precede CD80/86-mediated costimulation (priming).^{18,23} This raises the possibility that MAdCAM expressed on the surface of HEVs can participate in the activation and proliferation of migrating T cells as they pass into the inductive sites (PP, MLN) of the GALT.

$\alpha_4\beta_7^+$ /CD4⁺ T cells play a central role in the mucosal transmission of HIV and SIV.^{24–28} These cells, which can be found circulating in the blood and in all the peripheral lymph nodes, are also present in the genital mucosa.²⁹ The baseline levels of the circulating $\alpha_4\beta_7^+$ /CD4⁺ T cells are stable, but varies among individuals.^{27,30} Studies from the *Rhesus macaque*/SIV model of HIV transmission have shown that $\alpha_4\beta_7^+$ /CD4⁺ T cells are among the first cells infected and depleted following transmission,^{31,32} a finding that is consistent with the depletion of these cells early after the infection in humans.³³ Moreover, their frequency in mucosal tissues has been correlated with the susceptibility to infection.^{26,28} We recently reported that a monoclonal antibody (mAb) specific for $\alpha_4\beta_7$ (anti- $\alpha_4\beta_7$ mAb), when delivered intravenously, protected 6/12 *Rhesus macaques* from vaginal infection by a highly infectious and pathogenic strain of SIV (SIVMac251).²⁴ The capacity of anti- $\alpha_4\beta_7$ mAb to protect *Rhesus macaques* from infection underscores the critical role that $\alpha_4\beta_7$ -expressing cells play in mucosal transmission. However, it is unclear where along the pathway between the vaginal mucosa and the GALT $\alpha_4\beta_7^+$ /CD4⁺ T cells are first infected, and how the anti- $\alpha_4\beta_7$ mAb blocks the transmission.

Considering the critical role of cellular activation in HIV infection, and recognizing that $\alpha_4\beta_7^+$ /CD4⁺ T cells are infected early in the transmission, we asked whether MAdCAM-mediated signaling through $\alpha_4\beta_7$ could enhance the susceptibility of CD4⁺ T cells to infection and promote viral replication. In addition, we asked whether anti- $\alpha_4\beta_7$ mAb used to block SIV transmission could inhibit MAdCAM-mediated viral replication by inhibiting costimulation.

RESULTS

MAdCAM ligation of Integrin- $\alpha_4\beta_7$ promotes HIV replication

To determine whether MAdCAM-mediated cell signaling could promote viral replication, we carried out in vitro infections of freshly isolated CD4⁺ T cells, obtained from healthy donors with the CCR5-tropic isolate HIV SF162. To mimic the antigen-mediated stimulation through the T cell receptor (TCR), we utilized the well-established procedure of culturing cells with an anti-CD3

monoclonal antibody (anti-CD3).³⁴ Primary CD4⁺ T cells were added to the wells pre-coated with anti-CD3 or anti-CD3 in combination with recombinant human MAdCAM-1 Ig (MAdCAM). After 48 h, cultures were inoculated with HIV in the presence/absence of a pre-determined optimal concentration of anti- $\alpha_4\beta_7$ mAb. The cell supernatants were sampled 4 and 6 days post the infection, and viral replication was assessed by p24 antigen ELISA. While anti-CD3 alone induced relatively low levels of viral replication, cellular stimulation with anti-CD3 in the presence of MAdCAM resulted in robust HIV replication (Fig. 1a). Addition of anti- $\alpha_4\beta_7$ mAb abrogated the high levels of viral replication observed with anti-CD3 + MAdCAM, while the addition of a control antibody had no effect. Similar results were obtained with primary cells from six independent donors (Fig. 1b) and in experiments utilizing a CXCR4-tropic HIV isolate (Supplementary Figure 2). We therefore conclude that signaling directly through $\alpha_4\beta_7$ by MAdCAM promotes viral replication in vitro.

MAdCAM ligation of $\alpha_4\beta_7$ promotes CD4⁺ T cell proliferation

To better understand the signaling events mediated through $\alpha_4\beta_7$ and the antiviral effect of anti- $\alpha_4\beta_7$ mAb, we evaluated the capacity of MAdCAM to enhance cellular proliferation using a standard CFSE dye dilution assay. A culture protocol similar to that described above was employed. While anti-CD3 mAb alone generated minimal cellular proliferation, the inclusion of MAdCAM in the culture increased proliferation significantly (Fig. 2a, b). Proliferation was inhibited by the addition of anti- $\alpha_4\beta_7$ mAb, but not by a control mAb. Phenotypic analysis of the proliferating CD4⁺ T cells using a non-competitive mAb against the integrin- β_7 revealed that proliferation was limited largely to the β_7^+ population, again implicating a central role of $\alpha_4\beta_7$ in MAdCAM-mediated cellular proliferation (Fig. 2c). Further analysis revealed that the relative proportion of CD45RO⁻ cells decreased in the presence of MAdCAM-Ig, while the majority of CFSE-diluted cells expressed CD45RO (Fig. 2c). This observation suggests that MAdCAM-mediated costimulation induced proliferation of the CD45RO⁻ cell population and drove these cells toward a CD45RO⁺ cell phenotype. With this in mind, we sought to assess directly the manner in which MAdCAM costimulation impacts the

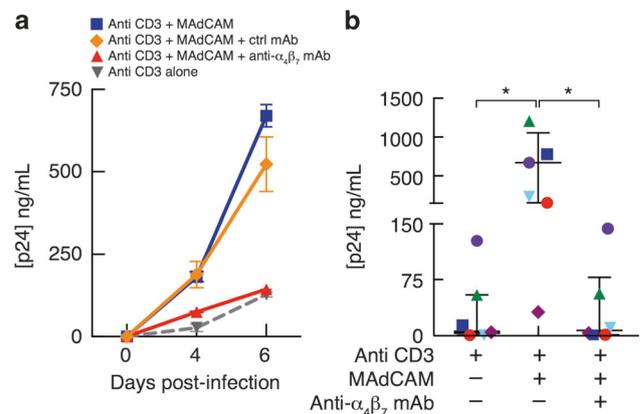


Fig. 1 MAdCAM stimulation promotes HIV replication. **a** Primary CD4⁺ T cells cultured for three days in the presence of stimulatory ligands—either anti-CD3 alone or anti-CD3 + MAdCAM, in the presence of anti- $\alpha_4\beta_7$ mAb or a control mAb (Synagis). Three days post the stimulation, cells were infected with the HIV isolate SF162, and viral replication was assessed by measuring the concentration of p24 antigen in cell supernatants 4 and 6 days post the infection. **b** Replication of HIV SF162 in CD4⁺ T cells isolated from six independent donors in the presence of stimulatory ligands, as described in (a). The concentration of viral p24 antigen in culture supernatants on day 6 post the infection is shown. * $P < 0.05$ (two-tailed parametric paired *t*-test)

