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Targeting $\alpha_4\beta_7$ integrin reduces mucosal transmission of simian immunodeficiency virus and protects gut-associated lymphoid tissue from infection

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$\alpha_4\beta_7$ integrin–expressing CD4$^+$ T cells preferentially traffic to gut-associated lymphoid tissue (GALT) and have a key role in HIV and simian immunodeficiency virus (SIV) pathogenesis. We show here that the administration of an anti-$\alpha_4\beta_7$ monoclonal antibody just prior to and during acute infection protects rhesus macaques from transmission following repeated low-dose intravaginal challenges with SIV_{mac251}. In treated animals that became infected, the GALT was significantly protected from infection and CD4$^+$ T cell numbers were maintained in both the blood and the GALT. Thus, targeting $\alpha_4\beta_7$ reduces mucosal transmission of SIV in macaques.

CD4$^+$ T cells residing in GALT are a predominant target of HIV and SIV during the acute phase of infection, and their depletion has been implicated in HIV and SIV disease[1–3]. CD4$^+$ and CCR5$^+$ T cells that traffic to GALT typically express $\alpha_4\beta_7$ integrin, which functions as a gut-homing receptor[4]. $\alpha_4\beta_7$ mediates gut-homing by binding to mucosal vascular addressin cell adhesion molecule (MAdCAM), a ligand expressed on venules that service GALT[5]. Of note, some strains of HIV and SIV bind to $\alpha_4\beta_7$ (refs. 6–8). These findings suggest that the $\alpha_4\beta_7^+$ subset of CD4$^+$ T cells may play a key part in transmission and that targeting could disrupt early steps in infection and possibly interfere with disease progression.

To explore that possibility, we used a recombinant rhesus anti-$\alpha_4\beta_7$ monoclonal antibody (mAb) ($\alpha_4\beta_7$-mAb)[9]. Previously, in a high-dose intravenous or intrarectal challenge study, we showed that $\alpha_4\beta_7$-mAb treatment before and after challenge mediated a significant decrease in gut tissue proviral DNA and a one- to two-log reduction in mean plasma viral loads in both studies[10,11]. Notably, whereas 10 of 12 controls died of AIDS within 2 years, all ten treated animals remained healthy with CD4$^+$ T cell counts >500/μl 5 years after infection[10,11].

In this study, we evaluated the efficacy of $\alpha_4\beta_7$-mAb therapy in preventing transmission. We employed a nonhuman primate model based on repeated low-dose intravaginal challenges, which more faithfully mimics HIV transmission. We found that intravenous administration of $\alpha_4\beta_7$-mAb reduced surface exposure of $\alpha_4\beta_7$ on CD4$^+$ T cells in the cervicovaginal canal and either prevented or delayed infection. When prevention failed, both viral DNA loads in GALT and the rate of peripheral CD4$^+$ T cell depletion were markedly reduced as compared to in the control IgG–treated group. The results of these studies suggest that $\alpha_4\beta_7$ antagonists might be useful for prophylaxis or treatment of HIV infection.

The protocol used for the entire study is illustrated in **Supplementary Figure 1**. We conducted baseline studies designed to optimize the collection and analyses of cervical tissue (cytobrush) and gut tissue samples in 24 uninfected female macaques (phase 1). The results are summarized in **Supplementary Figure 2a**,**b**. We then divided the macaques into two groups (n = 8), each of which was administered two sequential intravenous injections, at 3-week intervals, of either rhesus recombinant $\alpha_4\beta_7$-mAb or a recombinant isotype-matched control at 50 mg per kg body weight (mg/kg), as previously described[10]. We monitored plasma and cervicovaginal lavage (CVL) fluid $\alpha_4\beta_7$-mAb concentrations[10] and confirmed that this regimen maintained plasma levels at or above 25 μg/ml and detectable levels in the CVL fluid (**Supplementary Fig. 3a**,**b**). We collected peripheral blood, rectal biopsies and cytobrush samples weekly and analyzed them to determine the efficiency and kinetics of the binding of the $\alpha_4\beta_7$-mAb. Cells from all three compartments were bound by the $\alpha_4\beta_7$-mAb (**Supplementary Fig. 2c**) but did not significantly alter the proportion of T cells expressing the α_4 integrin subunit on the same cells (**Supplementary Fig. 2d**), indicating that treatment did not eliminate $\alpha_4\beta_7^+$ cells but rather masked the relevant epitope.

To determine whether masking $\alpha_4\beta_7$ affects susceptibility to infection, the 24 female macaques were divided into two equivalent groups on the basis of the frequencies of CD4$^+\alpha_4\beta_7^+$ T cells and regulatory T cells in blood, gut tissues and cervicovaginal cells (**Supplementary Fig. 4**), age and reproductive history and genetic characteristics (**Supplementary Tables 1** and **2**). We gave each group five injections of either $\alpha_4\beta_7$-mAb or the control mAb at 3-week intervals. Three days after the first injection, we challenged the animals intravaginally with a low dose of SIV_{mac251}. Under the predetermined protocol, we

