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CCR5 blockade is well tolerated and induces changes in the tissue distribution of CCR5+ and CD25+ T-cells in healthy, SIV-uninfected rhesus macaques

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Abstract

Background—CCR5 is a main co-receptor for HIV, but also homes lymphocytes to sites of inflammation. We hypothesized that inhibition of CCR5 signaling would reduce HIV-associated chronic immune activation.

Methods—To test this hypothesis, we administered an antagonistic anti-CCR5 monoclonal antibody (HGS101) to five uninfected rhesus macaques (RMs) and monitored lymphocyte dynamics in blood and tissue.

Results—CCR5 blockade resulted in decreased levels of CCR5+ T-cells in blood and, at later timepoints, in lymph nodes. Additionally, the levels of CD25+ T-cells increased in lymph nodes, but decreased in blood, bone marrow, and rectal mucosa. Finally, a profile of gene expression from HGS101-treated RMs revealed a subtle, but consistent, *in vivo* signature of CCR5 blockade that suggests a mild immune modulatory effect.

Conclusions—Treatment with anti-CCR5 antibody induces changes in the tissue distribution of CCR5+ and CD25+ T-cells that may impact on the overall levels of immune activation during HIV and SIV infection.

Keywords

immune activation; trafficking; HIV

Introduction

The chemokine receptor CCR5 plays a key role in the pathogenesis of HIV and SIV infections by serving as the main co-receptor that these viruses use for entry in association with the CD4 molecule [23, 24, 31, 53, 100]. As such, CCR5 expression is an important determinant of the pattern of infected cells *in vivo* during primate lentiviral infections. In

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particular, high CCR5 expression in memory/activated CD4⁺ T-cells residing in the mucosa-associated lymphoid tissue (MALT) is responsible for the high level of infection and severe depletion of these cells during the early stages of pathogenic HIV and SIV infections of humans and rhesus macaques (RM) [61, 74, 103]. For these reasons a number of CCR5 inhibitors have been developed as a new class of anti-retroviral drugs that appear to improve the efficacy of the conventional therapeutic regimens [27, 37, 38, 45, 46, 95].

In addition to its role as a virus entry co-receptor, CCR5 mediates a number of important immune system functions. Cells expressing CCR5 traffic to sites of inflammation upon binding the CCR5 chemokine ligands CCL3/MIP1- α , CCL4/MIP-1 β , and CCL5/RANTES [59, 93]. Dysregulation of CCR5-mediated lymphocyte trafficking has been associated with a number of inflammatory conditions, including rheumatoid arthritis, organ transplant rejection, and multiple sclerosis [78, 91, 99]. In addition, a less severe or delayed disease phenotype for these conditions has been observed in CCR5 knockout mice and in patients with a distinct *CCR5* gene deletion that prevents surface CCR5 expression (*CCR5 Δ 32*) [2, 8, 40, 116]. Collectively, these data indicate that the CCR5 signaling pathway mediates a potent pro-inflammatory effect. In the setting of pathogenic HIV and SIV infections, the pro-inflammatory effect of CCR5 may be one of the factors responsible for the chronic immune activation that is consistently observed in infected individuals [88].

SIV infection of natural host species, such as the sooty mangabeys (SMs), is typically non-pathogenic despite levels of virus replication that are similar to, if not higher than, those observed in HIV-infected individuals [77, 89]. Since SIV infection of SMs is associated with low levels of immune activation [21, 89, 96, 97], we have proposed that this immune-attenuated phenotype is a key determinant of the benign nature of SIV infection in these animals [87, 88]. Additionally, we have shown that SMs express very low levels of CD4⁺CCR5⁺ T-cells in peripheral blood and mucosal tissues as compared to humans and RMs [72]. These observations led to the hypothesis that low levels of CCR5 expression on SM CD4⁺ T-cells may protect these animals from the chronic immune activation associated with pathogenic HIV and SIV infections [72]. In this view, interventions aimed at blocking CCR5 signaling during pathogenic HIV and SIV infections may demonstrate an additional, non-virological beneficial effect by reducing the overall levels of immune activation.

To explore the potential immunological roles of CCR5 in RMs infected with SIV, we have performed an *in vivo* CCR5 blockade in five healthy, SIV-uninfected animals using the HGS101 monoclonal antibody. This study revealed that CCR5 blockade is well tolerated, results in specific changes in the tissue distribution of CCR5⁺ and CD25⁺ T-cells, and induces an identifiable “signature” in the profile of gene expression of circulating leukocytes.

Methods

Animals

Five healthy, SIV-uninfected adult female rhesus macaques (RMs) were used for this study. The animals were housed at the Yerkes National Primate Research Center of Emory University and maintained in accordance with National Institutes of Health guidelines; the studies were approved by the Emory University and the University of Pennsylvania Institutional Animal Care and Usage Committees (IACUC). Due to complications related to blood collection (i.e., post-phlebotomy hematoma) and unrelated to HGS101 treatment, one animal was euthanized during the study (shortly after day 59). At the end of the study, the remaining four RMs were returned to the colony in normal health conditions.