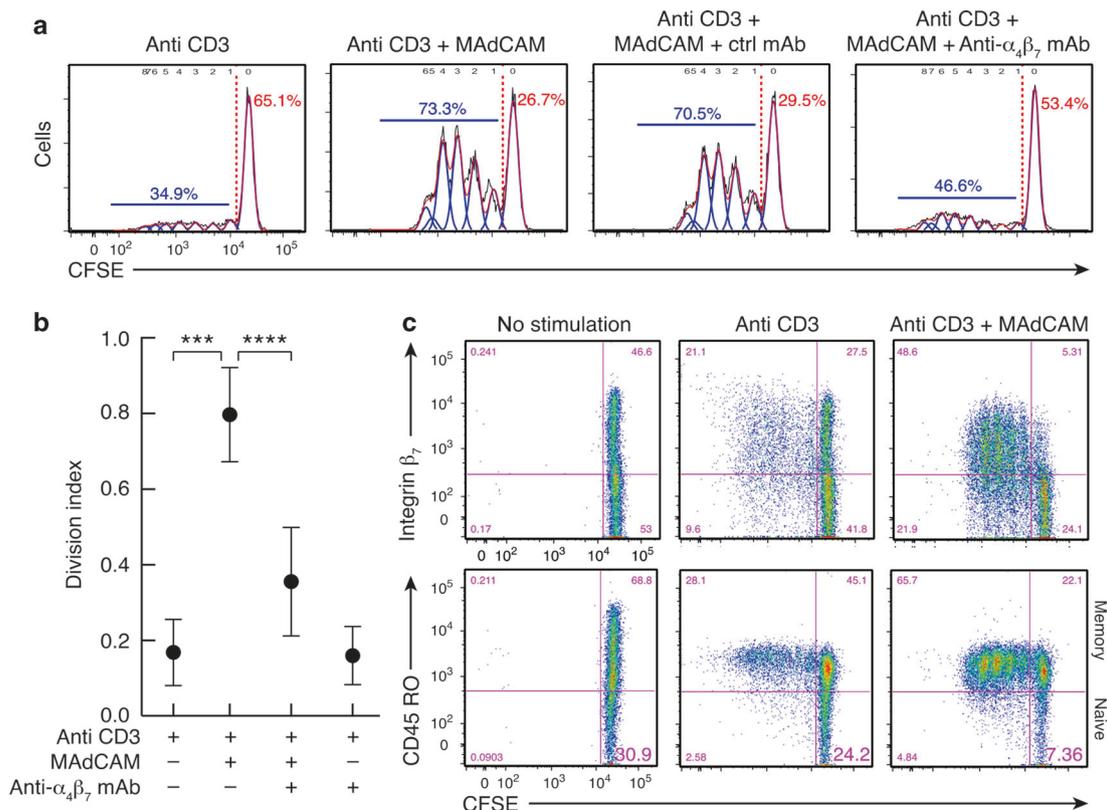


Fig. 2 The anti- $\alpha_4\beta_7$ mAb inhibits MAdCAM-mediated CD4⁺ T cell proliferation. **a** Cellular proliferation in primary CD4⁺ T cells cultured for 5 days in the presence of anti-CD3 alone, anti-CD3 + MAdCAM, anti-CD3 + MAdCAM + a control mAb (Synagis), or anti-CD3 + MAdCAM + anti- $\alpha_4\beta_7$ mAb. Proliferation was measured by CFSE dye dilution: red, draw model sum; blue, draw model components; black, total division profile. The percent of diluted CFSE labeled cells (divided cells) is shown in blue, above the blue bar, and undiluted (undivided) cells is shown in red. **b** Division of primary CD4⁺ T cells isolated from five independent donors and stimulated with ligands as in **a**, represented by a division index (measure of the average number of divisions). * $P < 0.0001$ (two-tailed parametric paired *t*-test). **c** The expression of Integrin- β_7 (y-axis, top row) and CD45RO (y-axis, bottom row) on CD4⁺ T cells stimulated for 5 days with anti-CD3 or anti-CD3 + MAdCAM is shown by CFSE dye (x-axis)

recently activated CD45RO⁻/CD4⁺ T cells, and whether this mode of costimulation supports viral replication in this subset of cells.

MAdCAM ligation of $\alpha_4\beta_7$ activates naive CD4⁺ T cells

It is well-established that shedding of CD62L from the surface of naive CD4⁺ T cells represents an early marker of cellular activation.^{35,36} In this regard, we set out to assess the manner by which MAdCAM costimulation influences the expression/shedding of CD62L on both CD45RO⁻ and CD45RO⁺ CD4⁺ T cells, and whether these effects were impacted by anti- $\alpha_4\beta_7$ mAb. Using the same protocol described above for assessing proliferation, we analyzed the amount of CD62L shed from CD4⁺ T cells by quantifying the loss of CD62L expression. We observed a significant reduction in the surface expression of CD62L on the CD45RO⁻ subset of CD4⁺ T cells five hours post exposure to anti-CD3 and MAdCAM (Fig. 3a). Anti- $\alpha_4\beta_7$ mAb inhibited this effect. When gating on the $\alpha_4\beta_7^+$ /CD45RO⁻ CD4⁺ T cell population, we found loss of CD62L expression by ~tenfold in the presence of anti-CD3 plus MAdCAM, relative to anti-CD3 alone in cells from four independent donors (Fig. 3b). Loss of expression also occurred on CD45RO⁺/CD4⁺ T cells, but to a much lesser extent. To further interrogate the memory CD4⁺ T cell subset, we subdivided the CD45RO⁺ cells into integrin β_7^+ and integrin β_7^- populations, with representative gating shown (Fig. 3c). In line with our previous observation, significant CD62L shedding was apparent only on those cells expressing $\alpha_4\beta_7$, and not on the $\alpha_4\beta_7^-$ population (Fig. 3d). In all of these experiments, the loss of expression of CD62L in the presence of MAdCAM was fully abrogated by anti- $\alpha_4\beta_7$ mAb.