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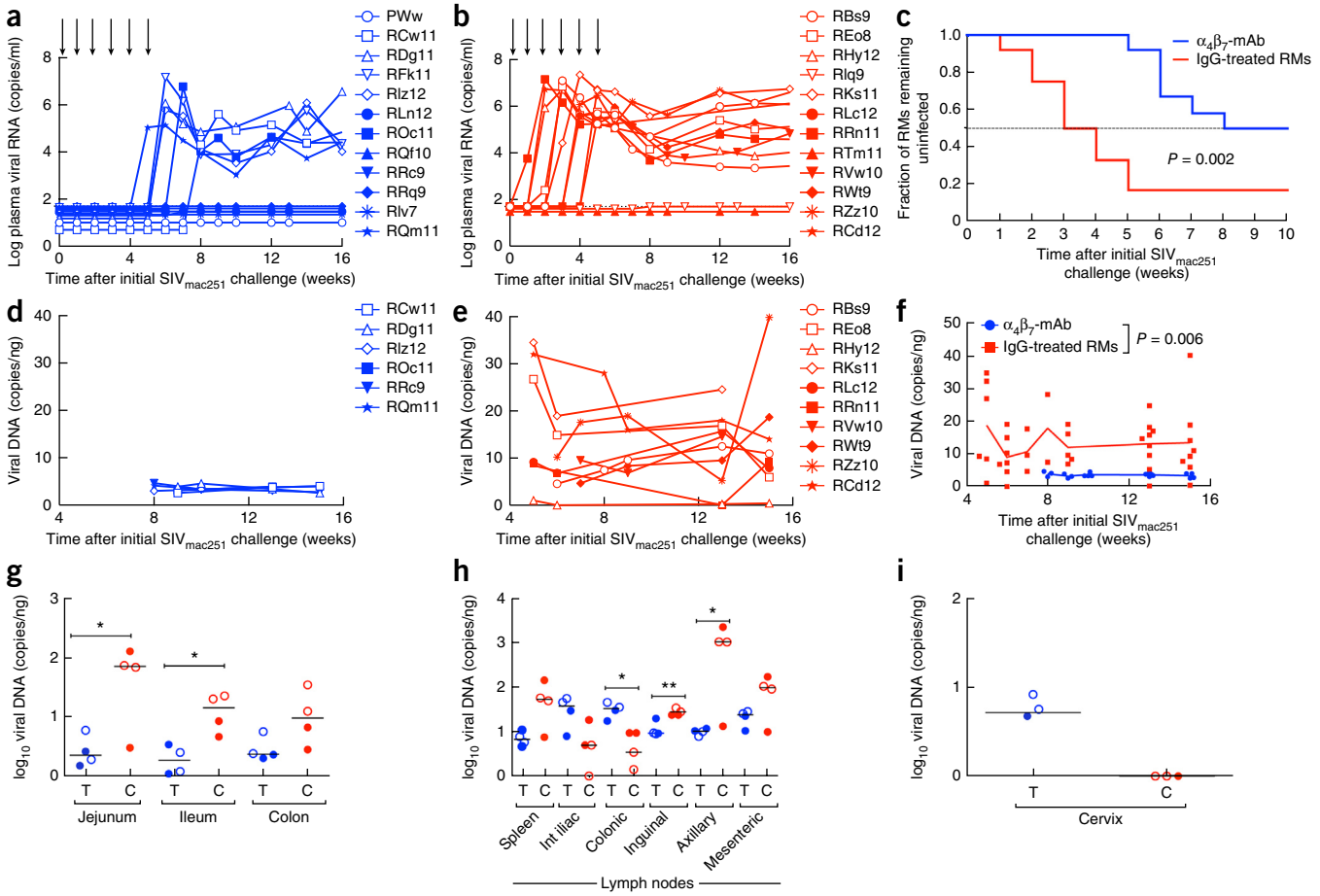

Figure 1 Kinetics of plasma viral load and tissue and organ specific pro-viral DNA loads. (**a**,**b**) Plasma viral loads from two groups of macaques ($n = 12$ for each group) that received 50 mg/kg of either a $\alpha_4\beta_7$-mAb (**a**) or a rhesus IgG (**b**) intravenously at 3-week intervals following the initiation of six once-weekly low-dose intravaginal challenges with SIV_{mac251} (arrows). (**c**) Kaplan-Meier curves based on plasma viral RNA ($n = 12$ each group; log-rank test; $P = 0.002$). (**d**,**e**) Proviral DNA load (number of copies per ng DNA) in rectal biopsies from $\alpha_4\beta_7$-mAb–treated (**d**) or IgG control–treated (**e**) macaques ($n = 6$ for $\alpha_4\beta_7$-mAb, $n = 10$ for IgG control). (**f**) Median values of data in **d** and **e** (random mixed effects (RME) model; $P = 0.006$). (**g**,**h**) Two infected macaques from each group ($\alpha_4\beta_7$-mAb in blue (T) control IgG in red (C)) were killed at 2 weeks after infection (filled circles) and two killed at 16–18 weeks after infection (open circles). Proviral DNA load (number of copies per ng DNA) in jejunal, ileum and colon tissue samples from both groups ($n = 4$; Mann-Whitney *U*-test; $P < 0.01$) (**g**) and spleen and internal iliac, colonic, inguinal, axillary and mesenteric lymph nodes ($n = 4$; Mann-Whitney *U*-test; $P < 0.01$) from both groups (**h**). Proviral DNA load (number of copies per ng DNA) from cervical tissue samples from three macaques from each group (**i**). Macaque identifiers in **a**,**b**,**d** and **e** are specified. $^*P < 0.05$, $^{**}P < 0.01$.

rechallenged the animals every week until 10 of 12 control animals became infected and then compared their plasma viral RNA loads (**Fig. 1a**,**b**). By week 5, 10 of 12 control animals became viremic, and we discontinued the challenges. In contrast, only 1 of 12 treated animals became viremic by week 5. An additional five animals developed viremia by week 8, whereas 6 of 12 remained uninfected. All uninfected animals were subsequently found not to be intrinsically resistant to infection (**Supplementary Fig. 5a**,**b**).

We used Kaplan-Meier analysis (**Fig. 1c**) to verify that $\alpha_4\beta_7$-mAb treatment significantly increased the number of challenges required for infection ($P = 0.002$, log-rank test). The hazard ratio was 4.3, with 95% confidence limits of 1.5–12.2 and $P = 0.007$ using the proportional-hazards regression model. In all, 71 challenges were needed to infect 6 of 12 mAb treated macaques, whereas only 44 challenges sufficed to infect 10 of 12 controls, implying a 2.7-fold decreased infection risk with each challenge in the $\alpha_4\beta_7$-mAb–treated group ($P < 0.05$ by Fisher's exact test).

