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Characterization of an Anti-lymphocyte Function-associated Antigen-1 Antibody in a Simian Immunodeficiency Virus–Pig-tailed Macaque (*Macaca nemestrina*) Model

Craig A Fletcher,[1,*] Zhaohao Liao,[1] Robert J Adams,[1] Darryl L Carter,[2] Odell D Jones,[3] Harry G Davis,[3] Joseph L Bryant,[3] and James EK Hildreth[4]

The simian immunodeficiency virus (SIV)/pig-tailed macaque (*Macaca nemestrina*) model of acquired immune deficiency syndrome (AIDS) is a powerful system in which to study cell adhesion molecules and retroviral pathogenesis in vivo. Preliminary experiments were conducted to examine the role of lymphocyte function-associated antigen 1 (LFA-1) in early SIV infection in vivo by using an LFA-1 monoclonal antibody (MHM.23) specific to human LFA-1. In vitro studies revealed that at concentrations of ≥20 μg/ml, MHM.23 blocked LFA-1-mediated adhesion and T-cell activation (>90%) of pig-tailed macaque peripheral blood mononuclear cells (PBMCs). In addition, SIVmac239 infection of macaque cells was inhibited in a dose-dependant manner by MHM.23. Administration of MHM.23 to pig-tailed macaques inhibited LFA-1–ICAM-1-mediated activity in vivo and maintained binding on macaque cells for ≤4 d. Our in vitro studies indicated that at an MHM.23 concentration of 20 μg/ml, macaque PBMCs were completely saturated. Our in vivo studies determined that 5 mg/kg MHM.23 intravenously every 24 h was required to maintain saturating levels and inhibit LFA-1–ICAM-1 function in pig-tailed macaques.

Abbreviations: AIDS, acquired immune deficiency syndrome; CCR, chemokine coreceptor; ELISA, enzyme-linked immunosorbent assay; cRPMI, complete Roswell Park Memorial Institute media; Fc, crystallization fraction; HIV, human immunodeficiency virus; ICAM-1, intracellular adhesion molecule 1; Ig, immunoglobulin; IL-2, interleukin 2; LFA-1, lymphocyte function-associated antigen 1; MCF, mean channel fluorescence; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; SIV, simian immunodeficiency virus; VCAM, vascular cell-associated adhesion molecule

Human immunodeficiency virus type 1 (HIV-1) enters susceptible cells by fusion of its envelope with the plasma membrane after binding to the CD4 molecule and interaction with a G-protein-coupled chemokine coreceptor (CCR), CXCR4 or CCR5. The identification of CCRs as coreceptors for HIV-1 clarified many questions that existed regarding the cellular tropism of this virus. It has been known for some time that retroviruses can incorporate host proteins into their membranes during the budding process. An early study from our laboratory revealed a key cellular adhesion molecule, lymphocyte function-associated antigen-1 (LFA-1), as the first molecule other than CD4 to play a direct role in HIV-1-induced syncytium formation in primary T cells.[7] Our laboratory and others have published seminal studies demonstrating the role of cell adhesion molecules, in particular LFA-1, in the biology of HIV and established the role of these proteins in HIV binding, infectivity, and resistance to neutralization by anti-viral antibodies.[4,8]

LFA-1 plays a key role in initiating the immune response by providing adhesion in the formation of the immunologic synapse via binding to its ligand, intracellular adhesion molecule (ICAM)-1, on leukocytes.[19] LFA-1 on HIV-1 could potentially target virus particles to sites of immune activation; this action would allow HIV-1 to interact with susceptible cells of the immune system. This 'virological synapse' in which CD4, chemokine receptors, and cell adhesion molecules aggregate within a localized patch on the cell membrane may indeed facilitate HIV transmission.[12] SIV appears to be minimally pathogenic in its natural hosts, the African green monkey and sooty mangabey, but infection progresses to cause an AIDS-like disease when inoculated into Asian-origin macaques. Macaques infected by certain strains of SIV develop a spectrum of clinical symptoms that closely resembles AIDS in humans, including CD4 cell depletion and central nervous system diseases.[8] Cell adhesion molecules may contribute significantly to the pathogenesis of HIV-1 infection and AIDS, and the true effects of these molecules in AIDS pathogenesis are best evaluated through in vivo studies. SIVmac or SIVsm (macaque or sooty mangabey strains, respectively) infection of asian macaques is an excellent model for this purpose. The chemokine receptor CCR5 is the major coreceptor for HIV and SIV; however, CXCR4-using HIV-1 variants evolve in up to 50% of AIDS patients. However, many HIV-2 and SIVmac isolates (i.e. 316, 1101, 17E-Fr) can infect cells without CD4 at least to some extent, whereas HIV-1 isolates are predominantly CD4-dependent.[5]

The most common mode of transmission of HIV-1 throughout the world is heterosexual intercourse. Male-to-female transmis-

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[1]Department of Comparative Medicine, Johns Hopkins School of Medicine, Baltimore, Maryland; [2]Nora LLC, Baltimore, Maryland; [3]Division of Animal Models, Institute of Human Virology, Baltimore, Maryland; [4]Meharry Medical College, Comprehensive Center for Health Disparities Research in HIV, Nashville, Tennessee.
[*]Corresponding author. Email: cfletch@jhmi.edu

sion primarily occurs across the barrier of the vaginal epithelium. The precise mechanism by which HIV-1 achieves the initial breach of the pseudostratified epithelial mucosa during male-to-female transmission remains unknown. However, elegant studies performed in the SIV–macaque model have provided convincing evidence that dendritic cells and Langerhans cells of the vagina, especially those within the epithelium, are the initial targets of SIV infection.[10,15] It is also likely that the lateral transmission of HIV-1 from dendritic cells to T cells is mediated by LFA-1, because this adhesion molecule is required for formation of the immunological synapse between T cells and dendritic cells. In addition, studies indicate that aberrant homing of infected lymphocytes to peripheral lymph nodes (PLN) may be an important component of AIDS pathogenesis.[13] Because LFA-1 is required for T cell homing to lymphoid tissue and T cell activation, blocking the function of this cell adhesion molecule during the early stages of infection likely will have a profound effect on the establishment of infection by HIV and SIV transmission through mucosal routes.

