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Baboon CD8 T cells suppress SIVmac infection in CD4 T cells through contact-dependent production of MIP-1 α , MIP-1 β , and RANTES

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Abstract

Simian immunodeficiency virus (SIV) infection in rhesus macaques is often characterized by high viremia and CD4 T cell depletion. By contrast, SIV infection in African nonhuman primate natural hosts is typically nonpathogenic despite active viral replication. Baboons are abundant in Africa and have a geographical distribution that overlaps with natural hosts, but they do not harbor SIVs. Previous work has demonstrated baboons are resistant to chronic SIV infection and/or disease *in vivo* but the underlying mechanisms remain unknown. Using *in vitro* SIVmac infections, we sought to identify SIV restriction factors in baboons by comparing observations to the pathogenic rhesus macaque model. SIVmac replicated in baboon PBMC but had delayed kinetics compared to rhesus PBMC. However, SIVmac replication in baboon and rhesus isolated CD4 cells were similar to the kinetics seen for rhesus PBMC, demonstrating intracellular restriction factors do not play a strong role in baboon inhibition of SIVmac replication. Here, we show CD8 T cells contribute to the innate SIV-suppressive activity seen in naïve baboon PBMC. As one mechanism of restriction, we identified higher production of MIP-1 α , MIP-1 β , and RANTES by baboon PBMC. Contact between CD4 and CD8 T cells resulted in maximum production of these chemokines and suppression of viral replication, whereas neutralization of CCR5-binding chemokines in baboon PBMC increased viral loads. Our studies indicate baboon natural restriction of SIVmac replication is largely dependent on CD4-extrinsic mechanisms mediated, in part, by CD8 T cells.

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DECLARATION OF INTERESTS

None

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Keywords

baboon; AIDS; cytokine; chemokine; CCR5

1. Introduction

Human immunodeficiency virus (HIV) resulted from multiple cross-species transmission events of simian immunodeficiency viruses (SIV). Accidental or experimental SIV infection of a nonnatural nonhuman primate (NHP) host, like SIVmac infection of rhesus macaques, causes a disease resembling human Acquired Immunodeficiency Syndrome (AIDS), characterized by high viremia, CD4 T cell depletion, and opportunistic infections [1]. In contrast to macaques, SIV infection of natural NHP hosts, including SIVagm infection of African green monkeys and SIVsmm infection of sooty mangabey monkeys, typically does not cause disease despite active viral replication [2, 3]. Understanding the mechanisms that underlie differences in disease outcomes for natural and nonnatural NHP hosts have proven valuable for the study of AIDS progression. Nevertheless, neither animal model is able to resist or clear SIV infection.

Baboons (*Papio hamadryas sp.*) are abundant in Africa, along with natural NHP hosts, but are not endemically infected with SIV. This is unexpected when considering the geographical distribution of baboon subspecies vastly overlaps with that of natural NHP hosts, primarily African green monkeys. Furthermore, African green monkeys comprise a significant portion of a baboon's diet [4]. Despite exposure to SIV through co-habitation and predation, baboons still remain largely SIV-negative. Field studies in Tanzania and Ethiopia have documented some baboons to be positive for antibodies to SIVagm, but the prevalence was less than 1% [5]. Interestingly, one of the seropositive baboons harbored fragments of an integrated SIVagm variant, suggesting the animal had been infected although was not producing virus [6].

Previous studies have interrogated SIV tropism for baboons *in vivo* and *in vitro*. Benveniste *et al.* challenged baboons with an SIV strain from pig-tailed macaques (SIVMne) and reported no clinical signs of disease, undetectable virus in circulation and tissues, and lack of seroconversion up to 1 year post-inoculation [7]. Earlier *in vitro* studies demonstrated baboon lymphocytes are susceptible to infection with SIVmac, but virus growth was less efficient than in rhesus macaque lymphocytes [8, 9]. In support of this finding, Cranage *et al.* showed baboons can support persistent SIVmac infection *in vivo*; however, baboons did not progress to disease or develop any tissue pathology at the microscopic level [10]. The processes by which baboons resist infection and/or disease *in vivo* and restrict virus growth *in vitro* remain unclear. Previous studies have investigated immune correlates of viral suppression in a baboon model of HIV-2 infection, a virus genetically close to SIVsmm and SIVmac [11, 12]. However, these animals were challenged with dual-tropic HIV-2 strains, which do not model the CCR5-tropic SIVs baboons would encounter in the wild. Further investigation in an appropriate SIV-baboon system could uncover mechanisms of natural SIV resistance in baboons that can be applied towards the development of novel antiviral strategies against HIV.

In this study, we used *in vitro* infections to identify the key baboon cell types involved in SIV suppression and to elucidate a mechanism of viral restriction. Here we report that SIVmac has an equal capacity to bind, enter, and replicate in baboon and rhesus macaque isolated CD4 cells. However, virus growth is dampened in baboon PBMC, where other immune cell types are present. Restriction in baboon PBMC is mediated, in part, by contact of CD4 cells with CD8 T as well as by high production of MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5, chemokines that compete with the virus for access to the entry co-receptor, CCR5.

2. Materials and Methods

2.1. Animals and cell separation

Whole blood in EDTA was obtained from SIV seronegative baboons (*Papio hamadryas sp.*, $n = 74$) and Indian rhesus macaques (*Macaca mulatta*, $n = 57$) from the Southwest National Primate Research Center (SNPRC) at the Texas Biomedical Research Institute (TBRI). Distribution of age and gender of the animals is shown in Supplementary Table 1. Animal care and treatments were all in accordance with protocols approved by the TBRI Institutional Animal Care and Use Committee (IACUC). Animals were serologically screened for simian T-lymphotropic virus (STLV) and SIV antibodies by Luminex assay. Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation using Lymphocyte Separation Medium (Cellgro, Corning). Cells were washed twice with PBS before phenotyping by flow cytometry. CD4 cells were sorted from freshly isolated PBMC by positive selection using magnetic beads coated with anti-CD4 (clone L200) antibodies, as per the manufacturer's instructions (IMagTM Human CD4 T Lymphocyte Enrichment Set-DM, BD Biosciences). Purity of the positive fraction was assessed by flow cytometry using a clone of anti-CD4 antibody that differed from that used for sorting (CD4-APC, clone 13B8.2, Beckman-Coulter).