Although we found no statistical difference between plasma viral RNA loads in control versus treated groups (**Fig. 1a**,**b**), the amount of proviral DNA in colorectal tissues differed significantly (**Fig. 1d**,**e**). Gastrointestinal tissue (GIT) biopsies from six treated and infected animals harbored, on average, median 3.5 copies of proviral DNA per ng of DNA, whereas biopsies from ten untreated and infected controls exhibited a median 12.8 copies per ng DNA ($P = 0.006$) (**Fig. 1f**). We confirmed that disparity in a survey of tissues collected from four viremic animals from both $\alpha_4\beta_7$-mAb– and IgG control–treated groups post-mortem. Proviral DNA was 5- to 25-fold more abundant in DNA sampled from jejunum, ileum and colon of control as compared to treated infected animals (**Fig. 1g**), indicating that $\alpha_4\beta_7$-mAb mediated reductions in GALT infection. In contrast, proviral loads in spleen and various other lymph nodes revealed no consistent disparity between the two groups (**Fig. 1h**). Although proviral DNA was undetectable in most other solid organs, it was detected in ecto- and endocervical tissues from three of three treated, infected animals, but not in ecto- and endocervical tissues of three control infected animals, sampled post-mortem (**Fig. 1i**). Thus, for animals that became infected despite treatment, $\alpha_4\beta_7$-mAb was associated