HGS101 treatment

HGS101, a fully human monoclonal anti-CCR5 antibody that binds to the 2nd extracellular loop (ECL-2) and acts as a signal antagonist, is manufactured by Human Genome Sciences (Rockville, MD). The antibody was administered by intravenous infusion every fifteen days at a dose of 10 mg/kg for a total of ten times. Treatment began at day 0, and the last administration was given at day 135. Since HGS101 infusion was conducted after collection of blood and tissue samples, both day -32 and day 0 are considered baseline time points.

Blood and tissue processing

Peripheral blood, rectal biopsies, lymph node, and bone marrow samples were longitudinally collected and processed as previously described [35, 96].

Immunophenotyping and flow cytometry

Multiparametric flow cytometry was used to analyze mononuclear cell populations isolated from blood, lymph node, rectal biopsy, and bone marrow samples according to standard procedures and using human monoclonal antibodies that are cross-reactive with RM samples. The following antibodies were used to identify and characterize lymphocyte populations: anti-CD4 Pacific Blue (clone OKT4, eBioscience), anti-CD8 PE-Texas Red (clone 3B5, Invitrogen), anti-CD3 Alexa 700 (clone SP34-2, BD Biosciences), anti-CCR5 APC (clone 3A9, BD Biosciences), anti-CD25 APC-Cy7 (clone M-A251, BD Biosciences), anti-CD69 APC-Cy7 (clone FN50, BD Biosciences), anti-Ki67 FITC (clone B56, BD Biosciences). A cocktail of anti-CD14 PerCP-Cy5.5 (clone M5E2, BD Biosciences), anti-CD16 PerCP-Cy5.5 (clone 3G8, BD Biosciences), anti-CD20 PerCP-Cy5.5 (clone 9F5, BD Biosciences) was used to exclude non-T-cell populations from analysis of T-cells. Anti-IgG4 FITC (clone HP6025, Beckman Coulter) was used to identify *ex vivo* HGS101-bound cells. Flow cytometric acquisition and analysis of samples was performed on at least 100,000 events on an LSR II flow cytometer using the DiVa software package (BD Biosciences). The software program FlowJo (Tree Star) was used for analysis of acquired data.

Detection of HGS101-bound T-cells in samples from HGS101-treated RMs

Rhesus macaque PBMCs were isolated from whole blood by density gradient centrifugation. Cells were then incubated with or without 100 µg of HGS101 at 4°C for one hour. Cells were washed to remove unbound reagent and stained with anti-human IgG4 FITC (clone HP6025, Beckman Coulter) for 30 min at 4°C, then washed again. Cells were then incubated at 4°C for 30 min with anti-CD3 Alexa 700 (clone SP34-2, BD Biosciences), anti-CD4 Pacific Blue (clone OKT4, eBioscience), anti-CD8 PE-Texas Red (clone 3B5, Invitrogen), and anti-CCR5 APC (clone 3A9, BD Biosciences). Following this incubation, cells were washed, fixed in 1% formaldehyde, and analyzed on an LSR II flow cytometer.

Plasma concentration of HGS101

Plasma concentration of HGS101 was determined for time points at which whole blood was collected, and was performed as follows: a 96-well assay plate was coated overnight at 4°C with 2 µg/mL of an anti-idiotypic antibody specific for HGS101 (clone 4G3B) and then blocked with 5% BSA for 1.5 hours at 22°C. Plates were washed and samples diluted at 1:200 and 1:20,000 were added to the plate then incubated for 2 hours at 22°C. Plates were washed and anti-human IgG4-HRP at 1:50,000 was added and then incubated for 1 hour at 22°C. Concentrations of HGS101 in samples were extrapolated from a standard curve present on each plate.

In vitro incubation of rhesus macaque PBMCs with HGS101

Rhesus macaque PBMCs were incubated with or without 100µg/mL of HGS101 at 37°C. Aliquots were taken at 0, 3, 6, 18, 24, and 42 hours, washed, and then stained for CD3, CD4, CD8, CCR5, and CD25 for 30 min at 4°C. Antibody reagents were the same as in the above section describing immunophenotyping and flow cytometry.