2.2. Flow cytometry

PBMC were stained with various combinations of the following monoclonal antibodies: CD3-V500 (clone SP34.2, BD-Biosciences), CD4-PerCp-Cy5.5 (clone L200, BD-Biosciences) or CD4-APC, clone 13B8.2, Beckman-Coulter), CD8-FITC (clone 3B5, Invitrogen, ThermoFisher), CCR5-PE (clone 3A9, BD-Biosciences). After 30 min of incubation at 4°C, cells were washed with cold PBS then fixed in PBS containing 1.6% methanol-free formaldehyde (Polysciences). Data was collected on a three-laser CyAn ADP (Beckman-Coulter) and analyzed on FlowJo version 10 software.

2.3. PBMC and CD4 cell infections

Prior to infection, freshly isolated PBMC or CD4 cells were cultured for 48 hr in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 25 mM HEPES, 1% non-essential amino acids (NEAA), and 1% L-glutamine. For some experiments, cells were stimulated for 48 hr with 5 μ g/mL Phytohemagglutinin-L (PHA-L) (Sigma-Aldrich). Cells were resuspended in fresh medium and infected with SIVmac251 at a M.O.I. of 0.01 TCID₅₀/cell by MagnetofectionTM (OZ Biosciences) according to the manufacturer's protocol. Briefly, cells were pelleted in 5 ml

polypropylene tubes. Virus was incubated with cationic magnetic nanoparticles (ViroMag R/L, OZ Biosciences) at 4°C for 15 min before addition to pelleted cells. The final volume in the tube after addition of virus to cells did not exceed 500 µL. Tubes were placed at 37°C for 2 hr over a magnet to concentrate the nanoparticle-associated virus onto the cell pellet. Cells were washed twice to remove unbound virus. Cultures were maintained at $1-2 \times 10^6$ cells/mL in complete RPMI-10 with 50 U/mL IL-2 (NIH AIDS Reagent Program). For some infections, cells were pre-treated and cultured in 1 µM of the CCR5 antagonist maraviroc (NIH AIDS Reagent Program). Chemokines were inhibited using polyclonal goat IgG neutralizing antibodies against MIP-1α, MIP-1β, and RANTES (R&D Systems). Infected cultures received 2 µg/mL of each antibody; an equal volume of normal goat serum was added as a control. Twice weekly, half the media was changed and treatments replenished appropriately. Supernatant was saved for quantification of viral loads and cytokines.

For single-cycle infections, CD4 cells were infected at a M.O.I. of 1 TCID₅₀/cell. Cells were incubated over a magnet with nanoparticle-associated virus at 4°C for 1 hr to allow binding but restrict entry. Cells were washed twice with ice cold PBS before harvesting an aliquot of cells for analysis. Remaining cells were resuspended in media and placed at 37°C for 1 hr to promote entry and progression through replication stages. Cells were then cultured in 200 nM of the protease inhibitor darunavir (NIH AIDS Reagent Program) to prevent subsequent rounds of infection. Cells were harvested at 12, 24, and 48 h.p.i. Cellular RNA was extracted with the RNeasy Mini Kit (Qiagen) then treated with DNase (TURBO DNA-free™ kit, Ambion, ThermoFisher). The Gentra Puregene Cell Kit (Qiagen) was used to extract and RNase-treat cellular DNA. Viral RNA was isolated from culture supernatants using the QIAamp Viral RNA Mini Kit (Qiagen). Before isolation, supernatant samples were spiked with a known amount of Qβ bacteriophage (Attostar) to control for efficient extraction and amplification of recovered RNA.

2.4. CD4, CD8 T, and NK cell co-cultures

CD8 T and NK cells were positively sorted from CD4-depleted PBMC by flow cytometry. CD4 depleted-PBMC were stained with antibodies to CD3-APC (clone Sp34.2, BD-Biosciences), CD8-Pacific Blue (clone 3B5, Invitrogen, ThermoFisher), and NKG2A-PE (clone Z199, Beckman-Coulter). CD8 T cells were sorted as CD3⁺CD8⁺CD4⁻ and NK cells as CD3⁻NKG2A⁺. Sorting was performed on a four-laser FACS Aria III (BD-Biosciences). Data from the sorting was collected on FACSDiva version 8 software and analyzed on FlowJo version 10 software. Cells were cultured overnight at 37°C before downstream application. CD4 cells were infected in isolation at a M.O.I. of 0.01 as described in section 2.3. After washes to remove unbound virus, sorted CD8 T and NK cells were added to uninfected or infected CD4 cell cultures in a 48-well plate in various combinations. The ratio of CD4 to CD8 T cells was 1:1 and CD4 to NK cells was 1:0.3. Ratios were selected to reflect the relative abundance of these cell types in baboon PBMC based on previous flow cytometry data from our laboratory [13]. For contact-dependency studies, CD8 T and NK cells were added to the surface of a 0.4 µm permeable membrane (Transwell®, Corning) in a 24-well plate. CD4 cells were cultured in the lower compartment. Segregation between the two compartments was confirmed by flow cytometry. The input and concentration of each cell type was equal across all conditions.

2.5. Luminex assays

Viral loads were monitored by measurement of SIV p27 in culture supernatant. Briefly, samples were left undiluted or diluted with RPMI-10 at 1:5, 1:10, or 1:100. Samples were lysed in buffer containing 0.2% Tween 20 for 1 hr at room temperature then incubated with capture beads coupled to an anti-SIV Gag p27 antibody (clone 55-2F12, NIH AIDS Reagent Program) at 4°C overnight. The following day, samples were washed then incubated for 1 hr at room temperature with plasma from an SIV⁺ rhesus macaque (containing anti-SIV antibodies). Lastly, samples were exposed to goat anti-human IgG-PE (Santa Cruz Biotechnology) antibody for 1 hr at room temperature before reading. The concentration of p27 in samples was determined by generating a standard curve from a SIVmac239 viral stock of known concentration [14].