Therefore, we propose to inhibit the function of LFA-1 in vivo by administration of a function-blocking monoclonal antibody (mAb). One of the main limitations to studying the role of adhesion molecules in the SIV–macaque system is the relative lack of mAbs specific for pig-tailed macaque (*Macaca nemestrina*) adhesion molecules. Efforts have been focused on producing and characterizing a suitable monoclonal antibody against pig-tailed macaque LFA-1 and establishing conditions for in vivo administration. This preliminary work focuses on characterizing the ability of an LFA-1 mAb previously produced in our laboratory to affect and subsequently inhibit pig-tailed macaque adhesion molecule function. Our in vitro studies demonstrate that anti-human LFA-1 mAb MHM.23 can bind and block function of pig-tailed macaques LFA-1 and is the best choice for future in vivo macaque studies. According to our initial pharmacokinetic studies, MHM.23 dosing was well-tolerated and a 5-mg/kg intravenous dose every 24 h is needed to maintain saturation and inhibit macaque LFA-1 function in vivo.

Materials and Methods

Animals and virus. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee at the Johns Hopkins School of Medicine, a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. Two captive-bred male pig-tailed macaques (animals PT650 and PT676; ages, 11 and 9 y) of similar weight were used in the dosing studies. The macaques received monkey chow (LabDiet 5038, PMI Nutrition International, St Louis, MO) along with daily food treats for environmental enrichment. Both macaques were seronegative for simian type D retrovirus, simian T lymphocyte-associated virus 1, and SIV. Ketamine hydrochloride (approximately 10 mg/kg body weight) was administered intramuscularly to facilitate venopuncture. The animals received 5 mg/kg of purified mAb in phosphate-buffered saline (PBS) by slow-push bolus (30 s). For the following described studies, venous blood (5 ml) was collected and processed appropriately at 1, 5, 24, 48, 96, and 168 h after mAb injection. White blood cell counts were measured using a hematology analyzer (CellDyne, Abbott Laboratories, Abbott Park, IL). Absolute leukocyte counts and other standard hematologic parameters (for example, red blood cell count, platelets) also were determined.

All SIV experiments were conducted using SIVmac239 (GenBank accession number, M33262).

Antibodies. The murine mAb MHM.23 (an immunoglobin [Ig] G_1 kappa) anti-human LFA-1β used in this study was produced in our laboratory as previously described.[3] All hydridomas were maintained in 10% HY medium (Life Technologies, Grand Island, NY). IgG was purified from hybridoma culture supernatants or ascitic fluid by protein G affinity chromatography as previously reported.[5,6]

Cells. Our studies used peripheral blood buffy coats as a source of peripheral blood mononuclear cells (PBMCs) for in vitro studies. Blood was obtained from healthy volunteers who had been screened for transmitted pathogens at the Johns Hopkins University Hemapheresis Laboratory. Because there were no identifiers on the samples provided, we could not determine the gender, age, or race of the donors; therefore our protocol was approved by the Johns Hopkins Clinical Investigation Review Board. For all experiments described, PBMCs were isolated from buffy coats from normal pig-tailed macaques or human blood by centrifugation on Ficoll–Hypaque gradient (Pharmacia Biotech AB, Uppsala, Sweden) as described previously.[1] The cells were used 3 d after stimulation with 3 μg/ml phytohemagglutinin (PHA; Sigma, St Louis, MO) and maintained in complete Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies) supplemented with 10 mM N-2-hydroxyethylpiperazine-N -2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, and 10% fetal calf serum (all from Life Technologies) and interleukin 2 (IL-2; 50 U/ml, Boehringer Mannheim Biochemicals, Indianapolis, IN) for ≤14 d.

Flow cytometry. Macaque and human PHA-stimulated buffy coat cells were washed and resuspended at 2×10^6 cells/ml in PBS containing 10% normal goat serum. We mixed 100 μl cells with 100 μl purified MHM.23 (20 μg/ml) and incubated 45 min on ice. The cells were washed with PBS twice and stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (20 μg/ml, Jackson ImmunoResearch Laboratories, West Groove, PA) in PBS containing 10% normal goat serum. Cells then were washed and fixed with 4% paraformaldehyde in PBS and analyzed on a flow cytometer (Epics XL, Beckman Coulter, Miami, FL). Mouse isotype-matched immunoglobulins (Jackson ImmunoResearch) were used as a negative control for nonspecific binding.