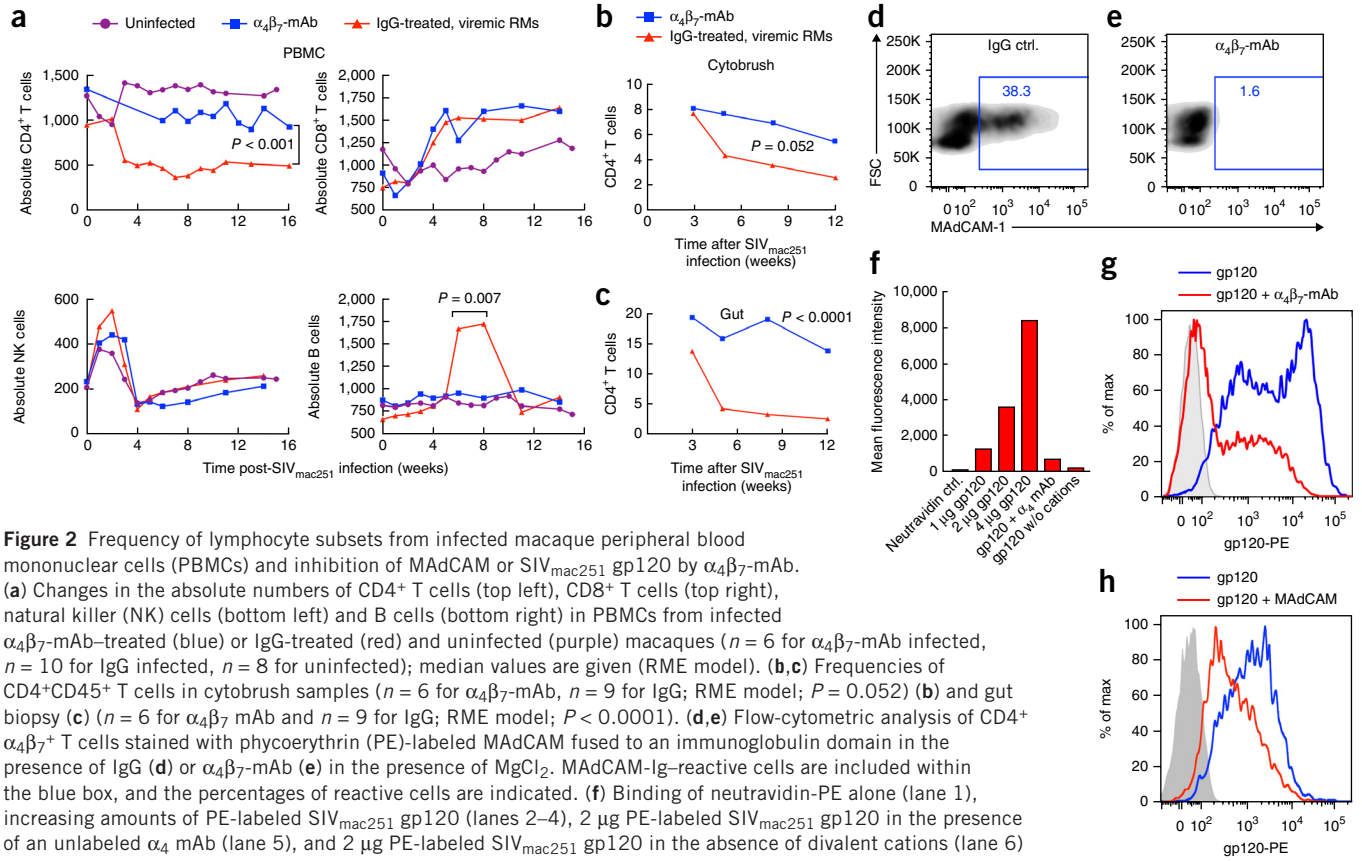

Figure 2 Frequency of lymphocyte subsets from infected macaque peripheral blood mononuclear cells (PBMCs) and inhibition of MAdCAM or SIV_{mac251} gp120 by $\alpha_4\beta_7$-mAb. (**a**) Changes in the absolute numbers of $CD4^+$ T cells (top left), $CD8^+$ T cells (top right), natural killer (NK) cells (bottom left) and B cells (bottom right) in PBMCs from infected $\alpha_4\beta_7$-mAb–treated (blue) or IgG-treated (red) and uninfected (purple) macaques ($n = 6$ for $\alpha_4\beta_7$-mAb infected, $n = 10$ for IgG infected, $n = 8$ for uninfected); median values are given (RME model). (**b**,**c**) Frequencies of $CD4^+CD45^+$ T cells in cytobrush samples ($n = 6$ for $\alpha_4\beta_7$-mAb, $n = 9$ for IgG; RME model; $P = 0.052$) (**b**) and gut biopsy (**c**) ($n = 6$ for $\alpha_4\beta_7$ mAb and $n = 9$ for IgG; RME model; $P < 0.0001$). (**d**,**e**) Flow-cytometric analysis of $CD4^+$ $\alpha_4\beta_7^+$ T cells stained with phycoerythrin (PE)-labeled MAdCAM fused to an immunoglobulin domain in the presence of IgG (**d**) or $\alpha_4\beta_7$-mAb (**e**) in the presence of $MgCl_2$. MAdCAM-Ig–reactive cells are included within the blue box, and the percentages of reactive cells are indicated. (**f**) Binding of neutravidin-PE alone (lane 1), increasing amounts of PE-labeled SIV_{mac251} gp120 (lanes 2–4), 2 μg PE-labeled SIV_{mac251} gp120 in the presence of an unlabeled α_4 mAb (lane 5), and 2 μg PE-labeled SIV_{mac251} gp120 in the absence of divalent cations (lane 6) as controls to $CD4^+\alpha_4\beta_7^+$ T cells. (**g**) Flow-cytometric profile of $CD4^+\alpha_4\beta_7^+$ T cells stained with neutravidin-PE alone (gray) or PE-labeled SIV_{mac251} gp120 in the absence (blue) or presence (red) of unlabeled $\alpha_4\beta_7$-mAb. (**h**) $CD4^+\alpha_4\beta_7^+$ T cells stained with neutravidin-PE alone (gray) or labeled SIVmac251 gp120 in the absence (blue) or presence (red) of unlabeled MAdCAM-Ig. Experiments shown in **f**–**h** were performed in the presence of an anti-CD4 mAb.

with a persistence of infected cells at the portal of infection and a substantial decrease in SIV-infected cells within GALT (**Fig. 1d**). $\alpha_4\beta_7$-mAb–treated animals that became infected maintained higher $CD4^+$ T cell counts in blood ($P < 0.001$; **Fig. 2a**), cytobrush specimens ($P = 0.0052$; **Fig. 2b**) and gut tissues ($P < 0.0001$; **Fig. 2c**) but had no effect on other cell lineages, confirming that $\alpha_4\beta_7$-mAb treatment did not markedly alter the frequencies of major immune cell types in cervicovaginal tissues.

We next wanted to explore potential mechanisms of the protection we observed in $\alpha_4\beta_7$-mAb–treated animals. Analysis of SIV-specific humoral and cellular responses failed to show any detectable immune responses that could have contributed to the SIV infection resistance of these 6 infection-resistant animals, although samples from the SIV-infected animals analyzed in parallel showed readily detectable SIV-specific humoral and cellular responses. Thus, the observed protection was not mediated by cellular or humoral immune responses. $\alpha_4\beta_7$-mAb treatment may have reduced transmission by inhibiting $\alpha_4\beta_7$ binding to MAdCAM, thereby reducing homing of $\alpha_4\beta_7^+CD4^+$ T cells to GALT, and/or by interfering with any potential interactions between $\alpha_4\beta_7$ and SIV_{mac251} envelope protein that could mediate infection. To this end we found that $\alpha_4\beta_7$-mAb inhibits binding of $\alpha_4\beta_7$ to both MAdCAM and a gp120 derived from SIV_{mac251} (**Fig. 2d**–**g**). In addition, we determined that MAdCAM and SIV_{mac251} gp120 compete for binding to $\alpha_4\beta_7$ (**Fig. 2h**). Thus, the $\alpha_4\beta_7$-mAb possesses the capacity, in at least two ways, to interfere with intravaginal transmission of SIV_{mac251}.

The present study demonstrates that intravenous administration of $\alpha_4\beta_7$-mAb shortly before and for several weeks after multiple low-dose intravaginal SIV challenges in rhesus macaques substantially decreased the likelihood of viral transmission. Furthermore, in those treated animals that did become infected, GALT viral loads were markedly reduced. Macaques receiving $\alpha_4\beta_7$-mAb prophylactically were, on average, 63% less likely to become infected following any single intravaginal challenge than were controls. The profound destruction of gut $CD4^+$ T cells that typically occurs in acute SIV or HIV infection was prevented in treated but infected animals, which could contribute to the amelioration of the underlying causes of AIDS[1]. Our earlier studies[10,11] of $\alpha_4\beta_7$-mAb employed high dosages of SIV_{mac239} that were designed to infect all animals with a single inoculation. Under those conditions, $\alpha_4\beta_7$-mAb markedly impeded disease progression and mortality. The present study was designed to understand the role of $\alpha_4\beta_7^+$ T cells in sexual transmission by using repeated low-dose intravaginal challenges. Targeting $\alpha_4\beta_7^+$ T cells not only impeded intravaginal transmission but also reduced proviral DNA loads in GALT long after treatment was terminated, despite sustained viremia.

Treatment with $\alpha_4\beta_7$-mAb did not greatly alter the numbers of $CD4^+$ T cells within the cervicovaginal compartment, consistent with the absence of MAdCAM in the female genital tract (FGT) under normal conditions (MAdCAM is induced in the FGT by sexually transmitted diseases known to increase the susceptibility to HIV[12]). However, $\alpha_4\beta_7$-mAb masked >99.9% of the $\alpha_4\beta_7$ heterodimers on cells

in cervicovaginal compartments (**Supplementary Fig. 2c**). Masking $\alpha_4\beta_7$ in the FGT might prevent transmission by suppressing the spread of a nascent infection into the largest depot of vulnerable target T cells in the body by interfering with the physical interaction of virus with $\alpha_4\beta_7^+CD4^+$ cells, or by disrupting cell-cell interactions necessary for efficient viral transmission. In any case, these results suggest that SIVmac251 utilizes $\alpha_4\beta_7^+CD4^+$ cells at some key early point in transmission. This conclusion is further supported by the delayed infection observed in a number of treated animals. Of note, one of these animals did not become viremic until 3 weeks after challenges were discontinued. In addition, we detected proviral DNA in cervical tissues of the SIV-infected $\alpha_4\beta_7$-mAb–treated but not the control IgG–treated macaques in samples collected from these animals at autopsies performed either early or 16–18 weeks after infection (**Fig. 1i**).