Culture supernatants were assayed for nonhuman primate cytokines and chemokines as previously described [15]. The following analytes were included in the multiplex panel: G-CSF, GM-CSF, Gro- α , IFN- α , IFN- γ , IL-1 β , IL-1Ra, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17, IP-10, MCP-1, MIG, MIP-1 α , MIP-1 β , Perforin, RANTES, sCD40L, TNF- α , TNF- β . Samples were diluted with RPMI-10 at 1:3 or 1:5. Concentrations were determined using human cytokines and chemokines as standards. All plates were read on the Luminex® 100/200 xMAP system (Luminex Corp.) and data was analyzed using MasterPlex™ QT version 1.0 (MiraiBio Corp.)

2.6. Real-time PCR

The sequences for all primers and probes are listed in Table 1. Real-time RT-PCR reactions were prepared using the RNA Ultra Sense™ One-Step Quantitative RT-PCR system (Applied Biosystems, ThermoFisher). Viral loads in supernatant were determined by quantification of SIV *gag* using published primer and probe sequences [16]. Q β bacteriophage (internal control) was detected using primers and probe purchased from the manufacturer (Attostar). The final concentration of SIV *gag* primers and probe were 200 nM and 100 nM, respectively. 5 μ L of template RNA was added to a final reaction volume of 25 μ L. Real-time RT-PCR reactions were carried out as follows: 50°C for 20 min, 95°C for 2 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min. The concentration of SIVmac was determined using a standard curve generated from SIVmac genomic RNA ranging from 10 to 10⁶ copies/ μ L.

For measurement of cell-associated viral RNA and host gene expression, 30–50 ng of template RNA, as determined by spectrophotometry (NanoDrop™-2000), was added to each real-time RT-PCR reaction. Reaction volumes and cycle conditions were as described above. The sequences for *Oncostatin-M (OSM)* primers and probe were obtained from Bruce *et al.* (2005) [17]. Sequences of primers and probes for *MIP1A*, *MIP1B*, and *GAPDH* are listed in the Table 1. A final concentration of 200 nM was used for all primers and 100 nM for all probes. The amount of cell-associated SIVmac and chemokine expression was relatively quantified as 2^{Ct} using endogenous control genes *GAPDH* and *OSM*, respectively.

Real-time PCR reactions for quantification of SIV DNA replication stages were set up per the manufacturer's instructions (PlatinumR Quantitative PCR SuperMix-UDG, Invitrogen,

ThermoFisher). Reactions were carried out in a 25 μ L volume containing 30–50 ng of template DNA. PCR conditions were 50°C for 2 min, 95°C for 2 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min. Primers and probe targeting SIV 2-LTR were designed and used in the assay as described elsewhere [18]. The levels of SIV *gag* and 2-LTR DNA were represented as 2^{-C_t} , using *OSM* as an endogenous control.

Primers and probes were designed on Geneious R7 (Biomatters). All real-time PCR assays were run on an ABI 7500 system. Data was collected and analyzed on the accompanying SDS System Software version 1.5.1.

2.7. Statistics

Prism 6 (GraphPad Software) was used to create figures and perform statistical analysis. The statistical test used for each experiment is noted in the figure legends. Significance was defined as a *P* value of <0.05.

3. Results

3.1. SIVmac251 infection is restricted in baboon PBMC

Previous studies reported baboon PBMC are permissive to SIVmac and SIVMne *in vitro* [8, 9]. We sought to confirm these findings as well as compare the kinetics of SIVmac251 growth between unstimulated and PHA-stimulated PBMC. In accordance with the literature, we observed that although baboon PBMC are susceptible to SIVmac251 infection, virus growth is stunted relative to the kinetics seen in rhesus macaque PBMC (Fig. 1A). The levels of p27 in the supernatant of baboon cultures did not reach the same levels measured in rhesus macaque cultures until about 17 days post-infection (d.p.i.). PBMC from some baboon donors remained largely refractory to SIVmac251. Stimulation with PHA allowed cells from almost all baboon donors to replicate virus, but kinetics were still delayed compared to rhesus macaques (Fig. 1B). All rhesus macaque donors were permissive even without PBMC stimulation.

Restriction of SIVmac251 in baboon PBMC could be explained by a lower abundance of SIV target cells and entry receptors, specifically CCR5⁺CD4⁺ cells which are the major target of SIV infection. However, flow cytometry analysis confirmed that baboons and rhesus macaques had comparable levels of CD4⁺ and CD4⁺CCR5⁺ T cells (Fig. 2A). Furthermore, there were no significant differences in surface expression levels of CD4 or CCR5 on unstimulated CD4⁺ T cells, although PHA stimulation did reduce surface CCR5 on rhesus macaque cells (Fig. 2B). Differences in viral loads between species in the RPMI condition was not due to initial activation status either, as unstimulated baboon and rhesus macaque CD4 cells in PBMC had similar expression of activation markers CD69, HLA-DR, and CD25 (Fig. S1).

It is known that SIV entry can be achieved through alternative co-receptors, such as CXCR6 (Bonzo) and GPR15 (Bob) [19–21]. Therefore, we sought to confirm CCR5 as the principal co-receptor used by SIVmac251 in baboon CD4 cells. Treatment of baboon sorted CD4 cells (Fig. S2A) with maraviroc, a CCR5 antagonist [22], inhibited SIVmac251 growth to nearly undetectable levels, strongly implicating CCR5 as the major entry co-receptor (Fig. S2B).

Thus, despite having similar levels of target cells and entry receptors as rhesus macaques, SIVmac251 replication is less efficient in baboon PBMC.

3.2. SIVmac251 replication in a single-cycle infection is similar in baboon and rhesus macaque isolated CD4 cells

Our flow cytometry analysis revealed baboon and rhesus macaque CD4 T cells have similar levels of CD4 and CCR5 surface expression; however, this does not exclude the possibility that SIVmac251 has a weak capacity to engage baboon receptors. Therefore, we determined if SIVmac251 equally bound baboon and rhesus macaque CD4 cells in isolation.

Quantification of cell-bound viral RNA, while internalization was inhibited by low temperatures, showed no significant difference in the amount of surface-bound SIVmac251 between CD4 cells from both species (Fig. 3A).