Cell adhesion function. The cell adhesion function of cells from treated animals were tested in plate-based adhesion assays using vascular cell-associated adhesion molecule (VCAM)-Ig and ICAM-Ig as substrates.[14] Briefly, immunoadhesion constructs were created by using cDNA sequences containing the 2 N-terminal Ig domains of ICAM-1 and the 3 N-terminal domains of VCAM-1, which were subcloned into a human IgG crystalline fraction (Fc)-specific fusion protein vector (pIg Plus, Ingenius, Abingdon, UK) by polymerase chain reaction amplification with primers containing the appropriate engineered restriction sites. We coated 96-well enzyme-linked immunosorbent assay (ELISA) plates (Costar, Cambridge, MA) with Fc fragment-specific goat anti-human IgG (Jackson ImmunoResearch Laboratories) at 4 °C overnight. Supernatants were removed, and the plates were blocked by adding 3% heat-inactivated bovine serum albumin in PBS for 1 h at 37 °C. After washing, 100-μl aliquots of ICAM-Ig or VCAM-Ig hybridoma supernatant were added to each well for 1 h at 37 °C. The wells were then washed with cRPMI before adding 100 μl PHA-stimulated buffy coat cells (2×10^6 cells/ml) previously labeled with horseradish peroxidase (Sigma) and incubated with or without MHM.23 (or mouse isotype-matched IgG) on ice for 45 min. Aliquots of 100 μl cells were resuspended to 2.5 $\times 10^6$/ml in RPMI containing 10% normal goat serum and incu-

bated at 37 °C, 5% CO_2 for 30 min. The wells were then washed with warm Hanks buffered salt solution–10 mM N-2-hydroxyethylpiperazine-N -2-ethanesulfonic acid to remove unbound cells. Tetramethylbenzidene substrate (Sigma) with Triton X-100 (Sigma) was added to wells, which were evaluated with a plate reader (Microplate Reader 3550, BioRad, Hercules, CA) at 450 nm. For ex vivo examination of macaque LFA-1-mediated adhesion after MHM.23 treatment, whole blood was collected, and cells were isolated on Ficoll–Hypaque and tested immediately in the assays.

PHA proliferation assay. Macaque and human PBMCs were cultured in triplicate in 96-well round-bottom microculture plates. PBMCs (2×10^5) in 50 µl cRPMI were mixed with equal volume of MHM.23 mAb at various concentrations in cRPMI. After 30 min incubation at 25 °C, 50 µl cRPMI containing PHA was added for a final PHA concentration of 3 µg/ml. Cells were incubated for 3 d at 37 °C in a CO_2 incubator. Proliferation was measured by uptake of 1 µCi/well of [^{3}H]-thymidine during the final 18 h before termination of the incubation. Levels of thymidine incorporation were determined by liquid scintillation (TopCount NXT, PerkinElmer, Meriden, CT).

Infectivity assay. Day 3 PHA blasts (1×10^6 cells/ml aliquots) were incubated with SIVmac239 (10 ng of p27) for 24 h. After incubation, the cells were washed twice with RPMI to remove input virus and resuspended in cRPMI supplemented with IL-2 and incubated with or without MHM.23 (or mouse IgG) for 6 d at 37 °C, 5% CO_2. SIV replication then was determined by using an ELISA to measure p27 viral capsid antigen in the culture supernatant.[1]

In vivo cell-bound mAb. Whole-blood samples (200 µl aliquots) were incubated with PBS or excess free mAb (10 µg) in PBS for 30 min to determine the percentage of cells with bound mAb and the level of saturation. After treatment with PharmLyze (BD Pharmingen, San Diego, CA), PBMCs were pelleted, washed once with PBS, and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 30 min. For controls, untreated macaque cells were mixed with 100 µl purified MHM.23 IgG1 (20 µg/ml) or mouse isotype-matched immunoglobulins (used to quantitate nonspecific binding of antibodies). Cells then were fixed with 4% paraformaldehyde in PBS and analyzed on a flow cytometer (Epics XL, Beckman Coulter). Nonviable cells were excluded from analysis of 10,000 cells. MHM.23 cell binding was expressed as percentage positive and mean channel fluorescence.

Plasma mAb concentration. The concentration of mAb in the plasma of treated macaques was determined by microfuging a small amount of anticoagulated blood and testing dilutions of plasma in a plate-based capture ELISA (modified from 1 of our previous studies).[12] Goat anti-mouse IgG (Fc specific) was used for capture, and horseradish peroxidase-conjugated goat anti-mouse IgG (heavy and light chains; Jackson ImmunoResearch Laboratories) was used for detection. Standard curves were generated with mouse IgG and MHM.23 IgG titration to calculate plasma mAb concentrations.

Statistical analysis. Significant differences included the mean ± 1 standard deviation of the population, one-way analysis of variance (ANOVA), Welch ANOVA, and two-tailed Student *t* tests for parametric data. Experiments were done in triplicate unless stated otherwise, and the threshold for statistical significance was defined as $P = 0.05$. Thereafter, post hoc analysis (Tukey–Kramer test for paired comparisons) was used to identify significant differences among the groups. Data analysis was performed using the software package JMP-IN (version 4.04, academic license, SAS Institute, Cary, NC).

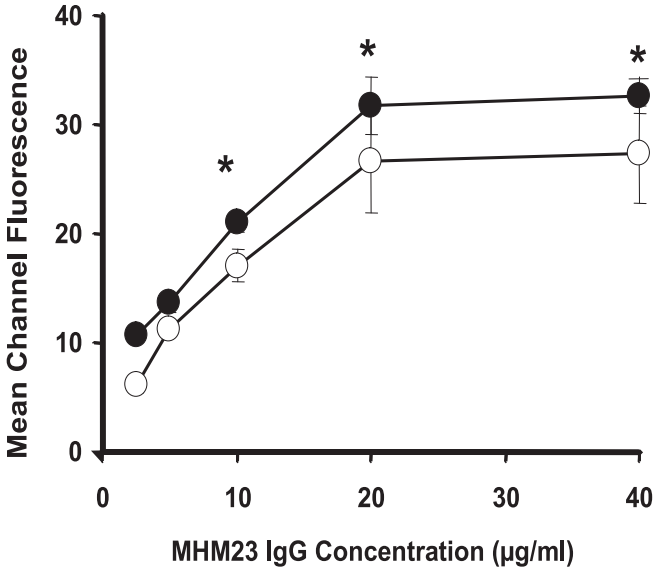

Figure 1. Binding of an anti-LFA-1 monoclonal antibody (MHM.23) to pig-tailed macaque cells. A binding curve was generated to demonstrate MHM.23 affinity to pig-tailed macaque LFA-1 using unstimulated PBMCs in flow cytometric analysis. The cells were incubated with MHM.23 and then stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (20 µg/ml). Nonviable cells were excluded from analysis of 10,000 cells. Nonspecific binding by an isotype-matched murine myeloma IgG was subtracted from the values shown. Channel fluorescence data (mean ± 1 standard deviation, n = 3) of human (▨) and macaque (▩) lymphocytes are shown. *, $P < 0.001$ compared with value for mouse isotype-matched control IgG at 40 µg/ml.