RNA purification, array hybridization and hemoglobin blocking

Total RNA purification and microarray hybridization were conducted as described previously [13]. Briefly, 2.5 ml of venous blood was collected into PAXgene Blood RNA tubes (Becton Dickinson, San Diego, CA) and stored at -80°C. Total RNA was purified with PAXgene Blood RNA kits (QIAGEN) according to manufacturer's protocol with on-column DNase digestion. RNA quality was assessed by Nanodrop analysis using Agilent Bioanalyzer capillary electrophoresis; all samples had a RNA Integrity Number (RIN) > 8.0. RNA was hybridized to Affymetrix GeneChip Rhesus Macaque Genome Arrays (Affymetrix, Santa Clara, CA). The Affymetrix 3 IVT Express Kit was used to amplify 0.5 µg of total RNA according to a modified protocol; nonspecific binding by hemoglobin transcripts was inhibited by the inclusion of a set of 5 peptide nucleic acid (PNA) oligonucleotides (Bio-Synthesis Inc.) specific for regions of hemoglobin a and b mRNA into the reverse transcription cocktail as described previously [13].

Microarray data analysis

Background adjustment, normalization and median polish summarization of CEL files was performed using the robust multi-chip average (RMA) algorithm [43]. RMA and downstream analyses were performed using Partek Genomics Suite software v6.4 (Partek Inc). Arrays with poor hybridization were excluded based on dissimilarity in PCA analyses and atypical intensity distributions. Due to the modest level of differential gene expression induced by HGS101, a pre-filtering step was performed prior to ANOVA; genes with high variance were identified by comparing average fold-changes of pre-treatment samples (day -32 and 0); genes in which the post-treatment average fold-change relative to day 0 did not exceed that of day -32 and day 0 at one or more time points were excluded from downstream analysis. To determine genes statistically changed after HGS101 treatment we performed 2-way ANOVA (individual animal, time after treatment). Genes whose expression was significantly changed were defined by exhibiting (i) an FDR-corrected p-values <0.001, and (ii) fold-change greater than 1.5X relative to day 0. Differentially expressed genes with similar expression patterns were organized using agglomerative clustering with Pearson dissimilarity distance metric and average linkage.

Annotation of the Rhesus Genome array with human Gene Symbol identifiers

Affymetrix annotation of the Rhesus GeneChip maps to the rhesus genome, and > 11,000 probesets have provisional Gene Symbols in NCBI Gene (ie. "LOC" symbols). To increase the mapping of the array, probesets lacking definitive annotation were cross-referenced to potential human orthologs identified by the online resources provided the laboratory of Robert Norgren (version 3, June 2010) (<http://www.unmc.edu/rhesusgenechip/>) and the InParanoid ortholog database (release 7.0, June 2009) (<http://inparanoid.sbc.su.se>) [11], using probeset IDs and Ensembl Gene/Protein IDs, respectively.

GEO Accession numbers

The microarray dataset was submitted to the GEO online repository according to MIAME (Minimum Information About a Microarray Experiment) standards (GEO accession number pending).

Statistical analysis

Changes in T-cell percentages over time were modeled using mixed effects models [58] in order to account for the inherent correlation between repeated measurements from the same animal. Linear and non-linear trends were tested for. Additionally, changes from baseline were computed, and significance adjusted for multiple comparisons. Log-transformations were applied to the data, as necessary, to meet model distributional assumptions.

Results

Experimental design

To investigate the role of CCR5 in T-cell activation and trafficking between lymphoid and mucosal tissues, we performed an *in vivo* CCR5 blockade in uninfected rhesus macaques (RMs). As shown in Figure 1A, five healthy, SIV-uninfected RMs were treated with the fully human anti-CCR5 antibody HGS101 at a dose of 10 mg/kg that was administered every fifteen days for a total of 135 days (i.e., ten administrations beginning at day 0 and ending at day 135). HGS101 is a known antagonist of human CCR5 signaling that was developed for treatment of conditions such as HIV infection and chronic inflammatory diseases. The treatment schedule was chosen based on available data on the pharmacokinetics of this compound in both human and non-human primates (Migone et al., unpublished observations), and to assess the long-term effects of blocking CCR5 signaling *in vivo*. In all animals, samples of peripheral blood, lymph nodes, bone marrow, and rectal mucosa (sampled via rectal biopsy, RB) were obtained at various time points (Figure 1A). Prior to this *in vivo* study, we confirmed that HGS101 binds to RM CCR5 with high affinity *in vitro* (Figure 1B). We also confirmed that HGS101 acts as a CCR5 antagonist in RM cells as pre-treatment with this antibody prevented the phosphorylation of CCR5 following MIP1-b/CCL4 *in vitro* stimulation at level comparable to the small molecule antagonist Maraviroc (data not shown). Note that, in this study, we did not use a group of control RMs treated with an “irrelevant” antibody due to budgetary and logistical constraints. While this design is admittedly not ideal, our previous extensive experience with *in vivo* administration of a “control” antibody in non-human primates [9, 33, 54, 55] made us confident that the observed immune modulatory effects of HGS101 are unlikely to be simply a consequence of an immunoglobulin administration.