To determine whether the activation of naive cells upon MAdCAM signaling was limited to CD62L loss of expression or was reflective of a broader degree of lymphocyte activation, we monitored the expression of additional cellular activation markers. We found that CD69 was upregulated within 5 h of anti-CD3 + MAdCAM treatment, in a manner that was entirely inhibited by anti- $\alpha_4\beta_7$ mAb (Supplementary Figure 3a). To evaluate the capacity of MAdCAM- $\alpha_4\beta_7$ interactions to specifically activate isolated naive CD4⁺ T cells, we purified the CD45RO⁻ subset of cells by negative bead selection and stimulated the cells with anti-CD3 + MAdCAM, as described above, and observed increased expression of two additional well-defined activation markers, Ki-67 and CD25 (Supplementary Figure 3b-c). Consistent with these observations, stimulation with MAdCAM drove naive CD4⁺ T cells into the S phase of the cell cycle (Supplementary Figure 4). Interestingly, we also noted that MAdCAM upregulated the expression of CCR5 (Supplementary Figure 3d). We conclude that MAdCAM interactions with integrin $\alpha_4\beta_7$ promote the rapid activation of both memory and naive CD4⁺ T cells subsets. The shedding of CD62L is particularly noteworthy insofar as it participates directly in, lymphocytes homing to gut-inductive sites through a direct interaction with MAdCAM.^{35,36}

Retinoic acid acts as a cofactor for MAdCAM costimulation of naive CD4⁺ T cells

The results presented above raised the possibility that anti-CD3 + MAdCAM treatment alone was sufficient to activate and induce the proliferation of CD45RO⁻ naive CD4⁺ T cells. To test this

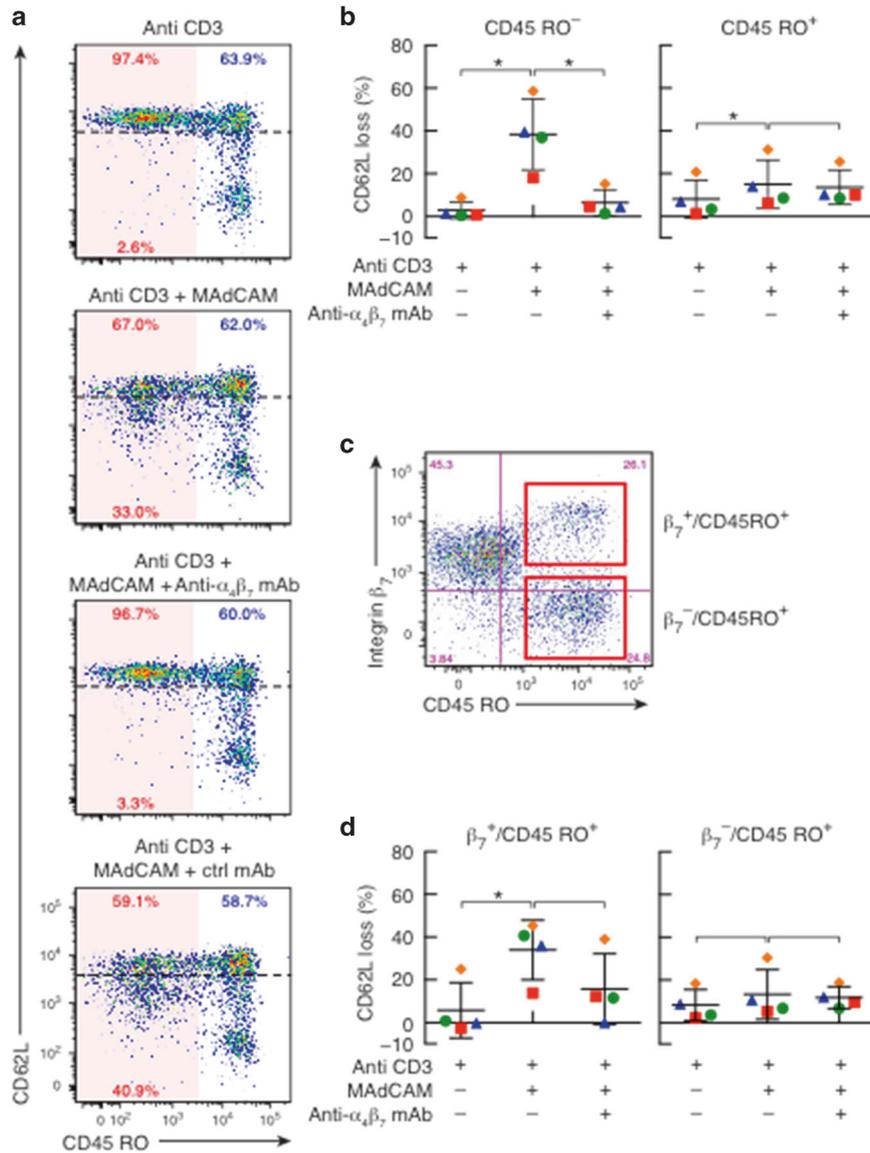


Fig. 3 MAdCAM stimulation promotes loss of CD62L expression. **a** Expression of CD62L (y-axis) and CD45RO (x-axis) on purified, primary, CD4⁺ T cells 5 h post the stimulation with anti-CD3 alone, anti-CD3 + MAdCAM, anti-CD3 + MAdCAM + anti- $\alpha_4\beta_7$ mAb, or anti-CD3 + MAdCAM + control mAb (Synagis). Cells below the black dashed line reflect loss of CD62L expression. In CD45RO⁻ or CD45RO⁺ cells, the percent of CD62L expressing cells is labeled in red or blue respectively. **b** The percent loss of CD62L expression (y-axis) in either CD45RO⁻ or CD45RO⁺ subsets of CD4⁺ T cells from four independent donors, treated as in (a). **c** A representative dot plot illustrating the expression of Integrin- β_7 (y-axis) by CD45RO (x-axis) on unstimulated CD4⁺ T cells, with red boxes around the β_7^+ /CD45RO⁺ and β_7^- /CD45RO⁺ subsets. **d** The percent loss of CD62L expression on purified CD4⁺ T cells subdivided into the CD45RO⁺/ β_7^+ and CD45RO⁺/ β_7^- populations as in c and treated as in (a). **P* < 0.05 (two-tailed parametric paired *t*-test)

possibility directly, we purified CD45RO⁻/CD4⁺ T cells by negative bead selection (96–99% purity) and carried out anti-CD3 + MAdCAM stimulation and CFSE dye dilution measurements, as described above. Although MAdCAM stimulation of the naive cells upregulated the markers of activation (shown above), cells stimulated by this manner failed to proliferate (Fig. 4a, b). To understand why naive cells in isolation failed to proliferate, we considered the fact that the average level of expression of $\alpha_4\beta_7$ on CD45RO⁻ cells is ~50–200-fold lower than that on CD45RO⁺/CD4⁺ T cells. Within PPs, the surface expression of $\alpha_4\beta_7$ on naive T cells is upregulated by retinoic acid (RA).³⁷ With this in mind, we exposed CD45RO⁻ CD4⁺ T cells simultaneously to anti-CD3 + MAdCAM and RA, and under these conditions we observed robust cellular proliferation (Fig. 4a, b). To determine whether the capacity of RA to enhance proliferation was specific to MAdCAM costimulation,

we treated CD45RO⁻/CD4⁺ T cells with anti-CD3 and RA in the absence of MAdCAM and observed no increase in proliferation over anti-CD3 alone (Supplementary Figure 5). We next asked whether RA could enhance other costimulatory signals. To this end, we exposed naive cells to a suboptimal amount of anti-CD28 and found that RA was unable to induce proliferation under this condition (Fig. 4a, b).