We next asked if baboon intracellular factors were responsible for SIVmac251 restriction. Sorted CD4 cells from rhesus macaque and baboon donors were infected in isolation with SIVmac251 and cultured in the presence of the protease inhibitor, darunavir, to confine the virus to a single replication cycle. A real-time PCR assay modeled after Butler *et al.* [23] was used to quantify SIV replication stages over the course of 48 hr. Total SIV DNA was amplified to quantify stages after reverse transcription. The 2-long terminal repeat (2-LTR) circle, a circular form of the HIV/SIV genome, serves as a marker of nuclear transport, as it is formed exclusively in the nucleus and its levels are proportional to the amount of imported viral DNA [24]. Baboon and rhesus macaque CD4 cells had comparable levels of total SIVmac251 DNA (Fig. 3B) and 2-LTR DNA (Fig. 3C) at 12, 24, and 48 h.p.i. Quantification of extracellular SIVmac251 genomic RNA revealed no significant differences in viral release between species (Fig. 3D). Total SIV DNA represents the contribution of nascent linear cDNA, circular DNA, and integrated DNA. As there was no asynchrony in progression from nuclear import to extracellular RNA and total SIV DNA levels were similar between species, it is likely that integrated DNA levels are also similar. Thus, the kinetics of SIVmac251 reverse transcription, nuclear import, integration, and egress are similar between baboon and rhesus macaque CD4 cells in isolation.

3.3. SIVmac251 growth in isolated CD4 cells is comparable between baboons and rhesus macaques

In our previous experiments we limited SIV infection of CD4 cells to a single round of replication. However, the antiviral host defense includes intracellular restriction factors, such as APOBEC3 and SERINC3/5, which act on subsequent rounds of replication [25–27]. We evaluated the potential contribution of such processes in restricting SIVmac251 in baboons by performing a multi-round infection in isolated CD4 cells. We observed that, in the absence of other cell types, SIVmac251 grows to the same efficiency in baboon and rhesus macaque CD4 cells (Fig. 4A). Noticeable, and in contrast to the previous observations in PBMC, the majority of baboon CD4 cell donors were permissive to infection without PHA stimulation.

When evaluating SIVmac251 replication kinetics in rhesus macaque cultures, it was clear that PBMC and CD4 cells from all donors were largely permissive to infection. There was

no evidence of strong contributions from other immune cell types in restricting viral replication in rhesus macaque PBMC, as SIV growth kinetics in PBMC were comparable to those in isolated CD4 cells (Fig. 4B). Similar to rhesus macaques, there was little variation in viral growth kinetics among most baboon CD4 cell donors (Fig. 4C); however, and differently from what was seen for rhesus macaque cells, roughly 50% of baboon PBMC donors were refractory to SIVmac251 infection when compared to CD4 cells. To address if restriction of SIVmac251 in baboon PBMC but not in isolated CD4 cells was consistent between donors, we contemporaneously infected PBMC and CD4 cells isolated from the same donor. In accordance with our previous data, PBMC from all baboon donors were susceptible to infection but grew the virus to varying titers by 17 d.p.i. ($4.50 \times 10^4 \pm 5.09 \times 10^4$ pg/mL of p27). In contrast, CD4 cell viral loads were more uniform across all donors and were roughly 2 logs higher than in PBMC at peak infection ($5.53 \times 10^6 \ll 2.37 \times 10^6$ pg/mL p27) (Fig. 4D).

Because a considerable number of wild and captive baboons harbor simian T-lymphotropic virus (STLV) [28–30], we evaluated if underlying STLV infection was associated with the observed variability in SIVmac251 growth kinetics between baboon donors. No significant difference was observed in SIVmac251 replication between PBMC from age-matched STLV- and STLV+ baboon donors (Fig. 4E), indicating STLV status does not impact the outcome of SIVmac251 infection in baboon PBMC.

Our findings from isolated baboon CD4 cell infections demonstrate a very permissive environment and suggest that intracellular restriction factors are likely not responsible for the inhibition of SIVmac251 replication observed in baboon PBMC.

3.4. CD8 T and NK cells restrict SIVmac251 infection in baboon CD4 cells

We hypothesized that inhibition of SIVmac in baboon PBMC could be mediated by other immune cell types present in PBMC but absent in sorted CD4 cultures. To mimic the baboon PBMC environment in a more controlled fashion as well as identify key SIV-suppressive cell types, we performed add-back studies in CD4 cultures. CD8 T and NK cells were of primary interest because of their canonical roles in antiviral defense. CD4, CD8 T, and NK cells were sorted from PBMC isolated from baboons and rhesus macaques (Fig. S3). CD4 cells were infected in isolation then co-cultured with autologous CD8 T and NK cells in various combinations, at ratios reflective of their relative abundance to CD4 cells in baboon [13] and rhesus PBMC. The input and concentration of CD4 cells was normalized across all conditions. As expected, CD4 cells cultured alone had the highest levels of p27 at peak infection. The presence of NK cells had varying degrees of impact on SIVmac251 replication between baboon and rhesus macaque donors (Fig. 5A and 5B). Interestingly, the addition of CD8 T cells to baboon CD4 cultures could reduce viral loads by an average of 50% whereas CD8 T cells in rhesus macaque CD4 cultures had no significant effect on reducing viral loads. It is important to note that all donors were SIV-naïve, and so restriction of SIVmac by baboon CD8 T cells was not due to a cellular memory response as has previously been reported for other viral strains [12].

We asked if the SIV-suppressive effects of CD8 T and NK cells in baboons were contact-dependent. Compared to cultures that had CD8 T or NK cells in contact, restriction of

SIVmac251 was diminished when CD8 T and NK cells were segregated from infected CD4 cells by a semi-permeable membrane (Fig. 5C). Here, we also observed CD4 cultures containing both CD8 T and NK cells had peak p27 levels that were, on average, 75% lower than in cultures containing CD4 cells alone. This finding was consistent across all baboon donors. Contact-dependent inhibition of SIVmac251 replication in baboon CD4 cells was not due to cytotoxic responses, as there was no significant loss of CD4 cells during co-culture with CD8 T or NK cells (data not shown). Altogether, these studies show baboon CD8 T cells require contact with CD4 cells to mediate control of SIVmac251 through a mechanism of innate immunity.