HGS101 is detectable in the plasma and on the surface of T-cells of treated RMs

The HGS101 treatment regimen was well tolerated by all RMs and none of the animals exhibited any signs of toxicity throughout the 135-day treatment period. As expected, in all treated RMs HGS101 was detectable in the plasma throughout the study at concentrations up to 200µg/ml within a few days of each administration, and remained consistently detectable through the next administration (Figure 1C). As HGS101 was first administered following sample collection at day 0, both days -32 and 0 are considered baseline time points for this study. Importantly, the specificity of HGS101 for CCR5 was confirmed by *ex vivo* staining for HGS101 that we detected only on T-cells expressing CCR5 (Figure 1D).

HGS101 does not reduce the levels of total or proliferating CD4+ and CD8+ T-cells in blood and tissues

We next sought to determine whether CCR5 blockade changes the levels of CD4+ and CD8+ T-cells in blood, lymph nodes, and mucosal tissues. Despite some minor fluctuations, treatment with HGS101 did not induce any substantial change in the percentage or absolute number of CD4+ or CD8+ T-cells in peripheral blood (Figure 2A). Similarly, no changes were observed in the fraction of CD3+CD4+ or CD3+CD8+ T-cells in lymph nodes, rectal biopsies, or bone marrow (Figures 2B, 2C, 2D). A key rationale for conducting this

experiment of *in vivo* treatment with HGS101 was to test the hypothesis that CCR5 blockade results in a decrease of the prevailing levels of T-cell activation. To this end, we next assessed the level of CD4+ and CD8+ T-cell activation in blood and tissues by measuring the fraction of T-cells expressing the proliferation marker Ki67 [82]. As shown in Figures 2E, 2F, 2G, and 2H, we did not find any evidence that CCR5 blockade results in a decline of proliferating T-cells in any of the examined tissues. In fact, we found a modest but significant increase in the levels of circulating Ki67+ T-cells ($p < 0.001$ for CD4+ and CD8+ T-cells in blood) (Figure 2E). Taken together, these data indicate that, in healthy RMs, treatment with HGS101 does not reduce the levels of either total or proliferating CD4+ and CD8+ T-cells in blood, lymph nodes, and rectal mucosa.

HGS101 treatment reduces the levels of CCR5+ T-cells in blood and lymph nodes

We next examined how treatment with HGS101 affects the levels of T-cells expressing CCR5. To conduct these experiments we used a CCR5-specific antibody that recognizes an N-terminal epitope of CCR5 that is not masked by binding of the HGS101 antibody, which recognizes an epitope in the second extracellular loop of CCR5 (Migone et al., unpublished observations). Interestingly, we observed that HGS101 administration was followed by a decline in the percentage and absolute number of CCR5-expressing CD4+ and CD8+ T-cells in blood ($p < 0.001$), with the most rapid declines occurring within the first 45 days of treatment ($p = 0.04$ for CD4+ T-cells and $p = 0.001$ for CD8+ T-cells) (Figure 3A). The decline in CCR5-expressing T cells was most evident by day 107 and remained very low six weeks following the last administration. These data suggests that while a reduction in CCR5 levels can be seen in peripheral blood within the first weeks, the cumulative effects of blocking CCR5 may be greatest after several months of sustained treatment. As shown in Figure 3B, HGS101-treated RMs experienced a similar decline in the percentage of CD4+CCR5+ and CD8+CCR5+ T-cells in lymph nodes ($p < 0.001$) that persisted up to 6 weeks after the end of treatment. A decrease in the fraction of CCR5+ T-cells in the context of HGS101 treatment may be explained by several mechanisms, including accelerated *in vivo* death of these cells, trafficking out of blood and lymph nodes into mucosal tissues, and down-regulation of CCR5 expression. To address these possibilities, we first measured the levels of CCR5+ T-cells in the rectal mucosa, and observed that HGS101 administration was not followed by a significant increase in the fraction of CD4+CCR5+ and CD8+CCR5+ T-cells (Figure 3C). To investigate whether HGS101 treatment results in down-regulation of CCR5 expression, we next performed *in vitro* experiments in which T-cells were treated with HGS101 and the level of surface CCR5 expression was assessed longitudinally. As shown in Figure 3D, HGS101 did not reduce surface CCR5 levels, and, in fact, appears to increase CCR5 expression, perhaps by preventing the physiologic internalization of the receptor. Of note, in this experiment we did not measure the rate of cell death, therefore no conclusion can be made with respect to the possibility that HGS101 depletes CCR5+ T-cells *in vivo*; however, no changes in viability were observed when cells were treated with HGS101 *in vitro* (data not shown). Collectively, these data indicate that HGS101 treatment reduces the levels of circulating and lymph node-resident CCR5+ T-cells, and that this reduction does not seem to be caused by enhanced trafficking to mucosal tissues or down-regulation of CCR5 surface expression.