Lehnert and colleagues have reported that MAdCAM and anti-CD28 can act synergistically.¹⁸ In fact, when we combined MAdCAM + anti-CD28, we observed levels of naive CD4⁺ T cell proliferation, comparable to that observed with MAdCAM + RA (Fig. 4a, b). However, the two conditions appear to involve phenotypically distinct cells, whereas RA increased the surface expression of $\alpha_4\beta_7$, no such increase was observed with anti-CD28 (Fig. 4c). This suggests that these two conditions, either addition

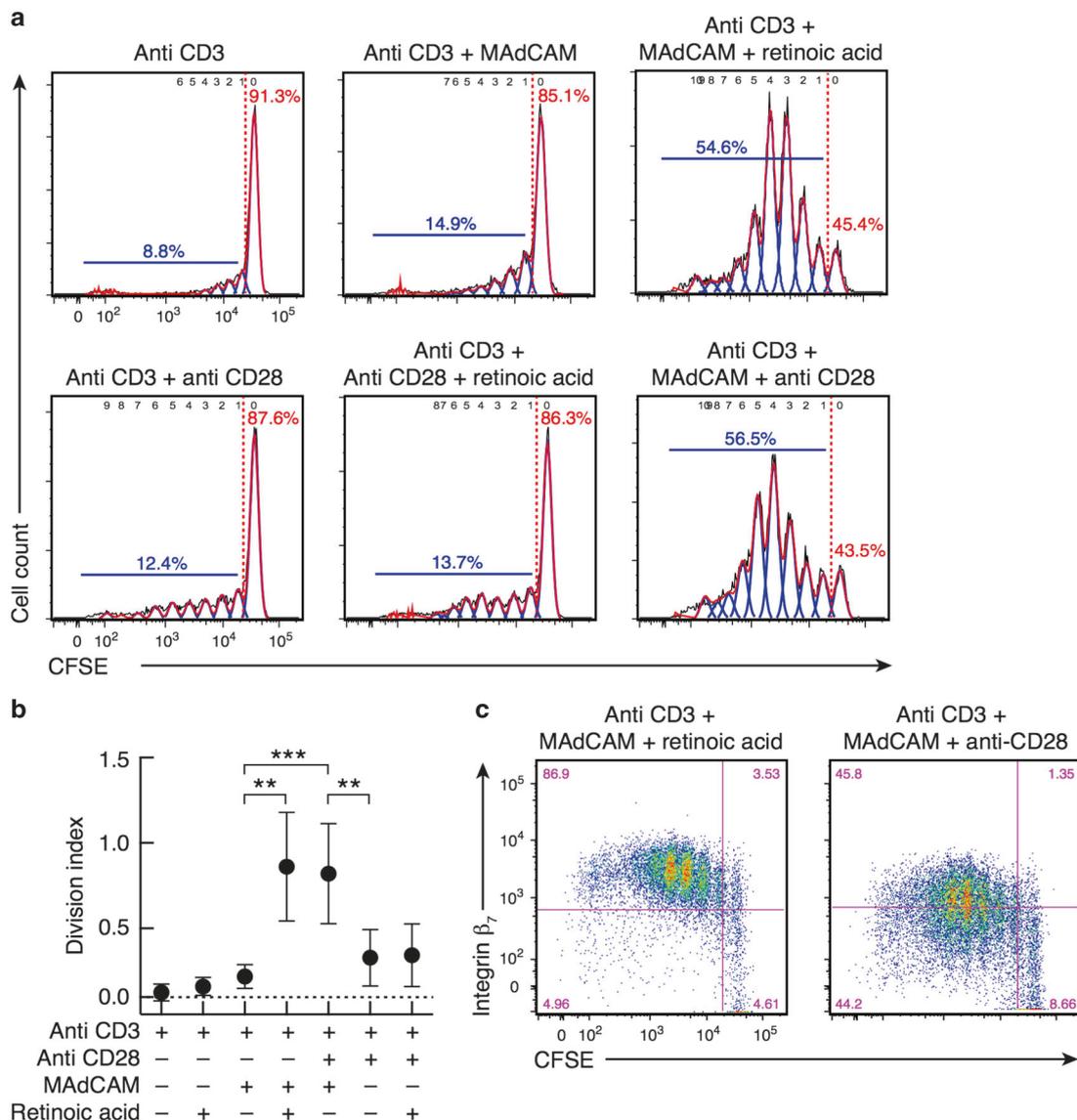


Fig. 4 RA combined with MAdCAM drives costimulation of naive $CD4^+$ T cells. **a** Purified naive $CD45RO^-/CD4^+$ T cells stained with CFSE dye (x-axis), then stimulated for 5 days with anti-CD3 alone or anti-CD3 + MAdCAM, with the addition of either RA or anti-CD28. The blue bar marks cellular proliferation in CFSE-diluted cells with the percent of either divided or undivided cells indicated in blue or red, respectively, as in (Fig. 2a). **b** Division index of purified naive $CD45RO^-/CD4^+$ T cells isolated from seven independent donors and stimulated as in (a). $*P < 0.008$ (two-tailed parametric paired *t*-test). **c** The expression of Integrin- β_7 (y-axis) by CFSE (x-axis), on purified naive $CD45RO^-/CD4^+$ T cells, stimulated for 5 days with anti-CD3 + MAdCAM + RA or anti-CD3 + MAdCAM + anti-CD28

of RA, or addition of anti-CD28, in the presence of MAdCAM, promote distinct patterns of cellular differentiation. Since certain T cell subsets that are relevant to the pathogenesis of HIV and SIV infection, including Treg and TH17 cells, express $\alpha_4\beta_7$,^{29,38,39} further investigation is required to understand the impact of MAdCAM on differentiation of naive T cells in a more complete way.