3.5. Baboon PBMC produce higher levels of CCR5-binding chemokines

Lastly, we analyzed the cytokine milieu in baboon cultures to aid in the identification of mechanisms responsible for SIVmac251 restriction. Our Luminex multiplex platform included 22 analytes that varied in classification and function (cytokines, chemokines, growth factors, cytolytic proteins, etc.). Early after infection, there were no significant differences in levels of most analytes between these species including key antiviral response regulators, TNF- α and IFN- γ (Fig. S4A). However, three chemokines that showed significantly different levels between rhesus macaque and baboon cells were MIP-1 α (macrophage inflammatory protein-1 alpha, CCL3), MIP-1 β (CCL4), and RANTES (regulated on activation, T cell expressed and secreted, CCL5), which were of early interest because they are the natural ligands for CCR5, the primary co-receptor used by HIV and SIV for entry into CD4 cells [31]. The levels of MIP-1 α , MIP-1 β , and RANTES were roughly 10-fold higher in baboon PBMC than in rhesus macaque PBMC at 3 d.p.i. (Fig. 6A). High production of these chemokines by baboon PBMC was independent of infection, as levels were similar in uninfected cultures. This trend was also evident before infection, particularly for MIP-1 α (Fig. S4B). Differences between baboon and rhesus macaque MIP-1 α and MIP-1 β production were confirmed at the mRNA level, indicating the high chemokine concentration measured in baboon cultures was not due to differences in binding to baboon or rhesus macaque molecules by the anti-human chemokine antibodies used in our Luminex assays (Fig. S5).

We then evaluated the contribution of MIP-1 α , MIP-1 β , and RANTES in suppressing SIVmac251 infection in baboon PBMC by inhibiting their activity with neutralizing antibodies. Our preliminary studies showed that neutralization of a single CCR5-binding chemokine had little to no effect on p27 levels (data not shown). However, simultaneous neutralization of all three chemokines in baboon cultures increased viral loads at 10 d.p.i. by roughly 2-fold in unstimulated PBMC (Fig. 6B) and 4 fold in PHA-stimulated PBMC (Fig. 6C); this effect could be seen at an earlier time in infection (6 d.p.i.) (Fig. S6). Furthermore, we observed a statistically significant correlation between increased CCR5-binding chemokine production and reduced viral loads in baboon PBMC (Fig. 6D). None of these findings were observed in rhesus macaque PBMC cultures, which also lacked an inverse correlation between chemokine production and viral loads (Fig. 6E).

Thus, the combined effects of MIP-1 α , MIP-1 β , and RANTES contribute to early restriction of SIVmac in baboon PBMC.

3.6. CD8 T and NK cells increase CCR5-binding chemokine levels in baboon CD4 cell cultures

Our add-back studies in isolated baboon CD4 cell cultures demonstrated CD8 T cells contribute to restriction of SIVmac251. CD4 T, CD8 T, and NK cells have all been reported to produce antiviral concentrations of MIP-1 α , MIP-1 β , and RANTES [31–36]. Therefore, we asked if CD8 T cells reduced viral loads by boosting production of CCR5-binding chemokines. Cytokine analysis showed baboon CD4 cells in isolation produced MIP-1 α , MIP-1 β , and RANTES (Fig. 7A). By contrast, the majority of CD8 T cells cultured alone produced undetectable levels of these chemokines. In some donors, the addition of just NK cells to CD4 cultures moderately elevated production of CCR5-binding chemokines (Fig. 7A). However, the concentrations of all chemokines increased in CD4 cultures by approximately 2- to 4-fold in the presence of CD8 T cells. The addition of CD8 T or NK cells to rhesus macaque CD4 cultures did not have a significant impact on elevating CCR5-binding chemokine production, and the levels of CCR5 binding chemokines were much lower than in baboons, as observed before for PBMC cultures (Fig 7B).

Lastly, we extended our analysis to transwell co-cultures where we observed contact between CD4, CD8 T, and NK cells provided maximum suppression of SIVmac251 (Fig. 5C). Interestingly, this condition also had the highest levels of MIP-1 α , MIP-1 β , and RANTES (Fig 7C). Segregation of CD8 T and NK cells from infected CD4 cells abrogated higher production of CCR5-binding chemokines, which was mirrored by a lack of SIVmac251 suppression. These findings support a model whereby interactions between baboon CD8 T and CD4 cells allow elevated production of CCR5-binding chemokines that suppress SIVmac growth by interfering with co-receptor binding.

4. Discussion

Although noninvasive studies in the wild have suggested SIVcpz infection is pathogenic for chimpanzees [37], studies of wild or captive African green monkeys, sooty mangabeys, and mandrills show a general lack of disease during SIV infection [2, 3, 38–40]. Nonpathogenic infection has long been a key area of HIV research to aid in the development of AIDS therapies. Studies in these animals could provide some insight into treating or preventing HIV-associated pathologies. Nevertheless, natural NHP hosts fail to control viral replication and thus are not an ideal model to uncover mechanisms for preventing or clearing infection. Although more than 90% of the African NHP species tested have been shown to be infected with an SIV [41], wild baboons do not harbor SIV, even though a significant portion of their diet comprises African green monkeys, a species where SIV prevalence is roughly 50% [42]. Baboon resistance to SIV challenge *in vivo* was first reported in 1988 [7]. Nearly 30 years later, the mechanisms underlying resistance still remain unclear. Using *in vitro* SIVmac infections, we sought to uncover mechanisms of SIV restriction in baboons that could explain two previously reported findings: (1) Baboon PBMC support SIVmac growth *in vitro* but replication kinetics are dampened (2) Baboons are susceptible to SIVmac *in vivo* but do not develop AIDS. A recent study identified immunogenetic factors in natural hosts that were highly divergent from rhesus macaques and so may have a strong role in preventing AIDS [43]. It would be tempting to speculate baboons resist disease during

SIVmac infection by these mechanisms identified in natural hosts. However, this study revealed baboons have more homology to macaques than to African green monkeys or sooty mangabeys in these immunogenetic factors heavily implicated in AIDS resistance. An earlier study by Apetrei *et al.* also showed baboon T cells share more immunophenotypic features with rhesus macaques than natural hosts [44]. Thus, baboons likely resist SIVmac-induced AIDS and infection with other SIV strains through mechanisms distinct from natural hosts, which may include direct control of viral replication.