Our findings are in accord with numerous studies that implicate $\alpha_4\beta_7$ integrin in promoting mucosal transmission of HIV and SIV[13–16]. The substantial decrease in the frequency of mucosal infection that we observed in $\alpha_4\beta_7$-mAb-treated animals implicates $\alpha_4\beta_7$ as a key determinant of SIV transmission. Evidence of lower tissue proviral loads and sparing of vital T cell subsets, even after treatment was discontinued, underscores the potential utility of $\alpha_4\beta_7$-directed intervention in SIV and HIV disease. Recent reports from clinical trials of new drugs targeting $\alpha_4\beta_7$ indicate their safety and efficacy in treating gastrointestinal inflammatory disorders[17–20] and raise the possibility that such therapies might prove efficacious in preventing and ameliorating HIV disease.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

The day-to-day scheduling of the experiments were carried out under the laboratory supervision of A.E.M., technically performed by S.N.B., B.K., P.D., T.V. and D.L. The experiments described in **Figure 2d–h** were performed by F.N. and J.H. The overall planning and direction of the studies was carried out by A.A.A. C.C., F.V. and P.J.S. in regularly scheduled consultation with E.N.K., J.M.M. and T.G.P. D.H. provided the statistical planning of the studies and performed the statistical analyses of the data obtained. K.A.R. consulted and provided the large-scale preparation of the recombinant $\alpha_4\beta_7$ monoclonal antibody and the normal rhesus IgG mAbs. M.B. and L.W. performed the major histocompatibility complex typing of the animals, and K.R. performed the Fc receptor typing of the animals. A.A.A. and T.G.P. prepared the draft of this manuscript with input from all the authors. A.S.F. provided helpful discussions and review and revision of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

1. Brenchley, J.M. & Douek, D.C. *Curr. Opin. HIV AIDS* **3**, 356–361 (2008).
2. Mehandru, S. *et al. J. Exp. Med.* **200**, 761–770 (2004).
3. Veazey, R.S. *et al. Science* **280**, 427–431 (1998).
4. Bargatze, R.F., Jutila, M.A. & Butcher, E.C. *Immunity* **3**, 99–108 (1995).
5. Erle, D.J. *et al. J. Immunol.* **153**, 517–528 (1994).
6. Arthos, J. *et al. Nat. Immunol.* **9**, 301–309 (2008).
7. Nakamura, G.R., Fonseca, D.P., O'Rourke, S.M., Vollrath, A.L. & Berman, P.W. *PLoS ONE* **7**, e39045 (2012).
8. Nawaz, F. *et al. PLoS Pathog.* **7**, e1001301 (2011).
9. Pereira, L.E. *et al. Cell. Immunol.* **259**, 165–176 (2009).
10. Ansari, A.A. *et al. J. Immunol.* **186**, 1044–1059 (2011).
11. Kwa, S. *et al. Blood* **118**, 2763–2773 (2011).
12. Kelly, K.A. & Rank, R.G. *Infect. Immun.* **65**, 5198–5208 (1997).
13. Cicala, C. *et al. Proc. Natl. Acad. Sci. USA* **106**, 20877–20882 (2009).
14. Kader, M. *et al. Mucosal Immunol.* **2**, 439–449 (2009).
15. Martinelli, E. *et al. J. Acquir. Immune Defic. Syndr.* **64**, 325–331 (2013).
16. McKinnon, L.R. *et al. J. Immunol.* **187**, 6032–6042 (2011).
17. Feagan, B.G. *et al. N. Engl. J. Med.* **369**, 699–710 (2013).
18. Jovani, M. & Danese, S. *Curr. Drug Targets* **14**, 1433–1443 (2013).
19. Sandborn, W.J. *et al. N. Engl. J. Med.* **369**, 711–721 (2013).
20. Danese, S. *et al. Ann. Intern. Med.* **160**, 704–711 (2014).

ONLINE METHODS

Animals. Adult female rhesus macaques (*Macaca mulatta*; range 5 to 17 years old, see **Supplementary Table 2**) of Indian origin were used for the studies reported herein. The animals were born and housed at the Yerkes National Primate Research Center (YNPRC) of Emory University (Atlanta, GA) and were maintained according to the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council and the Department of Health and Human Services guideline titled "Guide for the Care and Use of Laboratory Animals." These animals were fed monkey diet (Purina) supplemented daily with fresh fruit or vegetables and water *ad libitum*. The studies reported herein were performed under IACUC protocol #2001725 "Gut homing cells in SIV infection," which was reviewed and approved by the Emory University IACUC and the Biosafety Review Committee. The YNPRC has been fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International since 1985.

Genetic typing. PBMCs from each of the rhesus macaques were subjected to major histocompatibility complex class I typing according to protocols previously published[21,22]. In addition, each of these macaques was also typed for TRIM-5α, FcR and KIR polymorphisms using our standard laboratory protocols[21,23,24]. Results of the genotyping are provided in **Supplementary Table 1a–c**.