Retinoic acid combined with MAdCAM supports viral replication in recently activated naive $CD4^+$ T cells

Having shown that MAdCAM activates naive cells, we asked whether MAdCAM might enable the recently activated naive $CD4^+$ T cells to support viral replication. Considering that once inside PPs, these cells are exposed to RA, we also assessed the impact of RA on viral replication, exclusively in the $CD45RO^-$ naive $CD4^+$ T cell population. Naive cells from four healthy donors were stimulated with anti-CD3 and combinations of MAdCAM, RA, and

anti-CD28 (Fig. 5). As before, virus was added 48 h later. In general, the conditions that promoted proliferation of naive $CD4^+$ T cells in isolation (Fig. 4) also facilitated high levels of viral replication (Fig. 5).

All four donors supported substantial levels of viral replication in cells stimulated with MAdCAM + RA or MAdCAM + anti-CD28. The inclusion of all three, MAdCAM, RA, and anti-CD28, also supports high levels of viral replication. In two instances (donor 1 and 4), we observed viral replication in cultures stimulated with anti-CD3 + MAdCAM alone, without addition of RA or anti-CD28, despite our finding that this condition generally supports only low levels of cellular proliferation (Fig. 4a, b). The capacity of anti-CD3 + MAdCAM to support viral replication without added cofactors likely reflects the capacity of MAdCAM to mediate cellular activation. We cannot exclude, however, that a small fraction of the viral replication we observed may stem from the purity of our starting cultures, as they did include a fraction (<3%) of $CD45RO^+$

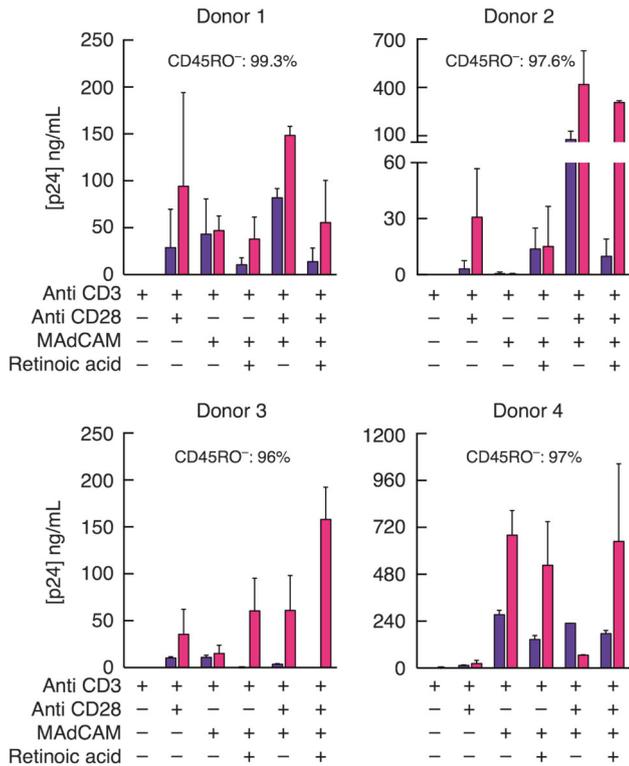


Fig. 5 Naive CD4⁺ T cells stimulated with RA and MAdCAM support viral replication. Replication of HIV SF162 in purified CD45RO⁻/CD4⁺ T cells from four independent donors, where cells were stimulated with anti-CD3 alone or in combination with MAdCAM, RA, or anti-CD28 as designated, for 3 days prior to infection. Viral replication was quantified by p24 AlphaLISA (y-axis) on days 4 (purple) and 7 (red) post-infection (x-axis). The purity of CD45RO⁻/CD4⁺ T cell cultures assessed on day 0, is shown

cells. We conclude that MAdCAM signaling through $\alpha_4\beta_7$ along with an additional signal (RA or anti-CD28) promotes viral replication in recently activated naive CD4⁺ T cells.

MAdCAM stimulation promotes viral replication in PBMCs from HIV-infected subjects

Because MAdCAM signaling through $\alpha_4\beta_7$ in both the naive and memory subsets of CD4⁺ T cells promotes viral replication in vitro and upregulates the surface expression of the key markers of cellular activation, we asked whether MAdCAM signaling through $\alpha_4\beta_7$ could promote replication of the virus in peripheral blood mononuclear cells (PBMCs) from HIV-infected subjects. PBMCs from six antiretroviral therapy naive subjects with viral loads ranging from 7631 to 54894 RNA copies/ml were evaluated. The subject cohort data are provided in Supplementary Table 1. Isolated PBMCs were CD8⁺ T cells depleted by positive bead selection to prevent any CD8-mediated cytolytic activity, and viral replication in the presence of our stimulatory ligands, anti-CD3, anti-CD28, MAdCAM, and combinations of MAdCAM with anti-CD28 and/or RA were tested. Cellular stimulation via the classical pathway with anti-CD3 + anti-CD28 promoted viral replication in three of the six subjects (Fig. 6). In four of the subjects, anti-CD3 + MAdCAM + RA facilitated viral replication. In two of the subjects, anti-CD3 + MAdCAM alone supported viral replication. In all six subjects, anti-CD3 + MAdCAM + anti-CD28 + RA supported viral replication. Although these stimuli are not as potent as non-specific stimuli that include mitogens, e.g., PHA/IL2, these results suggest that in all six subjects $\alpha_4\beta_7$ -expressing cells were

productively infected. This is consistent with previous reports that $\alpha_4\beta_7$ ⁺ memory CD4⁺ T cells are preferential targets.^{27,31} The fact that viral replication is induced by MAdCAM either with or without RA suggests that this is one of the mechanisms that could promote HIV replication in gut tissues.

DISCUSSION

During AI, GALT is a principal site of viral replication. Two features distinguish GALT from most other lymphoid tissues. First, trafficking of lymphocytes into these tissues is mediated by MAdCAM, and second, within GALT-inductive sites, dendritic cells convert vitamin A to RA. We find that MAdCAM alone, or in combination with RA promotes high levels of HIV replication. P24 values in MAdCAM stimulated culture supernatants 6 days post the stimulation, in most cases, exceeded 100 ng/ml. Of note, anti- $\alpha_4\beta_7$ mAb, which we previously demonstrated reduces mucosal transmission of SIV by 50 percent in a macaque model,²⁴ is able to inhibit this replication.