In this study, we used *in vitro* infections to identify key cell types and mechanisms responsible for SIVmac251 restriction in baboon PBMC. Although this *in vitro* system does not represent what may occur *in vivo*, PBMC infection has been shown to be an accurate predictor of the outcome of infection in rhesus macaques exposed to SIV [45, 46]. In accordance with our findings, other groups have reported dampened SIVmac growth kinetics in baboon PBMC relative to rhesus macaque PBMC [8, 9]. As PHA stimulation is often used to enhance *in vitro* retroviral infection by activating T cells and triggering cell division, we also compared SIVmac251 growth kinetics in unstimulated and stimulated PBMC from both species. PHA-stimulated baboon and rhesus macaque PBMC did show increased viral loads and growth kinetics relative to donor-matched unstimulated PBMC; however, SIVmac251 was still detectable in PBMC from both species even without stimulation. This allowed us to maintain cells in a natural state while interrogating innate antiviral responses that could otherwise be clouded by initial activation with PHA. Indeed, the difference in SIVmac251-suppressive activity between baboons and rhesus macaques was more apparent in unstimulated PBMC.

We evaluated if differences in the abundance of SIV target cells and entry receptors were responsible for dampened SIVmac251 infection in baboon PBMC. Pandrea *et al.* previously showed natural NHP hosts for SIV have a lower frequency of CD4⁺CCR5⁺ T cells and reduced CCR5 expression on CD4⁺ T cells compared to nonnatural NHP hosts. In this same study, the authors reported that baboons and rhesus macaques had comparable levels in both parameters [47]. We confirmed the latter findings in a larger baboon cohort as well as established similar levels of CD4⁺ T cells and surface CD4 expression on CD4⁺ T cells in baboon and rhesus macaque PBMC. Furthermore, we demonstrated CCR5 to be the main co-receptor used by SIVmac251 on baboon cells, since treatment with maraviroc prevented infection.

It is known that host intracellular restriction factors, such as APOBEC3G and TRIM-5 α , can block infection by interfering with different stages of viral replication; viruses, in turn, have adapted strategies to overcome mechanisms of host restriction [25]. As a consequence of this co-evolution, restriction factors accumulate species-specific amino acid differences which can impose barriers to cross-species transmission [48, 49]. Thus, we explored if baboon intracellular restriction factors were more effective against SIVmac251 than those from rhesus macaques. Here, in synchronized single-cycle infections, there were no significant differences in SIVmac251 replication kinetics between baboon and rhesus macaque CD4 cells, as measured by the levels of 2-LTR circles (indicating efficient nuclear transport) and viral loads in the supernatant (indicating a lack of interference with viral budding). In contrast to the delay in SIVmac251 replication seen in baboon PBMC, virus growth in a

multi-round infection was unrestricted in baboon isolated CD4 cells. Taken together, these observations indicate intracellular restriction factors, typically associated with species adaptation, do not play a significant antiviral role against SIVmac251 in baboons. Future work will be aimed at evaluating if these observations are applicable across other strains of SIV, such as SIVagm.

Unrestricted growth of SIVmac251 in baboon isolated CD4 cells raised the possibility that suppression of SIVmac251 in baboon PBMC is mediated by cell types removed during enrichment for CD4 cells. Our add-back studies established contributions from baboon CD8 T in restricting SIVmac251 growth in a contact-dependent manner. While CD8 T or NK cells alone could modestly reduce CD4 cell viral loads, the strongest inhibition of viral growth was seen in CD4 cultures containing both CD8 T and NK cells. While this dramatic response could be an additive effect, evidence of synergy between cell types was observed. For some baboon donors, the addition of NK cells alone had no impact on viral loads, but when NK cells were added to CD4 cultures containing CD8 T cells, SIVmac251 suppression was boosted beyond that observed by adding only CD8 T cells. These observations point to a potentially complex interplay between all cell types, whereby the antiviral or immunomodulatory activity of one cell type is activated or amplified in the presence of a different cell type.

Suppression of HIV and SIV replication by CD8 T and NK cells is well-known [50, 51]. CD8 T cells, in particular, have been reported to have a strong role in controlling SIV and HIV-2 infection in rhesus macaques and baboons, respectively [12, 52–54]. However, in these previous studies, significant suppression of viral replication *in vitro* was seen by CD8 T cells from infected but not uninfected animals, indicative of major contributions from an antigen-specific memory response; in addition, suppression was largely attributed to cytolytic activity [12, 52]. Our *in vitro* studies did not show any significant inhibition of SIVmac growth by CD8 T cells from SIV-naïve rhesus macaques. On the contrary, the strong viral inhibition by naïve baboon CD8 T cells demonstrates major contributions from components of innate immunity. Furthermore, we observed little to no CD4 cell loss in SIV-infected baboon cultures containing CD8 T cells, suggesting direct lysis of infected cells does not play a major role in the contact-dependent suppression of SIVmac251 growth by these baboon cell types. In support of our findings, several studies have reported contributions from non-cytolytic CD8 T cell responses in controlling HIV and SIV production [55, 56]. Currently, we are elucidating the role of these and other innate effector functions of baboon CD4 and CD8 T cells.

While interrogating the cytokine milieu, we identified elevated CCR5-binding chemokines as one mechanism of SIVmac251 restriction in baboon cells. When in culture, whether stimulated or not, baboon PBMC produced higher levels than rhesus macaques of CCR5 ligands MIP-1 α , MIP-1 β , and RANTES, which can all interfere with HIV and SIV entry by hindering access to docking sites or causing receptor internalization [31, 57, 58]. Neutralization of these chemokines increased viral loads in baboon PBMC but not in rhesus macaque PBMC, demonstrating their importance in limiting virus spread in baboon PBMC. Previous studies have shown CD4 T and CD8 T cells can serve as sources of MIP-1 α , MIP-1 β , and RANTES during HIV infection [31–36]. Analysis of baboon co-cultures

revealed CD4 cells in isolation could produce these chemokines, but levels were highest in CD4 cultures containing CD8 T cells, with some contribution from NK cells. Although baboon CD4 cells alone produce higher levels of CCR5-binding chemokines than any of the rhesus macaque conditions, additional production from CD8 T cells may be necessary to reach antiviral concentrations of these chemokines in baboon cultures, which could partially explain why SIVmac growth was unrestricted in isolated baboon CD4 cells when compared to rhesus macaques. The impact of soluble factors in restricting viral growth is commonly addressed using transwells. In this regard, we saw no restriction of SIVmac251 when CD8 T and NK cells were segregated from CD4 cells. However, we uncovered that contact between these cell types was required for elevated production of CCR5-binding chemokines, which was mirrored by a reduction in viral loads. Although CD4 cells can be a source of MIP-1 α , MIP-1 β , and RANTES in baboon PBMC, CD8 T cells may elevate levels to antiviral concentrations by also producing these chemokines or by promoting their production by CD4 cells. Identification of the major cell source(s) of CCR5-binding chemokines as well as the signals that boost their production in baboon PBMC will require future investigation.

