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Abbreviations used in this article: C/EBP, CAAA T enhancer binding protein; CT, cycle transcription factors such as STAT3 and C/EBP

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Abbreviations used in this article: C/EBP, CAAA T enhancer binding protein; CT, cycle threshold; DPI, d postinfection; GI, gastrointestinal;IEL, intraepithelial lymphocyte; LPL, lamina propria leukocyte; miRNA, micro-RNA; MTMR6, myotubularin-related protein 6; MUT, mutated; qRT-PCR, quantitative RT-PCR; RT, reverse transcription; TLDA, TaqMan low density arrays; TNPRC, Tulane National Primate Research Center; WT, wild-type.

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HIV replication and the cellular micro-RNA (miRNA) machinery interconnect at several posttranscriptional levels. To understand their regulatory role in the intestine, a major site of HIV/SIV replication, dissemination, and CD4+ T cell depletion, we profiled miRNA expression in colon following SIV infection (10 acute SIV, 5 uninfected). Nine (four up and five down) miRNAs showed statistically significant differential expression. Most notably, miR-190b expression showed high statistical significance (adjusted $p = 0.0032$), the greatest fold change, and was markedly elevated in colon and jejunum throughout SIV infection. In addition, miR-190b upregulation was detected before peak viral replication and the nadir of CD4+ T cell depletion predominantly in lamina propria leukocytes. Interestingly non–SIV-infected macaques with diarrhea and colitis failed to upregulate miR-190b, suggesting that its upregulation was neither inflammation nor immune-activation driven. SIV infection of in vitro–cultured CD4+ T cells and primary intestinal macrophages conclusively identified miR-190b upregulation to be driven in response to viral replication. Further miR-190b expression levels in colon and jejunum positively correlated with tissue viral loads. In contrast, mRNA expression of myotubularin-related protein 6 (MTMR6), a negative regulator of CD4+ T cell activation/proliferation, significantly decreased in SIV-infected macrophages. Luciferase reporter assays confirmed MTMR6 as a direct miR-190b target. To our knowledge, this is the first report, which describes dysregulated miRNA expression in the intestine, that identifies a potentially significant role for miR-190b in HIV/SIV pathogenesis. More importantly, miR-190b–mediated MTMR6 downregulation suggests an important mechanism that could keep infected cells in an activated state, thereby promoting viral replication. In the future, the mechanisms driving miR-190b upregulation including other cellular processes it regulates in SIV-infected cells need determination. The Journal of Immunology, 2014, 193: 1301–1313.
(Drosha and DGC8) to precursor miRNAs and subsequently in the cytoplasm by Dicer to generate the mature miRNAs (21). Recent studies have attributed more significant roles for these small RNA molecules in regulating the immune response that includes immune cell development, proliferation, activation, and differentiation (18, 22–26). Furthermore, several in vitro studies clearly show that HIV and the host miRNA machinery cross-talk at several posttranscriptional levels (27–31). The restraints exerted by the host miRNA machinery on HIV replication is clearly evident from the finding that viral replication kinetics are enhanced in PBMCs following siRNA-mediated knockdown of Drosha and Dicer (32). In addition, several recent miRNA profiling studies performed on purified CD4+ T cells, macrophages, and PBMCs of HIV-infected patients provide compelling evidence of their dysregulation in HIV infection (33–36). Recent studies also have described dysregulated miRNA expression in the brain (37) and plasma of SIV-infected rhesus macaques (38) including miRNAs that directly targeted SIV replication in macrophages (39). Accordingly, we hypothesized that SIV replication in the GI tract in association with the ensuing immune response induces the expression of miRNAs that have the potential to regulate viral replication, including host genes expressed during the immune response. Given the lack of information on the expression of miRNAs in the GI tract in response to SIV infection, we performed global miRNA expression profiling in the intestine of acutely SIV-infected macaques with a focus on the colon. Expression of nine miRNAs was significantly altered in the colon following acute SIV infection. Furthermore, miR-190b upregulation was detected as early as 7 d post-infection (Table I), tenaciously SIV-infected animals (Table I) and five uninfected control animals included 33 animals infected with pathogenic strains of SIV that received a complete necropsy. Tissue samples (colon and jejunum) from all control, SIV-infected, and non-SIV-infected macaques with diarrhea and colitis were collected in RNAlater (Life Technologies, Grand Island, NY) over several years (6–7 y) and stored at −20°C for total RNA extraction. Fourteen samples of the jejunum (6–8 cm from the pyloric ring), serial resections (2 cm), and eight samples of colon resections (2 cm) were collected from eight Indian-origin rhesus macaques (HC36, HB31, HF27, HB48, GK31, GA19, HR57 and HV95) at 6 wk before SIV infection and 21 and 90 d after SIV infection for TLDA and miR-190b RT-PCR confirmation studies. For histopathological evaluation, colon and jejunum tissues were collected immediately after euthanasia and fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 μm and stained with hematoxylin and eosin. Histological evaluation included the blinded fashion, and inflammation was scored semiquantitatively on a scale of 0–3 as follows: 0, within normal limits; 1, mild; 2, moderate; and 3, severe. In addition, the presence of crypt dilatation, villus blunting, diverticulosis, and amyloidosis were recorded (Table II).