Naive CD4⁺ T cells traffic continuously through PPs and MLNs via the MAdCAM-dependent pathway. In this regard, we asked whether MAdCAM costimulation could prime the naive CD4⁺ T cells for productive infection. In bulk cultures that included both CD45RO⁺ and CD45RO⁻ CD4⁺ T cells, MAdCAM costimulation appeared to drive the activation of both subsets. CD45RO⁻ naive cells were rapidly activated, as evidenced by the upregulation of CD69 and the shedding of L-selectin. Both of these effects were inhibited by the addition of anti- $\alpha_4\beta_7$ mAb. However, we were surprised to find that CD45RO⁻/CD4⁺ T cells, when costimulated with MAdCAM, failed to proliferate in a significant way. RA, which is generated in PPs and MLNs, exerts potent effects on the lymphocytes, including upregulation of the expression of $\alpha_4\beta_7$ and CCR5.^{40,41} It has also been reported that RA can act as latency reversing agent.⁴² This prompted us to ask whether its inclusion might enhance MAdCAM costimulation and hence permissiveness for HIV replication. This is noteworthy insofar as RA is often considered an “anti-infective” agent that enhances immunity against foreign microbes. Our observations are consistent with the above mentioned studies and the finding that, while vitamin A supplementation promotes resistance to other infectious agents, it paradoxically enhances HIV infection.⁴³ RA was unable to facilitate anti-CD28-mediated costimulation (Fig. 4). However, when we combined anti-CD28 with MAdCAM, anti-CD28 effectively substituted for RA. We conclude that MAdCAM primes the naive CD4⁺ T cells for proliferation, but such cells require an additional stimulus.

The apparent synergy between MAdCAM and RA is likely to influence the manner in which antigen-specific immune responses evolve within the GALT. Naive CD4⁺ T cells uniformly express intermediate levels of $\alpha_4\beta_7$, unlike $\alpha_4\beta_7$ ⁺ memory cells, which express high levels of $\alpha_4\beta_7$. This lower level deters naive lymphocytes from accessing the LP.¹⁶ In the course of MAdCAM/RA costimulation, $\alpha_4\beta_7$ was upregulated on proliferating cells. Such increased expression would facilitate the eventual trafficking of these cells to the gut effector sites including LP. In contrast, MAdCAM/CD28 costimulation promoted similar levels of proliferation, but without $\alpha_4\beta_7$ upregulation, reducing in a relative way the potential of such cells to migrate to gut effector sites. Thus, these two types of costimulation likely lead to differential trafficking of lymphocytes. In this regard, the three principal subsets of CD4⁺ T cells that express $\alpha_4\beta_7$ are TH17, Treg,^{29,38,39,44} and Tfh cells (Supplementary Figure 6). Determining whether MAdCAM/RA helps drive cells toward one or more of these functional CD4⁺ T cell subsets is the subject of ongoing studies. Finally, recognizing that in the PPs and MLNs, DCs are the source of both RA and CD80/86 signaling,⁴⁵ it is quite possible that in the context of an antigen-specific response, MAdCAM-primed naive CD4⁺ T cells are stimulated by both RA and CD80/86. In this regard, MAdCAM/

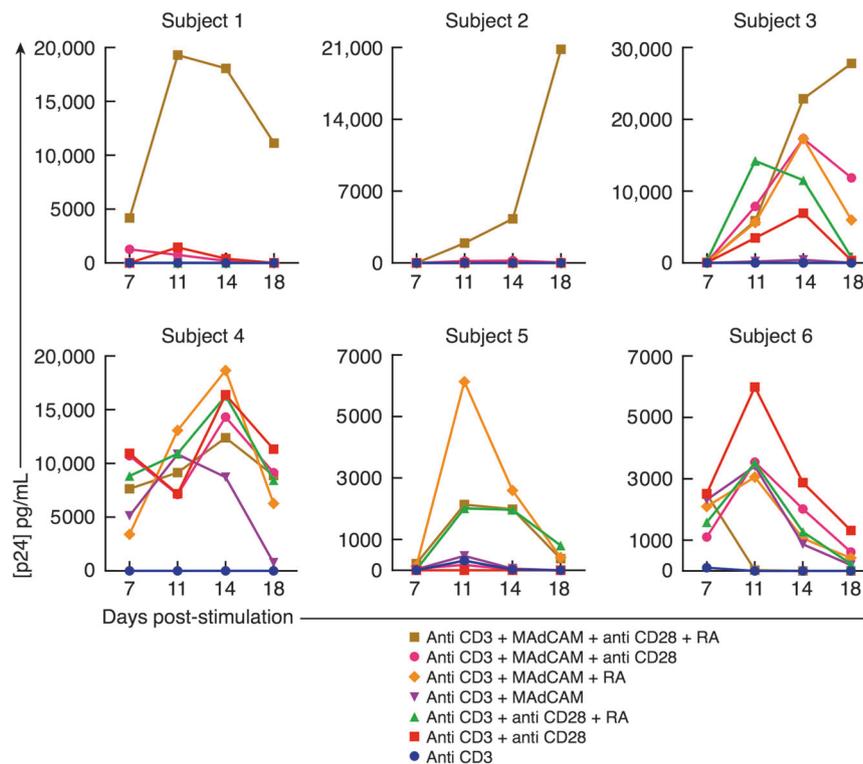


Fig. 6 MAdCAM stimulation of PBMCs from HIV-infected subjects promotes viral replication. PBMCs isolated from six HIV-infected donors were CD8⁺ T cell depleted, and cultured over ligand-coated plates. The stimulatory ligands include anti-CD3, anti-CD28, MAdCAM and RA in various combinations as designated. Viral p24 was measured by AlphaLISA (y-axis) from culture supernatants at day 7, 11, 14, and 18 (x-axis)

$\alpha_4\beta_7$ costimulation is distinct from the classical costimulation through CD28 in ways that suggest it may act in some manner as a 2nd costimulatory signal. To our knowledge, this is the first demonstration of RA as a factor that can enhance cellular proliferation. Of note, the combination of MAdCAM, anti-CD28, and RA was consistent in promoting replication of HIV in PBMCs of HIV-infected subjects (Fig. 6).

In addition to promoting proliferation, MAdCAM/RA treatment of CD4⁺ T cells also upregulates CCR5 (Supplementary Figure 3d). Because CCR5 functions as an entry receptor for most isolates of HIV, including those isolates that establish infection following mucosal transmission, its upregulation provides another way in which MAdCAM costimulation provides a conducive environment for the high levels of viral replication in the GALT during AI. We note that RA-mediated increases in CCR5 expression (Supplementary Figure 3d) coincided with the increases in the surface expression of $\alpha_4\beta_7$. There appears to be an interesting, but not well-understood relationship between these two cell surface receptors.