The ability of baboon PBMC to produce higher levels of MIP-1 α , MIP-1 β , and RANTES than rhesus macaque PBMC *in vitro* is, to our knowledge, a novel finding that may represent a mechanism for both limiting SIV replication and, by extension, preventing infection-associated pathology *in vivo*. Indeed, previous reports of pathogenic infections of baboons with HIV-2 employed a dual-tropic virus, which could circumvent CCR5 restriction [11, 59]. The relevance of CCR5 ligands in controlling HIV infection and disease in humans has been previously explored. Numerous studies have reported better clinical profiles in HIV-infected individuals whose cells produce higher levels of MIP-1 α and/or MIP-1 β *in vitro* [60–62]. A polymorphism in the *RANTES* promoter results in increased transcription activity and is associated with slower progression to AIDS [63, 64]. Naturally, chemokine-mediated inhibition of HIV and SIV entry has served as a springboard for the development of HIV therapeutics. Maraviroc, a CCR5-antagonist, was approved in 2007 for treatment of CCR5-tropic HIV infection; when used as a component of combined antiretroviral therapy, maraviroc could reduce viral loads by over 1 log [65].

We can only speculate on the conditions that favored the emergence of high CCR5 ligand production in baboons. As one possibility, specific underlying microbial infections could activate an immune response that includes elevated production of CCR5-binding chemokines. Studies have shown HIV patients infected with *Orientia tsutsugamushi* (bacterium that causes scrub typhus) [66, 67] or Hepatitis G/GB virus C [68, 69] have reduced viral loads and/or higher CD4 T cell counts. *In vitro* stimulation of PBMC with either pathogen increased secretion of RANTES [70, 71]. Although we found STLTV-1 status had no impact on the outcome of SIVmac infection in baboon PBMC, protection from SIV may be elicited by other infections currently endemic in wild and captive baboon colonies. Alternatively, baboons may have evolved a higher capacity for chemokine production to overcome now extinct microbes.

One limitation of using PBMC to identify SIV-suppressive cell types is the exclusion of other immune cell types, including granulocytes. Investigation into the potential impact of these cell types in baboon SIV resistance could uncover other antiviral mechanisms. In

addition, *in vitro* systems do not model the complexities of *in vivo* HIV and SIV infection. Our findings have provided insight into the key cell types responsible for baboon restriction of SIVmac251 *in vitro* and as such, will be the focus of future investigation *in vivo*.

To summarize, we have shown SIVmac251 growth is inhibited in the mixed-cell environment of baboon PBMC but not in isolated CD4 cells. By contrast, rhesus macaque PBMC and CD4 cells were equally permissive for viral growth. CD8 T cells provided suppression of SIVmac251 infection in baboon CD4 cells. We identified one mechanism of restriction that involves a high capacity for production of MIP-1 α , MIP-1 β , and RANTES, chemokines that limit SIV infection by interfering with viral binding to CCR5. Baboon resistance to SIV is likely mediated by multiple independent mechanisms but these studies have provided the foundation of our search for other factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- PBMC and CD4 cells from naïve rhesus macaques are equally permissive to SIVmac.
- SIVmac is restricted in PBMC but not in isolated CD4 cells from naïve baboons.
- Baboon, but not rhesus macaque, CD8 T cells suppress of SIVmac replication.
- Suppression by baboon CD8 T cells is due to components of innate immunity.
- CD8 T cells elevate levels of CCR5 ligands that interfere with SIV entry.

FIG 1.

SIVmac251 growth is restricted in baboon PBMC. PBMC were isolated from whole blood of rhesus macaques (open circles) or baboons (solid circles) then cultured for 48 hr in (A) RPMI-10 or (B) RPMI-10 containing PHA. PBMC were then infected with SIVmac251 at an M.O.I. of 0.01. Viral loads were quantified by measuring p27 in the supernatant by Luminex. In both panels, each curve represents one animal ($n = 10$). Repeated measures two-way ANOVA was used for statistical analysis (* $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$).

FIG 2.

Baboon and rhesus macaque PBMC have similar levels of SIV target cells and entry receptors. PBMC were isolated from whole blood of rhesus macaques (open circles, $n = 9$) and baboons (solid circles, $n = 8$). PBMC were phenotyped by flow cytometry following isolation (Fresh) and after 48 hr in culture with RPMI-10 (RPMI) or RPMI-10 containing PHA. A) Percentage of CD4⁺ T cells within lymphocytes and percentage of CCR5⁺ cells within CD4⁺ T cells. B) Median fluorescence intensity (MFI) of CD4 and CCR5 expression on CD4⁺ T cells. In all panels, lines indicate the median and each point represents one animal. Mann-Whitney tests were used for statistical analysis (*** $P < 0.001$). n.s.: not significant.

FIG 3.

The levels of SIVmac251 binding, intracellular replication, and egress are similar between baboon and rhesus macaque isolated CD4 cells. CD4 cells were sorted from baboon (dark bars) or rhesus macaque (light bars) PBMC and cultured for 48 hr before infection. Cells were infected with SIVmac251 at an M.O.I. of 1; viral binding was synchronized by keeping cells at 4°C for 1 hr. (A) Cells were washed twice before RNA extraction; SIV RNA was measured by real-time RT-PCR, using GAPDH mRNA to normalize RNA input and for relative quantification (2^{-C_t}) ($n = 5$). (B-C) After incubation at 4°C, cells were placed at 37°C for 1 hr to allow entry. A protease inhibitor, darunavir, was added to cultures to restrict the virus to a single replication cycle. Cells were lysed at indicated time points and SIV DNA was measured by real-time PCR using Oncostatin-M gene to normalize DNA input ($n = 3$). (D) Supernatant was harvested at 48 h.p.i. and SIV RNA was quantified by real-time RT-PCR ($n = 5$). In all panels, bars represent the mean $\pm$ SD. n.s.: not significant.