Global microRNA profiling using TaqMan Low Density Arrays

Total RNA was extracted from intact colon and jejunum samples using the miRNeasy total RNA isolation kit (Qiagen). RNA integrity was assessed by running an aliquot on a denaturing agarose gel, followed by staining with ethidium bromide to visualize intact 28S and 18S rRNA bands. For TLDA miRNA profiling, ∼350 ng total RNA from intact colon tissue was first reverse transcribed following the ABI microRNA TLDA reverse transcription reaction protocol. Briefly, two master mixes were prepared for each RNA sample representing either TLDA panel (panels A and B) and complementary DNA (cDNA) was prepared from the following components: including random priming, reverse transcription (RT) primers (10 times), 0.20 μM 2′-deoxynucleoside 5′triphosphates with 2′deoxyxymidine 5′triphosphate (100 μM), 1.50 μM MultiScribe reverse transcriptase (50 U/μl), 0.80 μl 10× RT buffer, 0.90 μl MgCl2 (25 mM), 0.10 μl RNase inhibitor, and 0.20 μl nuclelease-free water (20 μl/μl). Three microliters of total RNA (350 ng) was loaded into appropriate wells of a 96-well plate containing 4.5 μl of the RT reaction mix, and following a brief HIV1 reverse transcriptase was subjected to the following thermal cycling conditions on the ABI 7900 HT Fast PCR system: standard or max ramp speed, 16°C for 2 min, 42°C for 1 min, 50°C for 1 s (40 cycles), and 85°C for 5 min (hold). Approximately 2.5 μl of the resulting cDNA from each sample was mixed with a total of 22.5 μl preamplification reaction mix consisting of 12.5 μl TaqMan PreAmp Master Mix (2 times), 2.5 μl Megaplex PreAmp Primers (10 times), and 7.5 μl nuclelease-free water and preamplified on the ABI 7900 HT Fast PCR system, according to the TLDA miRNA preamplification protocol from the manufacturer (Life Technologies). The preamplification thermal cycling conditions were as follows: hold 95°C for 10 min; hold 55°C for 2 min; hold 72°C for 2 min; 12 cycles at 95°C for 15 s; and 60°C for 4 min. The preamplified product was first diluted 4-fold with 75 μl 0.1× TE (pH 8) mixed, following which 9 μl of the diluted PreAmp product was mixed with 450 μl 2× Taqman Universal PCR Master Mix with no uracil-N-glycosylase (Amperase) and 441 μl nuclelease-free water to bring the final volume to 1 ml. After proper mixing and centrifuging, 100 μl of the PCR mix was loaded into each port of the TaqMan Array Human MicroRNA A+B Card Set version 3.0. The TLDA cards were then centrifuged, sealed, and processed on the ABI 7900 HT Sequence Detection System using the 384-well TaqMan Low Density Array default thermal-cycling conditions.