HGS101 induces a complex anatomic redistribution of CD25+ T-cells

Given the known cross talk between CCR5 expression and IL-2 signaling through its receptor CD25 [19, 111], we next examined the effect of HGS101 treatment on the fraction of CD4+CD25+ and CD8+CD25+ T-cells in blood and tissues. In preliminary *in vitro* experiments, we found that HGS101 treatment does not affect the surface expression of CD25 (data not shown). As shown in Figure 4A, in HGS101-treated RMs, the fraction and absolute number of circulating CD4+CD25+ T-cells decreased significantly ($p = 0.001$). In

addition, we observed a decrease in the percentage of CD4+CD25+ T-cells in the bone marrow (Figure 4D) ($p=0.001$) and a decrease in the percentage of CD4+CD25+ and CD8+CD25+ T-cells in rectal biopsies ($p<0.001$ and $p<0.01$, respectively) (Figure 4C). In stark contrast, the levels of CD25+ T-cells in lymph nodes increased considerably during HGS101 treatment, and did not return to baseline levels until six weeks following the final administration (Figure 4B). Statistical analysis confirmed this quadratic trend with $p<0.001$ for both CD4+ and CD8+ CD25+ T-cells. Overall, these data indicate that CCR5 blockade results in an increased level of CD25+ T-cells in the lymph nodes associated with a decreased fraction of these cells in the rectal mucosa, as well as blood and bone marrow (for CD4+ T-cells).

HGS101 treatment induces specific changes in the transcriptional profile of leukocytes

To assess the effect of CCR5 blockade on the transcriptional profile of leukocytes, we next conducted a sequential analysis of gene expression by microarray in blood samples that were collected before and after HGS101 treatment. Figure 5A depicts the 203 genes whose transcriptional profile was most significantly altered during this study, as revealed using ANOVA. We found that several genes were up-regulated at day 35, though their increase in transcription was generally not sustained until day 92. The biological meaning of this transcriptional up-regulation that follows CCR5 blockade is uncertain, as it may at least in part reflect an immune response against the infused “foreign” immunoglobulin. For this reason, we focused our analysis on those genes whose expression was down-regulated after HGS101 treatment, and hypothesized that this down-regulation was for the most part a result of the inhibition of CCR5-mediated signaling. As shown in Figure 5B and Table 1, we identified 23 genes of interest that encode for proteins with various biological roles (cell cycle, apoptosis, lymphocyte signaling and activation, cell adhesion and migration, and general immune function) that were significantly down-regulated after CCR5 blockade. Interestingly, down-regulated transcripts included genes with documented or presumed involvement in B and T-cell development and activation such as *LOC701504*, *IGHV5-78*, *MS4A1*, *BLNK*, and *HLA-DRI1B* [3, 5, 14, 16, 34, 44, 52, 65, 73, 81, 106]. Additionally, this analysis revealed the transcriptional down-regulation of other genes potentially involved in general immune function and regulation of immune activation such as *HSPD1*, *IRF8*, *MAP2K2*, *SMAD2*, and *MAP2K1* [4, 22, 25, 26, 42, 64, 66, 70, 71, 90, 92, 98, 101, 102, 108, 112, 113, 117, 119]. Transcriptional down-regulation of genes involved in regulating cell cycle and proliferation (*CCNY* and *HSPA9*) [47, 50, 62, 104], as well as cell adhesion and migration (*KLF2* and *CYTH1*) [6, 18, 20, 29, 30, 41, 56, 57, 68, 76, 84, 85, 107, 109, 115]) was also observed during HGS101 treatment. Collectively, these data do not rule out a potential immune modulatory effect of CCR5 blockade.

We next investigated whether the transcriptional profile following HGS101 treatment could help explain some of the changes we observed by flow cytometry. Of note, this analysis did not reveal any significant change in the expression of *CCR5* and its ligands *CCL3*, *CCL4L1*, and *CCL5*, nor of *CD25* (*IL2RA*) and its ligand *IL2* (Fig 5C). In addition our analysis did not reveal any significant compensatory change in the transcriptional level of genes coding for any other chemokines or chemokine receptors that could have been triggered by CCR5 blockade. These data suggest that the observed changes in CCR5+ and CD25+ T-cell distribution *in vivo* after HGS101 treatment are related to altered T-cell trafficking as opposed to a transcriptional increase or decrease.

Discussion

Following CCR5 blockade *in vivo* in rhesus macaques (RMs) using the HGS101 antibody we observed: (i) decreased levels of both CD4+CCR5+ and CD8+CCR5+ T-cells in blood and lymph nodes; (ii) decreased levels of CD25+ T-cells in blood and rectal mucosa, with

concomitant increased levels of these cells in lymph nodes; and (iii) the presence of a subtle but significant set of changes in the transcriptional profile that is consistent with a mild immune-modulatory effect. Importantly, this study also showed that prolonged treatment with HGS101 was well tolerated by the animals, thus providing an encouraging safety result for future clinical studies in humans.