FIG 4.

SIVmac251 growth is similar in baboon and rhesus macaque isolated CD4 cells. (A) CD4 cells were sorted from baboon (closed triangles, $n = 10$) or rhesus macaque (open triangles, $n = 8$) PBMC, cultured for 48 hr in RPMI-10, then infected with SIVmac251 at a M.O.I of 0.01. (B-C) Species-matched compilation of viral loads data from infections in PBMC (shown in Fig. 1A) and sorted CD4 cells (shown in Fig. 4A). (D) PBMC and sorted CD4 cells from the same baboon donor ($n = 5$) were infected with SIVmac251 at a MOI of 0.01. (E) PBMC were isolated from STLV seronegative ($n = 5$) and seropositive ($n = 5$) baboon donors of the same age (4 y/o). PBMC were infected with SIVmac251 at a MOI of 0.01. Viral loads were quantified by measuring p27 in the supernatant by Luminex. In all panels, each curve represents one animal. Repeated measures two-way ANOVA was used for statistical analysis (* $P < 0.05$, **** $P < 0.0001$).

FIG 5.

CD8 T cells suppress SIVmac251 infection in baboon CD4 cells. Sorted CD4 cells were infected with SIVmac251 and cultured in isolation or with the addition of autologous CD8 T and NK cells. (A-B) The same number of SIV-infected CD4 cells from baboons ($n = 6$) or rhesus macaques ($n = 3$) were cultured alone, with CD8 T cells, or with NK cells. (C) CD8 T and NK cells were cultured with infected CD4 cells in contact or separated by a 0.4 μ m membrane (Transwell). In all panels, viral loads were quantified by measuring p27 in the supernatant by Luminex. Bar graphs display SIV p27 values normalized to the CD4 alone condition at peak infection (10–14 d.p.i.) (mean $\pm$ SEM). One-way ANOVA was used for statistical analysis (* $P < 0.05$, **** $P < 0.0001$). n.s.: not significant.

FIG 6.

Elevated production of CCR5-binding chemokines contributes to SIVmac251 restriction in baboon PBMC. (A) Identification of MIP-1 α , MIP-1 β , and RANTES in PBMC supernatants at 3 d.p.i. PBMC were isolated from baboons (dark bars) and rhesus macaques (light bars) and cultured for 48 hr in medium alone (RPMI) or medium containing PHA then infected with SIVmac251 at an M.O.I. of 0.01 ($n = 7$). Mann-Whitney tests were used for statistical analysis. Top and bottom of boxes represent first and third quartile, band is median, whiskers show minimum and maximum values. (B-C) PBMC were infected with SIVmac251 at an M.O.I. of 0.01. Cultures were treated with medium, normal goat serum, or goat polyclonal anti-chemokine neutralizing antibodies. Viral loads were assessed by measuring p27 in the supernatant by Luminex. Bar graphs display p27 MFI values normalized to the untreated condition at peak infection (10 d.p.i.) (mean $\pm$ SEM) (RPMI: $n = 6$, PHA: $n = 5$). Paired t-test was used for statistical analysis. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (D-E) Correlation analysis for total CCR5-binding chemokine concentration at 3 d.p.i. versus log of SIV p27 concentrations at 10 d.p.i. in baboon (closed circles, $n = 14$) or rhesus macaque (open circles, $n = 13$) PBMC. Line represents linear regression and each point represents a different donor.

FIG 7.

CD8 T and NK cells contribute to high levels of CCR5-binding chemokines in baboon cultures. Luminex cytokine assays performed on supernatants obtained from cultures at 3.d.p.i. (A) CD8 T and NK cells were cultured alone, with uninfected or infected CD4 cells in the indicated combinations ($n = 4 - 6$). (B) Infected CD4 T cells from baboons ($n = 6$) and rhesus macaques ($n = 3$) were cultured with autologous CD8 T and NK cells. Graph shows total CCR5-binding chemokine levels. (C) CD8 T and NK cells were cultured with infected CD4 cells separated by a 0.4 μ m membrane (Transwell) or in contact ($n = 6$). For box and whiskers plots, top and bottom of box represent first and third quartile, band is median, whiskers show minimum and maximum values. One-way ANOVA was used for statistical analysis (* $P < 0.05$).

Table 1

Sequence of primers used for RT-PCR and PCR.

Name ⁺	Sequence (5'f to 3'f)
SIV gag [16]	
SIVgag510F	GCC AGG ATT TCA GGC ACT GT
SIVgag592R	GCT TGA TGG TCT CCC ACA CAA
SIVgag535P	(FAM)-AAG GTT GCA CCC CCT ATG ACAT TAA TCA GAT GTT A-(TAMRA)
SIV 2-LTR [18]	
2LTRF	TAA GCT AGT GTG TGT TCC CAT
2LTRR	CTC CTG TGC CTC ATC TGA TAC A
2LTRP	(FAM)-AGC CGC CGC CTG GTC AAC TCG-(TAMRA)
MIPIA/CCL3	
MIP1A17F	CCG GCA GAT TCC ACA GAA TT
MIP1A247R	TCT GGA CCC ACT CCT TAC TG
MIP1A192P	(FAM)-ACC TGC CGG CCT CTC TTG GTT AGG-(TAMRA)
MIP1B/CCL4	
MIP1B13F	GTG ACT GTC CTG TCT CTC CT
MIP1B143R	ACA AAG TTG CGA GGA AGC TT
MIP1B86P	(FAM)-ACC CTC CCA CCT CCT GCT GCT-(TAMRA)
GAPDH	
G590F	CAA CAG CCT CAA GAT CGT CA
G697R	GTG GTC ATG AGT CCT TCC AC
G633P	(FAM)-TGC TTA GCA CCC CTG GCC AAG GT-(TAMRA)
OSM [17]	
OSMF	CCT CGG GCT CAG GAA CAA C
OSMR	GGC CTT CGT GGG CTC AG
OSMP	(VIC)- TAC TGC ATG GCC CAG CTG CTG GAC AA-(MGBNFQ)

⁺F = forward primer, R = reverse primer, P = probe