Quantitative Real-Time TaqMan Stem loop miRNA and SYBR Green RT-PCR

Expression of miR-190b was further investigated in both colon and jejunum through the course of SIV infection using the TaqMan microRNA predesigned and preoptimized assays (Life Technologies). Total RNA (500 ng for miR-190b and RNU48 and 100 ng for snoU6 [colon and jejunum], 250 ng for CD4+ T cells and primary intestinal macrophages) was reverse transcribed using the stem loop primers provided in the predesigned kit in a total reaction volume of 15 μl. The RNA to cDNA conversion reactions (5–75°C) have been previously used for miRNA quantitative RT-PCR (qRT-PCR) (42, 43). Approximately 4 μl cDNA was subjected to 40 cycles of PCR in a total volume of 20 μl on the ABI 7900 HT Fast PCR System (Life
Technologies) using the following thermal cycling conditions: 95°C for 10 min, followed by 40 repetitive cycles of 95°C for 15 s and 60°C for 1 min. As a normalization control for RNA loading, parallel reactions in duplicate wells to amplify RNU48 or snoU6 or RNU44 in combination with RNU48 (CD4+ T cells) were run in the same or different multwell plate. Although RNU48 worked well for intact intestine, we found snoU6 to be better when analyzing miRNA expression in distinct intestinal mucosal compartments such as epithelium and LPLs. RNU44 was used for CD4+ T cells because it has been previously used for normalization of miRNA expression in HIV-infected CD4+ T cells (44). Comparative real-time PCR was performed in duplicate wells including no template controls, and relative change in gene expression was calculated using the comparative ∆∆ cycle threshold (Ct) method.

Expression of MTMR6 in vitro cultured SIV-infected primary intestinal macrophages was evaluated by SYBR Green qRT-PCR assay. Approximately 1 μg total RNA was first reverse transcribed in a total volume of 50 μl using the SuperScript III First-Strand Synthesis System for RT-PCR kit (Life Technologies) following the manufacturer’s protocol. Each qRT-PCR (20 μl) contained the following: 2× Power SYBR Green Master Mix without uracil-N-glycosylase (12.5 μl), target forward and reverse primer (200 nM), and cDNA (4 μl). Forward and reverse primer sequence for MTMR6 and GAPDH is shown in Table III. The PCR amplification was carried out in the ABI 7900 HT Fast PCR System (Life Technologies) using the default thermal cycling conditions for SYBR Green assays. As a normalization control for RNA loading, parallel reactions in the same multiwell plate were performed using GAPDH. Relative changes in gene expression were calculated using the ∆∆Ct method. PCR efficiency analysis was performed using serial 10 fold RNA dilutions (500, 50, 5, and 0.5 ng of total RNA) and by using miR-190b, RNU48, snoU6, or cDNA derived from 40, 4, 0.4, and 0.04 ng for MTMR6 and GAPDH. The amplification curves for all assays were linear and based on slope values (∼3.09 to ∼3.24) all assays had 100–105% efficiency.

In situ hybridization and immunofluorescence for cellular localization of SIV and in vitro characterization of primary intestinal macrophages

In situ hybridization for detecting SIV RNA was performed using SIV-digoxigenin-labeled antisense riboprobes (Loxstrand Laboratories, Gaithersburg, MD). Briefly, 7-μm thick formalin-fixed, paraffin-embedded tissue sections were first deparaffinized, rehydrated in decreasing concentrations of ethanol, and pretreated in a microwave with citrate buffer (AgStar, Ashland, OR). The cells were first gated on singlets, followed by equipment (BD Biosciences) and analyzed with Flow Jo software (TreeStar, Ashland, OR). The cells were first gated on singlets, followed by lymphocytes, CD3+ T cells, and then on CD3+CD4+ T cell subsets.