The rationale for the current study was to investigate the non-virological effect of CCR5 signaling in AIDS pathogenesis. In particular, we proposed that blocking CCR5 could reduce the HIV- and SIV-associated immune activation by limiting the trafficking of activated T-cells that occurs in response to inflammatory signals, such as those mediated by CCL3, CCL4, and CCL5. In this first exploratory *in vivo* experiment, we elected to block CCR5 signaling in absence of SIV infection. This reductionist approach was chosen to allow for an assessment of the direct immunological effects of CCR5 blockade without the confounder represented by the intrinsic antiviral activity of HGS101. One limitation of this experimental system, however, is that our inability to observe the postulated effect of HGS101 in reducing T-cell activation might have been a consequence of low levels of immune activation present in SIV-uninfected RMs. For instance, the fact that HGS101 treatment did not reduce the levels of CCR5+ T-cells in the rectal mucosa may simply indicate that, in healthy RMs, CCR5+ T-cells do not use this molecule for homing to mucosal sites, and rather rely primarily on other receptors such as CCR9 and $\alpha 4\beta 7$ [39, 94]. Future experiments of HGS101 treatment in SIV-infected RMs, particularly using a non-CCR5-tropic virus, may reveal a more pronounced effect of CCR5 blockade in reducing the intestinal homing of activated CD4+ T-cells and the SIV-associated hyper-immune activation.

The most significant immunological effects of HGS101 treatment were (i) a decline in the percentages of CCR5+ T-cells in blood and lymph nodes and (ii) a complex perturbation of the tissue distribution of CD25+ T-cells. The HGS101 induced loss of CCR5+ T-cells in blood and lymph nodes could result from several mechanisms, including CCR5 surface down-modulation, enhanced migration to mucosal tissues, and antibody-mediated cytotoxicity. The possibility that CCR5 blockade caused a reduced surface expression of CCR5 (i.e., increased internalization of the receptor) is not supported by our *in vitro* experiments that revealed, if anything, increased CCR5 surface expression after HGS101 administration. Similarly, the possibility that CCR5 blockade induced heightened homing of CCR5+ T-cells to mucosal tissues is unlikely since no increase in the fraction of CCR5+ T-cells resident in RBs was observed during HGS101 treatment. While we did not observe any decrease in cell viability following *in vitro* HGS101 treatment, in the current *in vivo* study the rates of cell death were not directly measured. As such, the possibility that HGS101 treatment might have depleted at least a subset of CCR5+ T-cells *in vivo* cannot be ruled out. However, if cell depletion has occurred as a result of HGS101 treatment, it is likely not due to antibody dependent cell cytotoxicity or complement activation, as IgG4 antibodies are known to be functionally monovalent and do not normally induce these effects [1, 12, 17, 28]. Finally, it should be noted that although statistical analysis revealed that the reduction of CCR5+ T cells in blood and lymph nodes occurred throughout the study ($p < 0.001$ for blood and lymph nodes), the greatest reduction was observed at later time points. This may suggest that sustained treatment with HGS101 over several months is required to observe the greatest effects of CCR5 blockade.

The HGS101-induced perturbation in the tissue distribution of CD25+ T-cells consisted of a decline of this cell subpopulation in blood and rectal mucosa associated with an increased percentage of CD25+ T-cells in lymph nodes. The increased levels of CD25+ T-cells in lymph nodes may be related to an HGS101-associated increased fraction of T-cells expressing CD69 (data not shown), a molecule whose expression inhibits egress from lymph

nodes through its interaction with S1P1 [7, 86]. However, the increase of CD25⁺ T cells in lymph nodes was only observed at one time point, and thus, we caution against over-interpretation of this finding. An alternative, intriguing possibility is that CCR5 blockade resulted in reduced expression of CD25 on T-cells in blood and mucosal tissues. This hypothesis would be consistent with a previous study by Camargo *et al.* showing that CCR5 signaling results in enhanced expression of CD25 [19]. In this view, HGS101 treatment might have reduced CD25 expression in the anatomic compartment where CCR5 expression and signaling are more prominent, i.e., mucosal tissues. Whether and to what extent these effects of CCR5 blockade on the level of CD25⁺ T-cells in mucosal tissues may be useful to attenuate the HIV/SIV-induced hyper-immune activation remains to be ascertained.

HGS101 also induced a relatively subtle (i.e., involving a limited number of genes) but consistent set of changes in the transcriptional profile of blood leukocytes. As expected when using a molecule with an inhibitory effect, we observed that CCR5 blockade was associated with the down-modulation of the expression of a number of genes involved in the immune system development and function. This observation suggests that HGS101 had a mild immune modulatory effect *in vivo*, and raises the intriguing possibility that this effect may be amplified in the setting of heightened immune activation, such as during pathogenic HIV or SIV infection.