Study design. The overall objective of this study was to evaluate the efficacy of the $\alpha_4\beta_7$-mAb in influencing the infection susceptibility and kinetics of viremia and factors that impact disease in female rhesus macaques using a low-dose repeated-challenge model (LDRC). Even though several studies have been conducted using this model[25,26], there is still a paucity of data on the immunological composition of the female reproductive tract in nonhuman primates. The studies reported herein were therefore performed in three phases, each with a distinct objective (see **Supplementary Fig. 1**). Phase 1 was designed to optimize the procedure to be used for obtaining cytobrush specimens from the macaques and to determine the number and phenotypes of the cells from such cytobrush specimens in order to lay the foundation for studies under phases 2 and 3. Phase 2 was designed to determine whether the recombinant $\alpha_4\beta_7$-mAb administered at the doses previously used[27] reaches the vaginal tissues and to determine its pharmacokinetics. Phase 3 was the final study designed to assess the efficacy of the $\alpha_4\beta_7$-mAb to influence SIV infection using the LDRC model. Sample sizes for the phase 3 study were calculated using parameters obtained from our virus titration studies, which used six female rhesus macaques and was based upon statistical methods as outlined elsewhere[28,29]. For 80% power, a two-sided $\alpha < 0.05$, and assuming an infection probability of 0.2 among control animals (for example, 4 infections per 20 exposures), an effect size of 75%, and a susceptibility of 90–100%, it was determined that a total of 24 adult female rhesus macaques were required, 12 to receive rhesus IgG (controls) and another 12 to receive the anti-$\alpha_4\beta_7$ mAb (both the anti-a4b7 and the IgG control antibodies were obtained courtesy of the NIH Nonhuman Primate Reagent Resource, Massachusetts Biologicals, University of Massachusetts). The virus stock of SIV_{mac251} was obtained courtesy of N. Miller (NIAID, NIH). Data from the initial intravaginal titration studies suggested that we utilize 1 ml of a 1/20 dilution of the stock virus (5,000 $TCID_{50}$ containing 36 ng of p24). Following the collection of 3 pre-infusion baseline samples from each of the 24 macaques, the 12 control macaques were administered 50 mg/kg of rhesus IgG and the other 12 were administered 50 mg/kg of the $\alpha_4\beta_7$-mAb intravenously every 3 weeks (based on previous PK studies aimed at maintaining a trough plasma level of >5 μg/ml, data not shown). None of the animals were treated with Depo-Provera but were monitored for levels of progesterone and estradiol (Biomarkers Core Lab, YNPRC) in order to monitor cycling. Each of the 24 was then challenged 3 d later intravaginally with a 1/20 dilution of the stock virus in 1.0 ml of RPMI once weekly according to previously established procedures. Since infection by either the intravaginal or intrarectal route usually reveals that approximately 10% of animals are refractory to infection[30–32], it was reasoned that all 24 macaques would be exposed weekly until a majority of the control macaques (10/12) became infected. Infection was defined as plasma positive viremia at 2 consecutive weeks. Macaques were bled weekly for 6 weeks, biweekly until 12 weeks and monthly thereafter for a series of other studies as outlined below, including plasma viral load determinations. The green arrows in **Supplementary Figure 1** show weekly bleeds because the 24 macaques were divided into two groups and for practical reasons bled on alternative weeks. None of the macaques were biopsied or subjected to cervical brush sampling until they were confirmed as infected in order to avoid any trauma that might promote infection. Shortly after infection, cytobrush specimens and colorectal biopsy sampling was performed biweekly for 6 weeks and then monthly thereafter. While colorectal biopsy specimen collection included the procurement of 6–8 pinch biopsies using a standard bioptome as previously described[27], the cytobrush specimen collection procedure had to be optimized (see below). Cervicovaginal lavage (CVL) fluids from the macaques were collected as previously described[33] and aliquots kept at −80 °C until used for analyses.

Cytobrush specimen collection. The cytobrush procedure used was similar to that used for humans[34] modified and performed in consultation with R. Kaul (University of Toronto, Toronto, Canada). Briefly, a 3-ml syringe was cut transversally, the rubber plunger inserted within the barrel and the outside of the barrel coated with olive oil and introduced into the vaginal canal. The syringe plunger was then pulled out and the syringe barrel advanced gently against the cervix. A cytobrush+ (Medskin Medical, Cooper Surgical, Berlin, Germany) was then passed through the barrel of the syringe, introduced into the cervix with a rotating motion to collect cells. The cytobrush was then removed and placed in a 15-ml conical tube containing 2 ml of RPMI-1640 media containing DNase and 10 U/ml of sodium heparin and immediately placed on ice. The cytobrush was then placed successively in three tubes containing 3, 5, and 5 ml of media. Each tube was vortexed for 30 s, the cytobrush was then rinsed with 2 ml of media to remove any remaining cells and the content from all 4 tubes was pooled (15 ml) and centrifuged at 150*g*. The cell pellet was resuspended in 100 μl of media, an aliquot was used to determine cell count, and the cells were adjusted to 10,000 cells per 100 μl and subjected to flow-cytometric analyses as outlined below. Initial study of 64 such cytobrush samples from uninfected female macaques led to 41 that yielded >10,000 cells per sample (64%) that were sufficient in number to provide an initial profile of the phenotype of cells present in the cytobrush samples. The frequencies of $CD3^+$, $CD4^+$, $CD8^+$ and $NKG2a^+$ cells in the cytobrush samples were similar to those from the GIT biopsies of the corresponding animals (**Supplementary Fig. 2a**,**b**).

Viral loads. Plasma viral loads were monitored in samples every week for 4 weeks, every other week for 8 weeks and monthly thereafter, using bDNA quantitation (Siemens Inc., Berkeley, CA) on aliquots of EDTA-plasma by Siemens Inc. and expressed as number of copies/ml of plasma. Macaques were only considered infected when their plasma viral loads showed >1,000 vRNA copies/ml on 2 successive weeks, although the threshold for the assay being used was 50 vRNA copies/ml of plasma. Cellular proviral DNA loads were performed on aliquots of mononuclear cells isolated from colorectal biopsy tissue samples as described elsewhere[27]. Gastrointestinal tissue (GIT) proviral DNA loads were expressed as number of copies/ng DNA with a sensitivity of detection of 1 copy/ng DNA[27]. Plasma samples from all the macaques that were classified as negative (below level of detection) were subjected to ultrasensitive PCR analysis (detection limit 1 copy/ml) by M. Piatak (NCI, NIH, Frederick, MD) for confirmation. All samples recorded as negative were confirmed to be negative by his lab. In addition, a select number of CVL fluids collected from the macaques before the study (controls) and after confirmation of SIV infection were also subjected to RT-PCR analysis for the detection of virus. The CVL fluid (0.5 ml) was centrifuged at 450*g*, and the resulting cell pellet was used for RNA extraction and then subjected to RT-PCR using the same protocol as used for the plasma samples. The purpose was primarily to determine whether virus could be detected in such samples.