To further determine whether miR-190b upregulation was linked to SIV replication, we isolated peripheral blood CD4+ T cells and primary intestinal macrophages for in vitro SIV infection. CD4+ T cells from peripheral blood were isolated using the nonhuman primate–specific CD4+ T cell isolation kit (magnetic separation with an LS column) following the manufacturer’s recommended protocol (Miltenyi Biotech, Auburn, CA). Approximately 107 cells were first activated with 1 μg/ml Con A and cultured for 3 d in 10 ml RPMI 1640 medium containing 10% FBS, 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Following activation, cells were infected with 300 TCID50 SIVmac239 in the presence of 2 ng/ml IL-2 or left uninfected as controls. Syncytia formation was detected 48 h postinfection, at which time cells were pelleted and lysed for total RNA extraction.

For primary intestinal macrophage isolation, LPLs harvested in the previous step were transferred to T75 flasks containing RPMI 1640 medium supplemented with 10% FBS, 100 mM HEPES, t-glutamine, and 10,000 U penicillin/streptomycin. Under these culture conditions, macrophages will not attach and begin to apoptose by days 3–5. In culture, inflammatory macrophages or monocytes that have recently extravasated into the tissues and have not fully differentiated into macrophages will attach and proliferate forming monolayer after 2–3 d in culture (40, 47). In vitro–cultured primary jejunal macrophages isolated from SIV-infected (n = 5) and uninfected (n = 6) macaques were pelleted, activated with 20 ng/ml recombinant rhesus macaque GM-CSF (Cell Sciences, Canton, MA), and infected with 100 TCID50 SIVmac231 in a total volume of 200 μl for ∼2.5 h at 37°C. Cells were then plated in T25 flasks containing RPMI 1640 medium supplemented with 10% FBS, 100 mM HEPES, t-glutamine, and 10,000 U penicillin/streptomycin and cultured for 4 d post-SIV infection.

Flow cytometry to quantify intestinal CD4+ T cell dynamics

LPLs were isolated as described above and adjusted to a concentration of 107/ml. For T cell immunophenotyping, ∼100-μl aliquots were stained with appropriately diluted, directly-conjugated mAbs to CD3 (Pacific blue: SP54-2), CD4 (SK3: PerCP-Cy5.5), and CD8 (PE-TR: 3B5) (BD Biosciences, San Jose, CA). Samples were stained for 30 min in the dark at 4°C, fixed in 2% paraformaldehyde, and stored in the dark at 4°C overnight for acquisition the next day. Samples were acquired on a LSR II flow cytometry equipment (BD Biosciences) and analyzed with Flow Jo software (Tree Star, Ashland, OR). The cells were first gated on singlets, followed by lymphocytes, CD3+ T cells, and then on CD3+CD4+ T cell subsets.

Cloning of 3'–untranslated region of MTMR6 mRNA and Dual-Glo luciferase reporter gene assay

The 3'–untranslated region (UTR) of the rhesus MTMR6 gene contains a single predicted miR-190b binding site (TargetScan 6.2) (48) highly conserved across several species (Table IV). Accordingly, a short 42–44 nt long sequence representing the 3'–UTR containing the predicted miR-190b site (5'–TCTGTTTATAAAGTACATATC–3') was synthesized (IDT DNA Technologies, Coralville, IA) for cloning into the pmirGLO dual luciferase vector (Promega, Madison, WI). A second oligonucleotide with the binding site mutated (MUT) (5'–TCTGTTTATAAAGTACATATC–3') was synthesized to serve as a negative control. Both oligonucleotide sequences were synthesized with a Psnl site on the 5' and XbaI site on the 3' end for directional cloning. The pmirGLO vector was first cut with Psnl and XbaI restriction enzymes, gel purified, and ligated with either wild-type (WT) sequence containing the miR-190b binding site (pmirGLO-WT-MTMR6) or MUT sequence (pmirGLO-MUT-MTMR6). HEK293 cells were plated at a density of 5 × 105 cells well of a 96-well plate, at 50% confluence the cells were cotransfected with ∼100 ng pmirGLO-WT-MTMR6 or pmirGLO-MUT-MTMR6 UTR miRNA luciferase reporter vector and 100 nM miR-190b mimic using the DharmaFect Duo transfection reagent (ThermoFisher Scientific). In separate wells, cells also were transfected with pmirGLO vector (Promega) as a normalization control. After 48 h, the Dual Glo luciferase assay was performed according to the manufacturer’s recommended protocol using the Bio-Tek, Winooski, VT. The normalized Firefly to Renilla ratio was calculated to determine the relative reporter activity. Experiments were performed in six replicates and repeated thrice.
miR-190b upregulation in intestine during SIV infection