Results

Binding of anti-LFA-1 mAb (MHM.23) to pig-tailed macaque cells. There is a lack of commercially available mAbs designed specifically to recognize pig-tailed macaque surface antigens, especially adhesion molecules. Previous studies from our laboratory and others revealed that VLA-4 and LFA-1 are present on macaque cells and virus particles derived from infected cells.[14,16] We examined whether a monoclonal antibody to the β subunit of human LFA-1, MHM.23, could bind to LFA-1 on PBMCs isolated from pig-tailed macaques. Antibody cross-reactivity was assessed by flow cytometry of unstimulated pig-tailed macaque cells. The mean channel fluorescence (MCF) data shown in Figure 1 demonstrate that MHM.23, which was originally generated against human LFA-1, also recognized pig-tailed macaque LFA-1. This experiment was done 3 times in triplicate with various macaques' PBMCs. The MHM.23 binding curve revealed that saturation of macaque and human PBMCs was achievable at a concentration of approximately 20 µg/ml ($P < 0.001$ versus mouse isotype-matched control at 40 µg/ml).

MHM.23 blocks LFA-1-mediated adhesion of macaque PBMCs. To determine whether MHM.23 blocks macaque PBMC LFA-1–ICAM-1 mediated adhesion to prevent leukocyte adhesion and migration, a plate-based cell adhesion assay was conducted. Macaque PBMCs were incubated with MHM.23 IgG or mouse IgG and then plated in wells coated with ICAM-Ig or VCAM-Ig (negative control) substrates to allow for integrin-mediated adhesion. As expected, control antibody did not block the binding of macaque PBMCs to ICAM-1 immunoadhesion substrate (Figure 2). In contrast, MHM.23 specifically inhibited LFA-1-mediated

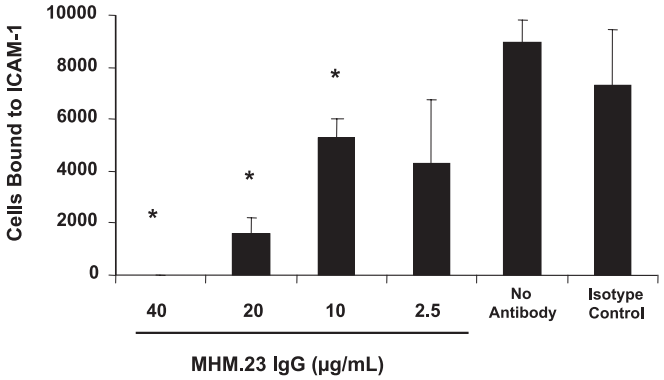

Figure 2. MHM.23 blocks LFA-1-mediated adhesion of macaque PBMCs. To determine whether MHM.23 could specifically block macaque PBMC LFA-1–ICAM-1 mediated adhesion, a cell-based assay was conducted, in which 96-well plates were coated with ICAM-Ig and VCAM-Ig (not shown) and used in cell-capture studies with macaque PBMCs. Cells were labeled with horseradish peroxidase and incubated with or without MHM.23 and mouse isotype-matched IgG before their addition to the wells. The wells then were washed gently to remove unbound cells. Numbers of adherent cells (mean ± 1 standard deviation, n = 3) were determined by addition of Triton X-100 with tetramethylbenzidene substrate and evaluation with a plate reader. *, $P < 0.001$ (F = 626.4298) compared with value for mouse isotype-matched control at the same concentration (data not shown).

cell adhesion of pig-tailed macaque PBMCs in a dose-dependent manner (Figure 2). Adhesion of macaque PBMCs to ICAM-1 was completely blocked at the 40-µg/ml dose of MHM.23. Binding of macaque PBMCs to VCAM-Ig was not inhibited by MHM.23 therefore showing that cell adhesion mediated by LFA-1–ICAM-1 was specifically abrogated (data not shown). This experiment was done 3 times in triplicate with different macaques' PBMCs. The difference between the effects of MHM.23 and the isotype-matched control on binding of macaque PBMCs to ICAM-1 was statistically significant ($P < 0.001$, F = 626.4298, Welch ANOVA).

MHM.23 inhibits PHA-induced proliferation of macaque PBMCs. To determine whether MHM.23 blocks pig-tailed macaque T cell activation by inhibiting LFA-1 function, a PHA stimulation assay was performed. Mitogenic proliferation was measured by labeling T cells with [^{3}H]-thymidine beginning 18 h prior to termination of the incubation. Thymidine incorporation subsequently was determined by liquid scintillation counting. The activation-induced proliferation of both human and pig-tailed macaque PBMC was markedly inhibited (>90%) by MHM.23 at concentrations of ≥20 µg/ml. In contrast, mouse IgG at comparable concentrations did not inhibit T cell proliferation at comparable concentrations (data not shown). This experiment was done in triplicate using various macaques' PBMCs. One-way analysis of variance and post hoc testing (Tukey–Kramer paired comparisons) indicated the statistical significance ($P < 0.001$) of the difference between MHM.23 and the mouse isotype-matched control at each concentration.