This study indicates that *in vivo* CCR5 blockade is well tolerated and has mild but detectable immune modulatory effects. However, it would be interesting to compare, in terms of potential treatment of the HIV-associated immune activation, HGS101 with small molecule CCR5 inhibitors (i.e., maraviroc, vicriviroc, and aplaviroc) as well as immune suppressive drugs (i.e., cyclosporin-A, mycophenolate) that have no direct effect on HIV replication. To address this issue, further *in vivo* studies of HGS101 vs maraviroc vs immune suppression in RMs infected with both CCR5-tropic and non-CCR5-tropic virus strains, and in the presence or absence of antiretroviral therapy, may be warranted.

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Figure 1. HGS101 is detectable in the plasma and on the surface of T-cells of treated rhesus macaques (RMs)

A. Five age-matched SIV negative female RMs were infused with the CCR5-blocking monoclonal antibody HGS101 (×) at 10mg/kg every 15 days starting at day 0. Blood (♦), bone marrow (●), lymph node (□), and rectal biopsy (▲) samples were taken at the time points indicated. B. Representative flow cytometry plots showing rhesus macaque PBMCs incubated *in vitro* with HGS101 and then stained for HGS101 using an anti-human IgG4 antibody (and CCR5 staining with 3A9). Cells were pre-gated on CD3+, CD4+ and CD8+ T-cells. C. The mean plasma concentration of HGS101 in HGS101-treated RMs was determined at several time points pre-, during, and post-treatment period. ND denotes “not detectable.” D. Representative flow cytometry plots showing HGS101-bound T-cells in the whole blood of HGS101-treated and untreated rhesus macaques. Cells were pre-gated on CD3+, CD4+ and CD8+ T-cells, and HGS101 was detected using an anti-human IgG4 antibody (and CCR5 staining with 3A9).

Figure 2. HGS101 does not reduce the levels of total or recently proliferated (i.e., Ki67+) CD4+ and CD8+ T-cells in blood and tissues

A. Longitudinal analysis of the percentage (■) and absolute counts (cells/mm³) (▲) of CD3+CD4+ (top) and CD3+CD8+ T-cells (bottom) in the peripheral blood of HGS101-treated RMs. Longitudinal analysis of the percentage of CD3+CD4+ (■) and CD3+CD8+ (▲) T-cells in the lymph nodes (B), rectal biopsies (C), and bone marrow aspirates (D) of the same animals. Longitudinal analysis of the percentage of CD4+Ki67+ (■) and CD8+Ki67+ (▲) T-cells in peripheral blood (E), lymph nodes (F), rectal biopsies (G), and bone marrow aspirates (H).

Figure 3. In vivo HGS101 treatment reduces the levels of CCR5+ T-cells in blood, lymph nodes, and rectal mucosa

A. Longitudinal analysis of the percentage (■) and absolute counts (▲) of CD4+CCR5+ (top) and CD8+CCR5+ T-cells (bottom) in peripheral blood from HGS101-treated rhesus macaques. Longitudinal analysis of the percentage of CD4+CCR5+ (■) and CD8+CCR5+ (▲) T-cells in lymph nodes (B) and rectal biopsies (C) from the same animals. D. Longitudinal analysis of the percentage of CD4+CCR5+ and CD8+CCR5+ T-cells after in vitro HGS101-treatment (—) or control (----) in RM PBMCs incubated over 42 hour (two representative animals are shown).

Figure 4. HGS101 induces a complex anatomic redistribution of CD25+ T-cells

A. Longitudinal analysis of the percentage (■) and count (▲) of CD4+CD25+ T-cells in peripheral blood from HGS101-treated RMs. Longitudinal analysis of the percentage of CD4+CD25+ (■) and CD8+CD25+ (▲) T-cells was performed in lymph nodes (B), rectal biopsies (C), and bone marrow aspirates (D) from the same animals.

Figure 5. HGS101 treatment induces specific changes in the transcriptional profile of peripheral blood leukocytes

A. Hierarchical clustering of 203 genes differentially expressed in whole blood of RMs after in vivo administration of HGS101 as assessed at days -32, 35, 92, and 177 of treatment. B. Hierarchical clustering of 23 genes with immune-related function significantly altered by HGS101 treatment on days 35, 92, and 177 post-treatment. C. Heat map of immune related genes regulating leukocyte trafficking, homeostasis and apoptosis demonstrating no changes in expression in response to HGS101 treatment. Agglomerative clustering of genes was performed using Pearson dissimilarity and average linkage. The gene expression color scale is indicated at bottom left.