Polychromatic flow cytometric analyses. The frequencies of subsets of mononuclear cells in blood samples and cells isolated from pools of GI tissue biopsies and cytobrush specimens was determined using panels of monoclonal antibody reagents with a focus on analyses of $\alpha_4\beta_7$ expression and blocking as described elsewhere[27,35]. In brief, the PBMCs were isolated from heparinized blood, the mononuclear cells were isolated from pools of colorectal biopsies by techniques outlined elsewhere[27], and cells were obtained from cytobrush samples as

outlined above. Absolute values of each cell lineage in the blood were calculated from CBCs that were performed on an aliquot of each blood sample. A battery of rhesus macaque–reactive commercially purchased monoclonal antibody reagents conjugated with a variety of fluorochromes were used for polychromatic flow cytometric analysis of lymphoid cells using a LSR-II flow cytometer (B-D Immunocytometry Division, Mountain View, CA). Appropriate panels of the monoclonal antibodies were used to identify subsets of T cells, B cells and NK cells as described elsewhere[35]. The mAbs utilized for the studies are listed herein. The list describes the reagent, the commercial source of the reagent, the clone number and the dilution at which it was utilized. mAbs purchased from BD Biosciences (San Jose, CA) were Alexa700- and PE-Cy5-anti-CD3 (clone SP34-2, 1:100), FITC-, PE- and Pac Blue-anti-CD8 (clones SK1 and clone RPA-T8, 1:20), PerCP- and Pac Blue-anti-CD4 (clone L200, 1:20), FITC-anti-CD14 (clone M5E2, 1:20), FITC- and APC-Cy7-anti-CD16 (clone 3G8, 1:20), APC-anti-CD20 (clone 2H7, 1:20), PE-Cy7-anti-CD56 (clone NCAM16.2, 1:20), PE-Cy5-anti-CD95 (clone DX2, 1:20), FITC-anti-CD197 (clone 3D12, 1:20), FITC- and PerCP5.5-anti-HLA-DR (clone G46-6, 1:20), PE-Cy5-anti-CD45 (clone TU116, 1:20), PE-Cy5-anti-CD107a (clone LAMP-1, 1:40), FITC-anti-Lin- (cocktail), APC-anti-CD11c (clone S-HCL-3, 1:20), APC-anti-CD123 (clone 7G3, 1:20), PE-anti-CD21 (clone B-ly4, 1:20) and FITC-anti-CD27 (clone M-T271, 1:20). The mAbs purchased from Beckman Coulter (Brea, CA) were PE-anti-NKG2a (clone Z199, 1:20), PR-TR-anti-CD28 (clone CD28-2, 1:20) and PE-anti-NKp44 (clone Z23, 1:20). The mAb Alexa647-anti-IL-17A (clone eBio64DEC17, 1:20) was purchased from eBioscience (San Diego, CA), and the mAbs APC-anti-IFN-α (clone LT27:295, 1:10) and PE/APC-anti-CD25 (clone 4E3, 1:20) were purchased from Miltenyi (Auburn, CA). Select studies utilized PE-anti-Ki-67 (clone B56, 1:40) and PE-conjugated p11c-Mamu-A01*-MHC class I tetramer (1:100) (courtesy of the NIH tetramer core facility, Emory University School of Medicine, Atlanta, GA). A minimum of 40,000 events were analyzed for each cell lineage except for mDC/pDC which required a minimum of 100,000 events. The data obtained was analyzed using the FlowJo software (Treestar, Ashland, OR) software. NK cells were defined as cells that were $CD3^{-}CD8^{+}NKG2a^{+}$, which includes the four major subsets of NK cells. In the case of the cytobrush specimens, the antibody panel included Live/Dead; PE-Cy7 CD45; Alexa700 CD3; PerCP-Cy5.5 CD4; Pac Blue CD8; APC-Cy7 CD20; FITC CCR5; PE NKG2a; APC $\alpha_4\beta_7$; and PE-Cy5 α_4. Following gating on live cells, the cells were gated on $CD45^{+}$ cells (on average consisting of <10% of the cellular elements) and a minimum of 10,000 events analyzed per cytobrush specimen. Samples showing >2–3% $CD20^{+}$ B cells were excluded from analysis (reasoned to be contaminated with blood). Cells that stained positively for the anti-α_4 integrin but failed to stain with anti-$\alpha_4\beta_7$ was used as a measure of the blocking of $\alpha_4\beta_7$-expressing cells by the infused $\alpha_4\beta_7$-mAb as described previously[27].

Plasma and CVL levels of the $\alpha_4\beta_7$-mAb and anti-idiotypic antibodies. Aliquots of the plasma samples and the CVL fluid were analyzed for levels of $\alpha_4\beta_7$-mAb as described elsewhere[27]. Aliquots of the same plasma samples were also analyzed for levels of anti-idiotypic antibodies using standard ELISA.

***In vitro* and *in vivo* susceptibility to SIV infection.** PBMCs from the appropriate macaques to be tested were cultured for 2 d *in vitro* in media (RPMI 1640 medium supplemented with antibiotics, L-glutamine, 10% FCS) in the presence of anti-CD3/CD28–conjugated immunobeads + rHu-IL-2 (20 U/ml). The cells were then washed and cultured at 2×10^6/ml in either media (control) or inoculated with SIV_{mac251}, and the supernatant fluids were collected at varying time intervals up to day 14 and assayed for levels of p27, using a standard p27 ELISA. Two of the macaques originally from the control IgG group (RIq9 and RTm11) and two originally from the anti-$\alpha_4\beta_7$-mAb group (RRq9 and RVi7) that were resistant to IVAG infection were challenged intrarectally with 1 ml of the undiluted SIV_{mac251} stock virus. However, RIq9 and RTm11 were switched in this study to receive $\alpha_4\beta_7$-mAb and RRq9 and RVi7 switched to receive rhesus IgG according to the same dose and schedule as outlined under **Supplementary Figure 1** to neutralize any potential bias from the previous administration of the antibodies in the studies reported in **Fig. 1a**,**b**. Plasma samples from each of these four macaques were subsequently monitored for SIV using the same assay. Macaques were considered infected if the plasma from the monkey was positive (>10,000 viral copies/ml) at two consecutive weeks.