The anti- $\alpha_4\beta_7$ mAb employed in our studies is a “primatized” analog of vedolizumab, a therapeutic mAb used in the treatment of inflammatory bowel disease (IBD).⁴⁶ Vedolizumab interferes with the lymphocytes trafficking to the gut, which is believed to be the basis for its anti-inflammatory activity. However, MAdCAM-mediated lymphocyte trafficking and costimulation of lymphocytes through $\alpha_4\beta_7$ are likely to be closely linked. Likewise, the capacity of anti- $\alpha_4\beta_7$ mAb to block the trafficking and signal transduction through $\alpha_4\beta_7$ cannot be easily separated. In this regard, it is reasonable to consider whether the efficacy of vedolizumab in the treatment of IBD involves not simple alterations in trafficking, but also a reduction of MAdCAM-driven costimulation of $\alpha_4\beta_7^+$ /CD4⁺ T cells.

Although MAdCAM expression is generally limited to the gut in healthy adults, other infectious agents that elicit inflammatory responses induce MAdCAM expression outside of the gut.

MAdCAM is expressed in the liver of hepatitis C-infected individuals,⁴⁷ the female genital tract of chlamydia-infected women,⁴⁸ and the vaginal mucosa of HSV-2-infected macaques.^{45,49} MAdCAM is also widely expressed in peripheral lymph nodes of newborns.⁵⁰ Whether, during the course of AI, HIV, and SIV infection results in expression of MAdCAM outside of the GALT remains to be determined, however, if that is the case, such expression could potentially contribute to the dissemination of the virus, subsequent to the seeding of PPs and MLNs at the outset of acute infection.

We speculate two possible scenarios in which $\alpha_4\beta_7^+$ /CD4⁺ T cells encounter MAdCAM in a way that might facilitate viral replication in vivo. The first involves the direct interaction of these cells with HEVs proximal to the GALT (see schematic in Supplementary Figure 7). In this context, $\alpha_4\beta_7$ binds to MAdCAM as part of diapedesis, a multi-step adhesion cascade that immediately precedes extravasation of migrating lymphocytes into the PPs, MLNs, and LP.¹⁵ The second scenario stems from reports that follicular dendritic cells (fDCs) in the germinal centers can express MAdCAM⁵¹, while both Tfh CD4⁺ T cells and germinal center B cells express $\alpha_4\beta_7$ ^{52,53} (see schematic in Supplementary Figure 7d). Thus, there exists a potential for MAdCAM on fDCs to activate Tfh cells via interaction with $\alpha_4\beta_7$. It is well-established that fDCs trap HIV virions and transfer the virus to anatomically secluded Tfh CD4⁺ T cells.^{54,55} This trapping and transfer of virions is thought to contribute to the formation of viral reservoirs and to viral persistence. We recently reported that a primatized analog of vedolizumab facilitated the control of SIV rebound in ART-treated macaques following analytical treatment interruption.⁵⁶ Although the mechanism of the control in these macaques remains to be identified, it is possible that the anti- $\alpha_4\beta_7$ mAb interferes, in some manner, with $\alpha_4\beta_7$ signaling in germinal centers in a way that contributes at least in part to that control. Of note, we observed qualitatively distinct anti gp120 antibody responses that persisted in animals receiving the anti- $\alpha_4\beta_7$ mAb.

Viral replication in, and destruction of the GALT, is a defining feature of AI and a central event in HIV pathogenesis. In this report, we have shown that MAdCAM, a surface receptor whose expression is largely restricted to the GALT, promotes viral replication in $\alpha_4\beta_7^+$ /CD4⁺ cells. The role of RA together with MAdCAM in promoting infection of recently activated CD4⁺ T cells provides a plausible explanation for the central role that GALT play in AI. This capacity of MAdCAM to signal through $\alpha_4\beta_7$ in the gut tissues may contribute in a significant way to the gut-tropic nature of HIV. That the anti- $\alpha_4\beta_7$ mAb blocks this activity and inhibits viral replication suggests a novel intervention strategy that could potentially protect the GALT during AI and delay the eventual progression of HIV disease.

METHODS

Human blood and tissue samples

All primary CD4⁺ T cells utilized in these studies were isolated from PBMCs, collected from healthy donors through a NIH Department of Transfusion Medicine protocol that was approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health. Informed consent was written and was provided by study participants and/or their legal guardians. Studies on HIV-infected subjects were approved by the NIAID Institutional Review Board. PBMCs were isolated from these subjects by leukapheresis. HIV infection was determined by HIV-1/2 immunoassay (Abbott Laboratories, Abbott Park, IL) and Cambridge Biotech HIV-1 Western blot (Maxim Biomedical, Inc., Rockville, MD). All subjects acquired HIV infection in the USA and, therefore, are likely infected with clade B viruses, with no history of ART and no history of opportunistic diseases.

Primary cells and tissue culture reagents

Primary CD4⁺ T cells utilized in these studies were freshly isolated from healthy donor PBMCs and separated by lymphocyte separation medium (MP Biomedicals, Santa Clara, CA). Purified CD4⁺ T cells were obtained by negative selection (Stem Cell Technologies, Vancouver, Canada) to >95% purity, as determined by flow cytometry. CD4⁺ T cells were cultured in complete RPMI 1640 medium, with 2% L-glutamine-penicillin-streptomycin (Gibco Laboratories, Gaithersburg, MD) and 10% FBS (Gibco Laboratories, Gaithersburg, MD). Where designated, naive CD45RO⁻/CD4⁺ T cell cultures were generated by CD45RO positive selection of the bulk CD4⁺ T cells with Macs beads (Miltenyi Biotec, San Diego, CA), resulting in CD45RO⁻/CD4⁺ T cells at >96% purity. Purity of CD45RO⁻/CD4⁺ T cell cultures was validated in the control experiments that included anti-CD3 and retinoic acid, and no cellular proliferation was observed (Supplementary Figure 8) indicating the contamination by antigen-presenting cells did not contribute to the proliferative responses. CD8⁺ T cells were depleted in a similar manner, by positive bead selection (Stem Cell Technologies, Vancouver, Canada) to 97% purity.

Antibodies and flow cytometry

The following antibodies were used for flow cytometry: anti-Integrin β_7 PE (clone FIB504), anti-CD45RO APC and anti-CD45RO BV421 (clone UCHL1), anti-CD62L PE (clone DREG-56), anti-CD69 PE (clone FN50), anti-CCR5 APC (clone 2D7), and anti-Ki-67 FITC (clone MOPC-21) (BD Pharmingen, San Diego, CA). For intracellular Ki-67 staining, BD Perm Wash Kit and company protocol were utilized (BD Pharmingen, San Diego, CA). The anti- $\alpha_4\beta_7$ mAb heterodimer (clone Act-1) was gifted by Dr. Aftab Ansari. The Synagis antibody was gifted from Dr. Barton Haynes and used as a control in several experiments where designated. Data were collected on a FACSCanto II (BD Biosciences, San Diego, CA) and were analyzed using FlowJo and GraphPad Prism software.