Table 1

List of 23 HGS101-regulated genes with immune-related function

Probeset ID	Proposed Human Gene Symbol	Proposed Gene Description	Mean Fold Change over Baseline (Day 0)			Documented or presumed function in:					
			Day 35	Day 92	Day 177	p-value (Timepoint)	Cell Growth/Cell Cycle/Apoptosis	General Immune Function	B cell signaling/activation	T cell signaling/activation	Cell adhesion/migration
MmugDNA.39885.1.S1_at	IL12RB2	interleukin 12 receptor, beta 2	1.95	1.22	0.91	4.51E-06	[75]	[36]		[63]	
MmugDNA.43015.1.S1_at	SMAD2	SMAD family member 2	0.66	0.94	0.95	6.17E-05	[66]	[98, 112]		[57, 101]	
MmugDNA.16142.1.S1_at	KLF2	Kruppel-like factor 2 (Lung)	0.64	0.92	0.89	8.10E-05		[29, 85, 107]	[115]	[18, 57, 109]	[6, 20, 41, 67, 84, 85]
MmugDNA.3626.1.S1_at	TP53BP2	tumor protein p53 binding protein, 2	2.14	1.16	0.98	8.28E-05	[10, 48, 79, 110]				
MmuSTS.1985.1.S1_at	CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	2.06	0.97	1.02	1.46E-04	[83, 114]				
MmugDNA.609.1.S1_at	MAP2K2	mitogen-activated protein kinase kinase 2	0.58	0.88	0.87	1.62E-04	[102, 119]	[113]		[4]	
Mmu.12449.1.S1_at	SDC1	syndecan 1	1.52	1.16	1.15	1.77E-04	[102, 119]	[80]			[32, 80]
MmugDNA.12133.1.S1_s_at	BCL2L1	BCL2-like 1	1.53	0.89	0.95	2.05E-04	[15, 51]			[15]	
MmuSTS.2978.1.S1_at	MAP2K1	mitogen-activated protein kinase kinase 1	0.67	1.07	0.92	2.31E-04	[90, 102, 119]	[92, 113]			
MmugDNA.34.1.S1_at	BLNK	B-cell linker	0.63	0.59	0.77	2.32E-04			[5, 34, 65, 81]		
MmugDNA.3956.1.S1_at	HEMGN	hemogen	2.32	1.17	1.12	4.86E-04	[60]				
MmugDNA.36856.1.S1_at	HSPD1	heat shock 60kDa protein 1 (chaperonin)	0.64	0.91	0.66	4.86E-04	[22]	[70]	[25, 26]	[22, 71]	
MmugDNA.31389.1.S1_at	CYTH1	cytohesin 1	0.66	1.06	1.07	6.16E-04					[30, 56, 76]
MmugDNA.3654.1.S1_at	MARCH8	membrane-associated ring finger (C3HC4) 8	1.73	1.06	0.96	6.16E-04		[69]			
MmugDNA.12754.1.S1_s_at	MS4A1	membrane-spanning 4-domains, subfamily A, member 1	0.66	0.57	0.70	6.17E-04			[44, 73, 106]		
MmugDNA.20063.1.S1_at	WDR26	WD repeat domain 26	1.63	1.21	1.00	6.66E-04	[118]				
MmugDNA.11290.1.S1_at	BCLAF1	BCL2-associated transcription factor 1	0.71	1.00	0.63	7.02E-04	[49]				
MmugDNA.31268.1.S1_at	LOC701504	similar to Ig kappa chain V-III region HAH precursor	0.70	0.48	0.75	8.57E-04			[52]		
MmugDNA.24356.1.S1_at	IGHV5-78	immunoglobulin heavy variable 5-78 pseudogene	0.57	0.48	0.81	8.58E-04			[14]		
MmugDNA.8826.1.S1_at	IRF8	interferon regulatory factor 8	0.66	0.79	0.84	1.02E-03	[42]	[42, 64, 108, 117]			
MmugDNA.27974.1.S1_at	CCNY	cyclin Y	0.57	0.97	0.89	1.04E-03	[26, 47]				
MmunewRS.436.1.S1_s_at	HLA-DR1B	major histocompatibility complex, class II, DR beta 1	0.66	0.84	0.91	1.15E-03		[3, 16]			
MmugDNA.28475.1.S1_at	HSPA9	heat shock 70kDa protein 9 (mortalin)	0.62	0.81	0.86	1.20E-03	[50, 104, 105]				

Genes with documented or presumed immune-related function that were regulated by HGS101 treatment are listed here. Proposed human Gene Symbols were derived from the August 2010 Affymetrix annotation update, by ortholog mapping reported by InParanoid release 7, by ortholog mapping reported by InParanoid release 7, or from the annotation provided by the Norgren laboratory (<http://www.unmc.edu/rhesusgenechip/>). Mean gene expression ratios are expressed in linear form.

Presumed gene function was inferred from cited studies of homologous genes.