SIVmac239 replication is inhibited by MHM.23 (anti-LFA-1) in vitro. Functional host-derived integrins may affect the tropism and infectivity of HIV-1. Virus-associated proteins may facilitate HIV-1 infection of cells that express the appropriate ligands. This effect occurs with virus-associated HLA-DR1 binding to CD4 on target cells and with virus-associated ICAM-1 on target cells.[7] Conceivably, binding of LFA-1 on HIV-1 particles to ICAM-1 on target cells also may facilitate infection. To determine whether MHM.23 neutralizes SIVmac239 infection of macaque cells, in-

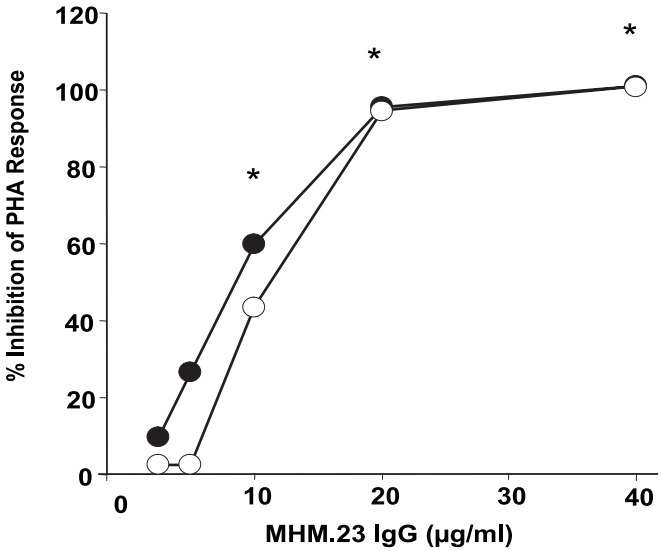

Figure 3. MHM.23 (anti-LFA-1 mAb) inhibits PHA-stimulated proliferation of pig-tailed macaque PBMCs. To determine whether MHM.23 could block pig-tailed macaque T cell activation by inhibiting LFA-1 function, a PHA stimulation assay was performed. Pig-tailed macaque (☒) and human (▩) PBMCs (2×10^5) were cultured with equal volume of MHM.23 at various concentrations. Cells were incubated for 3 d, and proliferation was measured by uptake of 1 µCi/well of [^{3}H]-thymidine during the final 18 h prior to termination of the incubation. The levels (mean ± 1 standard deviation, n = 3) of thymidine incorporation were determined by liquid scintillation counting. *, $P < 0.001$ versus value for mouse isotype-matched control IgG at the same concentration (data not shown).

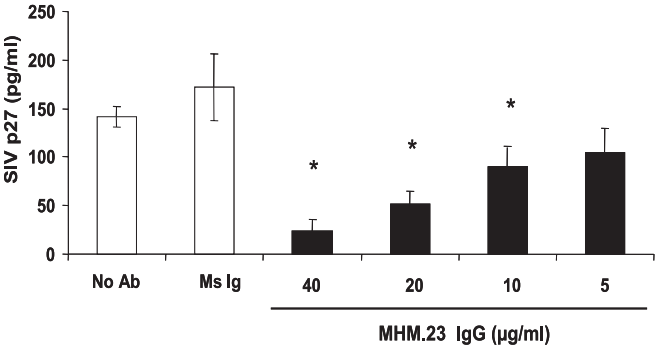

Figure 4. SIVmac239 replication is inhibited by MHM.23 in vitro. Cells were cultured in cRPMI + IL-2 with or without SIVmac239 (10 ng p27) for 24 h. Cells were washed to remove input virus, resuspended in cRPMI + IL-2 with or without MHM.23 and mouse isotype-matched control IgG, and incubated for 6 d. SIV replication was determined by using an ELISA to measure p27 (mean ± 1 standard deviation, n = 3) in the culture supernatant. *, $P < 0.001$ versus value for mouse isotype-matched control IgG at 40 µg/ml (data not shown).

fected PBMCs were incubated with various concentrations of MHM.23 or mouse isotype-matched IgG. Viral replication was monitored by measuring p27 in the culture supernatant.

MHM.23 inhibited SIVmac239 infection in a dose dependent manner compared to the mouse isotype-matched control (Figure 4). The difference was statistically significant ($P < 0.001$ versus mouse isotype-matched IgG at 40 µg/ml, one-way ANOVA) for MHM.23 at concentrations of ≥10 µg/ml. This experiment was done 3 times in triplicate using various macaques' PBMCs.

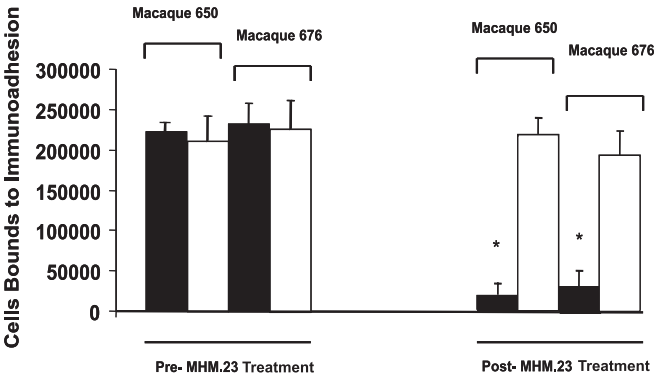

Figure 5. MHM.23 blocks pig-tailed macaque LFA-1 in vivo. Pig-tailed macaques 676 and 650 each received 5 mg/kg purified MHM.23 in PBS intravenously. Venous blood was collected before and 5 h after MHM.23 treatment and used in cell adhesion capture studies. Macaque PBMCs were labeled with horseradish peroxidase and added to 96-well plates coated with ICAM-Ig (☒) or VCAM-Ig (☒). The wells were washed gently to remove unbound cells, Triton X-100 with tetramethylbenzidene substrate was added to wells, and the number of bound cells (mean ± 1 standard deviation; each assay contained replicate wells, and assays were run 3 times) was evaluated using a plate reader at 450 nM. Data shown represents bound cells in replicate wells. *, $P < 0.001$ between pre- and post-MHM.23 treatment values.