Viral replication assay

The 96-well flat bottom cell culture-treated plates were coated overnight at 4°C with either 200 ng of anti-CD3 (clone OKT3, eBioscience, San Diego, CA), 200 ng of anti-CD28 (CD28.2, eBioscience, San Diego, CA), 200 ng of recombinant human MAdCAM-1 Fc chimera (R&D Biosystems, Minneapolis, MN), or combinations of these ligands in 100 μ l HBS (1 \times). In certain wells, 2 μ g of anti- $\alpha_4\beta_7$ mAb or a control mAb Synagis were added to test the specificity. The following day, plates were washed in PBS solution and primary CD4⁺ T cells were added over each well of the ligand-coated plates at a concentration of 2×10^6 cells/ml. Plates were subsequently cultured at 37°C, 5% CO₂ for 3 days in the presence of the ligand. Following stimulation, cells were infected with 2 μ l of HIV-containing infectious cell supernatant (viral concentration of 0.16 ng/ μ l, p24-based ELISA). After 12 h of incubation, plates were washed in PBS to remove excess virus, resuspended in RPMI (10%FBS), and cultured, whereby the cell supernatants were collected on days 4, 6, and 7 post the infection. The virus utilized for the in vitro experiments was prepared by cloning the full-length gp160 from the SF162 viral isolate (accession number EU123924) into the NL4.3 viral backbone. In one experiment, the full-length NL4.3 (accession number AF324493) was utilized, as designated. Viral stocks were produced by transient transfection of 293T cells and then passaged for one round through PBMCs. Experiments conducted on cells from HIV-infected human subjects were performed in a similar manner, such that the ligands were coated overnight in a 48-well flat bottom plate. In order to avoid CD8 suppressor activity^{57,58}, PBMCs from HIV-infected subjects were CD8⁺ T cell depleted by positive bead selection, and the CD8-depleted PBMCs were cultured over the ligand-coated wells at a concentration of 2×10^6 cells/ml. In both the in vitro HIV infection assays and viral replication assays utilizing PBMCs from the infected donors, RA (Sigma Aldrich, St. Louis, MO) was added to specific test wells at a concentration of 10 nM. Culture supernatants were collected at various time points post the infection, and virus replication was detected by measuring the concentration of p24 GAG antigen by ELISA (PerkinElmer, Santa Clara, CA) or p24 GAG AlphaLISA (PerkinElmer, Santa Clara, CA).

Cellular proliferation assay

The 96-well flat bottom cell culture-treated plates were coated overnight at 4°C with either 200 ng of anti-CD3, 200 ng of anti-CD28, 200 ng of MAdCAM-Fc chimera or combinations of these ligands. In certain wells, anti- $\alpha_4\beta_7$ mAb or a control mAb Synagis were added to test the specificity. The following day, primary CD4⁺ T cells were CFSE labeled using the CellTrace CFSE Cell Proliferation Kit, as per the manufacturer's instructions (Invitrogen, Grand Island, NY). CD4⁺ T cells were washed twice in PBS, resuspended in PBS containing 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) at a final concentration of 0.5 μ M, and incubated at room temperature for 10 min. The labeled cells were washed three times with RPMI 10% FBS, and 2×10^5 cells were added over each ligand-coated well. In certain wells, RA (Sigma Aldrich, St. Louis, MO) was also added at a concentration of 10 nM. Cells were incubated over the ligands at 37°C, 5% CO₂ for a minimum of 4 days in RPMI (10% FBS). After 5 days, cells were doubly stained with anti-CD45RO APC and anti-Integrin- β_7 PE. CFSE dilution was measured by flow cytometry. The division index for cells in each treatment group was calculated using FlowJo software.

Cell cycle analysis

The 96-well flat bottom cell culture-treated plates were coated overnight at 4°C with either 200 ng of anti-CD3, 200 ng of anti-CD28, 200 ng of MAdCAM-Fc chimera or combinations of these ligands, as in the cellular proliferation assay. The following day, purified naive CD45RO⁻/CD4⁺ T cells were added to the ligand-



coated wells. In certain wells, RA was also added at a concentration of 10 nM. Cells were incubated over the ligands at 37 °C, 5% CO₂ for a minimum of 4 days in RPMI (10% FBS). After 3 days, the cells were stained with 50 µg/ml propidium iodide (PI) in a buffer containing 4 mM sodium citrate and 0.3% Triton X-100 (Sigma Aldrich, St. Louis, MO). PI staining was measured by flow cytometry, and the data was analyzed using FlowJo software.

Animal lymphocyte staining

All animals were housed in compliance with the regulations under the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, at Tulane National Primate Research Center TNPRC; Covington LA. Inguinal lymph nodes were collected from adult, female *Rhesus macaques* at necropsy, cut in small pieces, and passed through a 40-µm cell strainer. Cells were washed and stained with live-dead fixable Aqua (Invitrogen, Grand Island, NY) and monoclonal antibodies against CD3 (clone SP34) and CD4 (clone L200) from BD Pharmingen (San Diego, CA), PD1 (clone eBioJ105) from eBioscience (San Diego, CA) and CXCR5 (clone 710D82.1), and integrin- $\alpha_4\beta_7$ (clone Act-1) from Mass Biologics (Mattapan, MA).

Statistical analysis

Parametric, paired two-tailed *t* tests for normally distributed data were calculated by GraphPad Prism and *p* values < 0.05 were reported.

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AUTHOR CONTRIBUTIONS

JA, CC, ASF, FN, LRG, JCR, and RO conceived and designed the study; FN, LRG, JCR, RO, IP, JH, DVR, DW, MW, and KJ optimized all reagents and performed cell stimulation and viral replication assays, and carried out all cellular phenotyping; DW performed virus stock production and quantification; EM performed biopsies and staining of Tfh cells from the macaque lymph node; MC and SAM collected clinical samples and provided cohort data for HIV-infected subjects; AAA, FV, and EM provided expert advice on experimental planning and data interpretation; JA, CC, FN, and LRG wrote the manuscript; ASF, AAA, FV, EM, SAM, and JCR read and commented on the manuscript.

ADDITIONAL INFORMATION

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