Necropsy studies. In efforts to determine what differences, if any, could be detected in *in vivo* organ or tissue localization of SIV, 4 animals from the control group and 4 from the group that received the $\alpha_4\beta_7$-mAb confirmed as SIV positive were subjected to necropsy (2 animals from each of the 2 groups were killed at 2 weeks post infection and data displayed in **Fig. 1** as filled circles and 2 from each of the 2 groups skilled at 16–18 weeks post infection displayed as open circles in **Fig. 1**) with 42 different tissues sampled. It is to be noted that we were only able to obtain cervical tissues from 3 of the 4 animals from each group due to technical error. Flash-frozen tissue samples were used to extract DNA, the DNA was quantitated and subjected to pro-viral DNA analysis, and the data were recorded as number of viral copies/ng DNA.

$\alpha_4\beta_7$-mAb inhibition of MAdCAM and SIVmac251 gp120. Specific inhibition of MAdCAM binding to $\alpha_4\beta_7$ by the $\alpha_4\beta_7$-mAb was carried out as follows. $CD4^{+}$ T cells were cultured in 1 μM retinoic acid (RA) to induce increased expression of $\alpha_4\beta_7$. Aliquots of 300,000 cells were stained with biotin/neutravidin-PE–labeled MAdCAM-Ig (0.5 μg) in the presence of a 5× molar excess of either an irrelevant unlabeled IgG control mAb, $\alpha_4\beta_7$-mAb or the anti-α_4 mAb clone 2B4 (R&D Systems), using a standard flow-cytometric staining protocol, with the inclusion of 1 mM $MgCl_2$/100μM $CaCl_2$ in the stain and rinse buffers. A recombinant gp120 corresponding to the sequence derived from SIV_{mac251} (GenBank accession number JQ086004) was constructed, expressed, purified and biotin labeled as described in detail in the supplementary methods of ref. 36. A total of 300,000 RA-cultured $CD4^{+}$ T cells were stained with 1–4 μg of biotin-labeled gp120 in the presence of1 mM $MnCl_2$/100μM $CaCl_2$. The α_4-mAb 2B4 was used to inhibit gp120 binding to $\alpha_4\beta_7$, and cells were stained in the absence of divalent cations as an additional way to inhibit cation-dependent binding of gp120 to $\alpha_4\beta_7$ (ref. 36). Inhibition of gp120 binding by the $\alpha_4\beta_7$-mAb to $\alpha_4\beta_7$ was demonstrated by preincubation of cells with a 5× molar excess of unlabeled $\alpha_4\beta_7$-mAb before the addition of biotin/neutravidin-PE gp120. Competition between gp120 and MAdCAM was demonstrated by simultaneously adding biotin/neutravidin-PE–labeled gp120 with unlabeled MAdCAM to $CD4^{+}$ T cells. All data were collected on a BD FACSCanto (BD Biosciences, San Jose, CA).

Statistical analyses. The Fisher's exact test (two-sided P value <0.05) was implemented to assess differences in infection probabilities of $\alpha_4\beta_7$-treated animals relative to IgG control animals. Kaplan-Meier survival curves and the log-rank test were used to plot and compare study group differences in cumulative time (# of exposures) to infection; proportional hazards regression was used to estimate the instantaneous hazard for infection. Means of subject-specific Pearson correlation statistics were computed. Mixed-effects regression models were implemented to test for group differences and trends using longitudinal, repeated measurements. Reported P values are based on two-sided testing, and a P value < 0.05 was considered statistically significant. Statistical analyses were performed using Prism GraphPad Software (version 5, CA) or SAS software, version 9.3 (SAS Institute, Cary, NC, USA). The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

21. Moreland, A.J. *et al. BMC Genomics* **12**, 295 (2011).
22. O'Leary, C.E. *et al. Immunogenetics* **61**, 689–701 (2009).
23. Lim, S.Y. *et al. PLoS Pathog.* **6**, e1000738 (2010).
24. Nguyen, D.C., Scinicariello, F. & Attanasio, R. *Immunogenetics* **63**, 351–362 (2011).
25. Butler, K. *et al. AIDS Res. Hum. Retroviruses* **29**, 1091–1094 (2013).
26. Subbarao, S. *et al. J. Med. Primatol.* **36**, 238–243 (2007).
27. Ansari, A.A. *et al. J. Immunol.* **186**, 1044–1059 (2011).
28. Hudgens, M.G. & Gilbert, P.B. *Biometrics* **65**, 1223–1232 (2009).
29. Regoes, R.R., Longini, I.M., Feinberg, M.B. & Staprans, S.I. *PLoS Med.* **2**, e249 (2005).
30. Letvin, N.L. *et al. J. Virol.* **81**, 12368–12374 (2007).
31. Promadej-Lanier, N. *et al. J. Acquir. Immune Defic. Syndr.* **53**, 574–581 (2010).
32. Veazey, R.S., Shattock, R.J., Klasse, P.J. & Moore, J.P. *Curr. HIV Res.* **10**, 79–87 (2012).
33. Spear, G., Rothaeulser, K., Fritts, L., Gillevet, P.M. & Miller, C.J. *PLoS ONE* **7**, e52992 (2012).
34. McKinnon, L.R. *et al. J. Immunol.* **187**, 6032–6042 (2011).
35. Pereira, L.E. *et al. Cell. Immunol.* **259**, 165–176 (2009).
36. Arthos, J. *et al. Nat. Immunol.* **9**, 301–309 (2008).

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