MHM.23 (anti-LFA-1 MAb) blocks pig-tailed macaque LFA-1 in vivo. Cell adhesion molecules, especially the integrin LFA-1, have the potential to be key players in the early stages of establishing HIV and SIV transmission through mucosal routes. Our in vitro studies on macaque LFA-1 function and SIV neutralization indicated that the anti-LFA-1 mAb MHM.23 warranted further evaluation to determine whether it mediated functional inhibition in vivo. Venous blood collected from 2 macaques before and after administration of 5 mg/kg purified MHM.23 was analyzed. All mAb treatments were well tolerated by macaques. MHM.23 caused a 2- to 3-fold elevation in the white blood cell count by 24 h and maintained these levels for 4 d (data not shown). We considered that MHM.23 causes demargination of leukocytes by blocking LFA-1–ICAM-1 ligation, and these data are a strong indication of the inhibitory effects of MHM.23 in vivo. To confirm this hypothesis, cells from MHM.23-treated macaques were examined ex vivo for the ability to bind to ICAM-Ig substrates. Cells from treated animals were unable to bind ICAM-Ig via LFA-1 mediated adhesion due to saturation of surface LFA-1 by the administered MHM.23 (Figure 5); the differences between pre-MHM.23 treatment and post-MHM.23 treatment were statistically significant ($P < 0.001$, two-tailed Student t test). In addition, as expected, very late antigen-4-mediated adhesion to VCAM-1 was uninhibited, indicating the specificity of MHM.23 in the abrogation of LFA-1 binding in vivo.

By 1 h post-treatment, 99% of monocytes were positive for MHM.23 binding and remained so through 96 h (Figure 6B). However, lymphocytes were >90% positive by 1 h post-treatment but rapidly declined in staining intensity to 6% to 12% by 96 h (background staining using mouse isotype-matched control IgG was 0.36% for lymphocytes and 0.66% for monocytes). MHM.23 demonstrated 2 distinct pharmacokinetic profiles, a rapid elimination phase and a delayed elimination phase (Figure 6A). The rapid elimination phase occurred within 24 h of administration. Saturation of binding sites as demonstrated by MCF (monocytes,

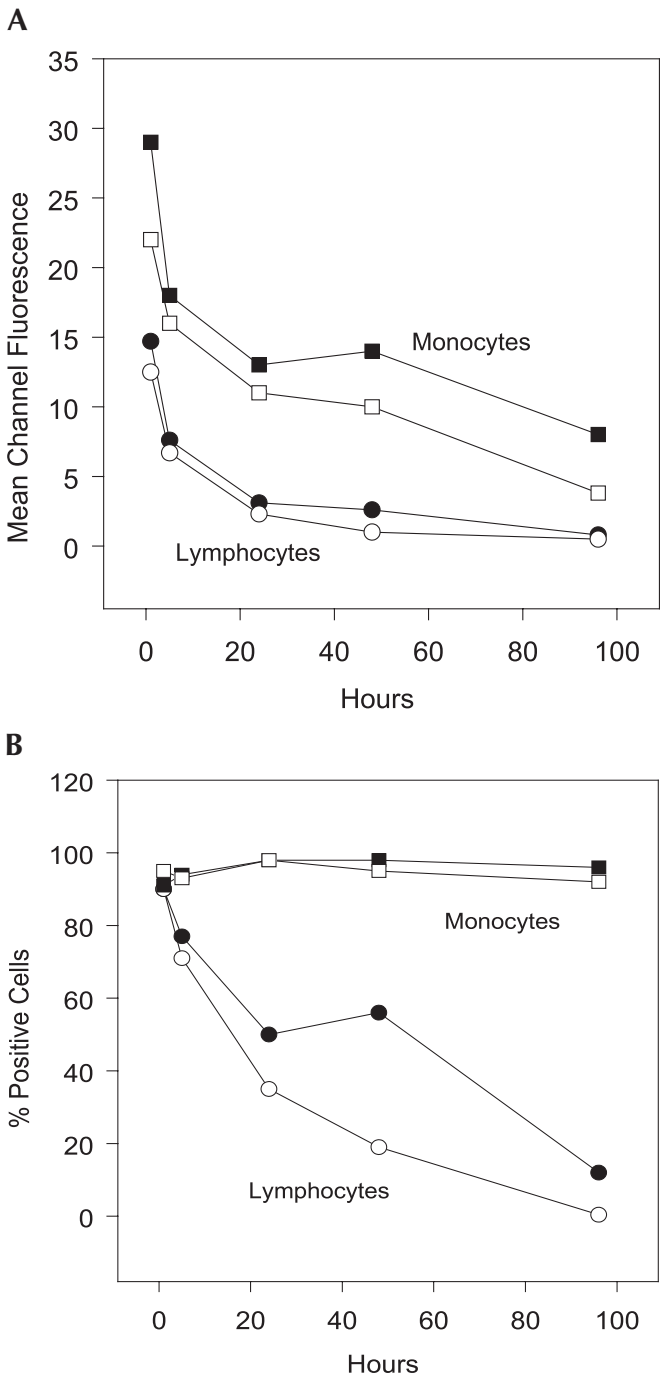

Figure 6. Binding of MHM.23 in pig-tailed macaques in vivo. Pig-tailed macaques 676 (☒) and 650 (☒) were injected intravenously with 5 mg/kg purified MHM.23. Blood was taken at 1, 5, 24, 48, and 96 h time points after administration. Antibody bound to leukocytes was detected by whole-blood flow cytometry using fluorescein isothiocyanate-conjugated goat anti-mouse IgG as a secondary antibody. (A) mean channel florescence staining and (B) percentage of positive staining and for MHM.23 binding are shown.

26; lymphocytes, 14) revealed that MHM.23 binding had declined rapidly during the first 24 h but plateaued at near-saturating levels for the monocytes for approximately 4 d (MCF background staining by using mouse isotype-matched control IgG was 0.3 for

monocytes and 0.4 for lymphocytes). MCF for lymphocytes was significantly less ($P < 0.05$) than that for monocytes, and this difference was probably due to there being decreased levels of surface LFA-1 present on T cells compared with monocytes. Despite the significant difference between MCF values, ex vivo examination revealed that LFA-1–ICAM-1 adhesion was inhibited in both populations (Figure 5).

Quantification of MHM.23 in macaque plasma after administration of 5-mg/kg intravenous dose. To quantify the actual concentration of MHM.23 in the plasma of treated macaques, a small amount of heparinized blood was microfuged, and various dilutions of plasma were tested in a plate-based capture ELISA. Goat anti-mouse IgG (Fc specific) and horseradish peroxidase-conjugated goat anti-mouse IgG were used for capture and detection, respectively. Standard curves were generated using an isotype-matched mouse myeloma and MHM.23, to calculate plasma mAb concentrations.

Our previous in vitro data showed that 20 µg/ml was needed to achieve saturation of LFA-1 binding sites on the surface of macaque cells. Ex vivo examination of PBMCs and plasma from treated macaques confirmed that saturation of LFA-1 binding sites was achieved after a single dose of MHM.23 IV (5 mg/ml). We calculated that plasma concentrations of MHM.23 were >20 µg/ml for approximately 24 h (Figure 7). Flow cytometric evaluation of ex vivo PBMCs revealed a direct correlation between MHM.23 plasma concentration levels (Figure 7) and cell saturation (Figure 6A). As stated previously the post-MHM.23 treatment MCF for lymphocytes declined rapidly when compared with that of monocytes. This difference may indicate down-modulation of surface LFA-1, which has been reported to occur in patients receiving therapeutic monoclonal antibody administration.[4] Compared with direct steric hindrance of LFA-1–ICAM-1 binding by MHM.23, receptor-mediated down-modulation of surface LFA-1 would achieve the same desired result, as was confirmed by the previously described cell-based adhesion assays (Figure 5). According to data collected from this dosing study, a 5-mg/kg intravenous dose every 24 h is needed to maintain saturation of LFA-1 in vivo and block LFA-1 function. These studies show that MHM.23 demonstrates a favorable pharmacokinetic profile that allows us to minimize dosing intervals while maintaining antigen saturation in vivo. This characteristic of MHM.23 may be particularly important in reducing the risk of macaque anti-mouse reaction and minimizing repeated sedation and stressful manipulation of the macaques when administering antibody.

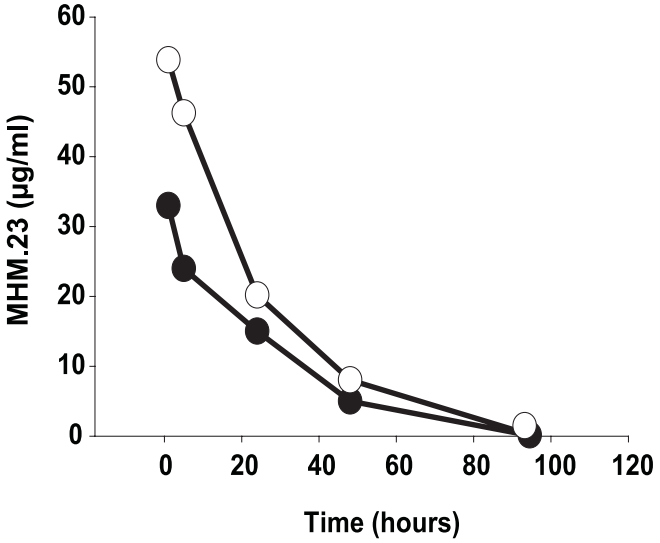

Figure 7. Quantification of MHM.23 in macaque plasma after administration of single 5-mg/kg intravenous dose. After administration of MHM.23 (5 mg/kg), plasma was isolated from pig-tailed macaques 650 (⊠) and 676 (⊠) to assay mAb concentrations. The concentration was determined by microfuging a small amount of anti-coagulated blood and testing dilutions of plasma in a plate-based capture ELISA. Goat anti-mouse IgG (Fc specific) was used for capture and horseradish peroxidase-conjugated goat anti-mouse IgG (heavy and light chains) was used for detection. Standard curves (not shown) were generated with a mouse isotype-matched IgG and MHM.23 and were used to calculate plasma concentrations.

Discussion

Compared with other steps in the virus life cycle, HIV-1 attachment and transmission is poorly understood. Previous in vitro studies have shown that adhesion molecules on HIV-1 particles contribute significantly to virus attachment, thereby providing insight into the physiologic significance of cell adhesion molecules, in particular LFA-1, in HIV-1 biology.[4,8,9] The preliminary experiments we report here lay the groundwork for directly testing the role of LFA-1 in virus transmission in vivo, and our efforts have been focused on producing and characterizing a suitable monoclonal antibody against pig-tailed macaque LFA-1 and establishing conditions for administering the antibody.

Our results indicate that the MHM.23 mAb generated against human LFA-1 can bind and inhibit pig-tailed macaque T cell function. MHM.23 effectively saturated LFA-1 on macaque PBMCs at concentrations similar to those needed to saturate LFA-1 on human PBMCs. This finding suggests that MHM.23 binds to pig-tailed macaque LFA-1 with an affinity similar to that of its binding to human LFA-1. In binding studies, the binding of MHM.23 to human LFA-1 was identical to that of the positive control mAb, MHM.5, to its ligand, major histocompatibility complex type 1 (data not shown). Compared with human T cells, unstimulated macaque T cells appear to have less LFA-1 on their surface, allowing for saturation of sites at lower doses of MHM.23. In cell adhesion and T cell proliferation assays with pig-tailed macaque PBMCs, MHM.23 demonstrated 80% to 100% inhibition of binding and proliferation at 20 to 40 µg/ml doses, respectively. These results are comparable to those of human PBMC studies with MHM.23.

Retroviral binding has been shown to be rapid, saturable, and temperature-dependent and to correlate with infectivity, implying that specific receptor usage is manifested at the attachment level.[18] Therefore, virus-associated cell adhesion molecules (for example, LFA-1, ICAM-1) may contribute toward determination of virus tropism. Neutralizing mAbs may interfere with the interaction between monomeric gp120 and $CCR5^+$ or $CXCR4^+$ $CD4^+$ T cells. We show here that MHM.23 blocked SIVmac239 infection of activated macaque PBMCs in a dose-dependent manner. The SIV we used in these experiments was produced in CEMx174 cells, and we have shown that SIV produced in these cells do in fact bear LFA-1.[2]

A great deal of HIV-1 and SIV infection and replication occurs in the gastrointestinal lymphoid tissue.[2,18] This lymphoid microenvironment may protect cells from undergoing cell death via integrin-mediated rescue from apoptosis (anoikis). Also upon viral entry, virion-incorporated cell adhesion molecule ligation is a strong possibility in preventing viral-induced apoptosis during docking and fusion. We hypothesize that modulation of integrin

function with anti-LFA mAb in vivo may decrease viral burden as well as seeding of peripheral lymph nodes by infected cells by inducing apoptosis of circulating HIV-infected cells. The in vitro data reported in this manuscript confirms that MHM.23 at 20 μg/ml was needed to achieve saturation of LFA-1 binding sites on the surface of pig-tailed macaque cells and was the best choice for further macaque pharmacokinetic studies. Ex vivo examination of PBMCs and plasma from treated macaques confirmed that we achieved saturation of LFA-1 binding sites after administration of a single intravenous dose of MHM.23.

Repeated administration of mouse monoclonal antibodies is known to provoke an anti-mouse immune response. Strategies have been developed to avoid, mask, or redirect this unwanted immune response; these strategies include making 'chimeric' antibodies by fusion of mouse variable regions to donor constant regions, 'de-immunization' by removal of T cell-stimulating epitopes, and 'humanization' by grafting mouse surface residues onto donor–acceptor antibody frameworks. Our dosing studies were 1 wk in duration (compared with the approximately 2-3 wk duration needed for an anti-mouse Ig response, thus limiting the macaque anti-mouse antibody response associated with recurrent antibody dosing.[17]

Compared with whole antibodies, the use of smaller antibody molecules such as F_{ab} fragments or microbial expression of single-chain variable domains (V_H and V_L) exhibits better pharmacokinetics for tissue penetration as well as provides full binding specificity, thus requiring less manipulation of the animals because of increased dosing intervals. However, native immunoglobulins provide high specificity and high affinity due to their multivalency and thus demonstrate better on-rates and target retention times than do monovalent recombinant antibody fragments. Alternatively, the half-life of antibodies can be extended by linkage to molecules that modify their pharmacokinetics, such as by fusion to polyethylene glycol (PEGylation) or lipids.

In light of several published studies on macaques and our participation in pig and dog studies, we expected the optimal dose of MHM.23 to be between 2 and 10 mg/kg, and therefore we chose 5 mg/kg for our dosing studies. Two macaques were used in the dosing studies reported here and a 5-mg/kg dose of purified MHM.23 was administered intravenously to each. The mAb treatments were well tolerated by the macaques. MHM.23 caused a significant leukocytosis by 24 h, which remained at these levels for 4 d before returning to predosing levels. The elevation of the white blood cell count was mostly due to severe neutrophilia caused by cell demargination via inhibition of LFA-1–ICAM-1 binding. This neutrophilia was an indication that MHM.23 blocked LFA-1 function in vivo.

In summary, our data show that an anti-LFA-1 mAb (MHM.23) alters (neutralizes) infectivity or replication of SIV in vitro. Experiments are being conducted to determine the postentry mechanisms involved in decreasing virus transmission. The inhibition of viral fusion with coreceptor substrate has been explored, with mixed results. Given that HIV fusion occurs through a number of critical steps, the potential for synergy between agents that inhibit HIV fusion exists. Direct cell-to-cell transfer is an efficient mechanism of viral dissemination within an infected host, and HIV-1 can exploit this mode of spread. There is recent evidence that HIV can drive cell-to-cell synapse formation, and LFA-1 is critical in the formation of this synapse.[11, 12] We hypothesize that modulation of integrin function with anti-LFA mAb may decrease cell-to-cell transmission of HIV. We suggest that HIV-1, and possibly human T lymphocyte-associated virus 1, use a combination of interactions between virus receptors and cognate immune receptors to activate and regulate cell-cell movement of virus. Therefore, blockade of LFA-1 may represent a novel therapeutic approach against primate lentivirus infection.

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