Quantification of plasma and mucosal viral loads

Total RNA samples from plasma, colon, and jejenum tissues of all SIV-infected animals were subjected to quantitative real-time TaqMan two-step RT-PCR analyses to determine viral loads. Briefly, primers and probes specific to the SIV long terminal repeat sequence were designed and used in the real-time TaqMan PCR assay. Probes were conjugated with a fluorescent reporter dye (FAM) at the 5’-end and a nonfluorescent quencher dye at the 3’-end. Fluorescence signal was detected with an ABI Prism 7900 HT sequence detector (Life Technologies). Data were captured and analyzed with Sequence Detector Software (Life Technologies). Viral copy number was determined by plotting Ct values against a standard curve \( y = 3.246 \times 10^{39.374} \) \( (r^2 = 0.998) \) generated with in vitro–transcribed RNA representing known viral copy numbers.

Data analysis and availability

TLDA-SDS run files from 10 SIV-infected and 5 uninfected control macaques were loaded onto Applied Biosystems Relative Quantification Manager Software version 1.2.2 and analyzed using automatic baseline settings and a manual threshold of 0.2. The results from the Relative Quantification manager analysis containing five columns (well, sample detector, task, and Ct values) was saved as a tab-delimited text file, which was later imported and analyzed using the DataAssist version 3.01 software (Life Technologies), a data analysis tool designed to compare samples using the \( \Delta \Delta CT \) method for relative quantification of gene expression. miRNA expression data were analyzed using global normalization (49, 50) as this method has been reported to be the most sensitive and accurate approach for high-throughput miRNA profiling using qRT-PCR compared with endogenous controls. In all experiments, the Ct upper limit was set to 33, meaning that all miRNA detectors with a Ct value \( \geq 33 \) were excluded. Multiple comparisons correction using Benjamini–Hochberg method for false-discovery rate was simultaneously applied to all 768 miRNA target probes (card A and B combined). TLDA data were deposited with Gene Expression Omnibus (accession number: GSE56624).

For miR-190b and MTMR6 qRT-PCR studies, one uninfected control macaque with the highest (for miR-190b) or lowest (for MTMR6) \( \Delta \Delta CT \) value served as the calibrator/reference and assigned a value of 1. All differentially expressed miRNAs or mRNAs in SIV-infected and uninfected macaques with diarrhea including other macaques in the control group are shown as an \( n \)-fold difference relative to this macaque. The stepwise calculation of fold change using this approach is shown in Supplemental Table I. miRNA fold change was also calculated using an average of all control animal \( \Delta \Delta CT \) values providing very similar results (Supplemental Fig. 2A, 2B). Accordingly, we have used the former approach as it facilitated graphing the control samples so that the variation within the control samples can be displayed (Supplemental Fig. 2A). Intestinal CD4+ T cell data and individual miR-190b qRT-PCR data in colon, jejenum, intestinal epithelium, and LPL compartments were analyzed using nonparametric Kruskal–Wallis test and posthoc analysis was done using Dunn’s multiple groups comparison employing the GraphPad Prism 5 software (La Jolla, CA). \( p < 0.05 \) was considered as significant. miR-190b and MTMR6 miRNA qRT-PCR data in SIV-infected macaques was analyzed by nonparametric Wilcoxon’s rank-sum test for independent samples using RealTime STATMiner package, a bioinformatics software developed by Integromics on Spotfire DecisionSite. A Spearmann’s nonparametric one-tailed correlation analysis was performed to determine the degree of association between tissue viral loads and miR-190b fold expression. Firefly/Renila ratios were statistically analyzed using an unpaired t test.

Results

Mucosal and plasma viral loads, CD4+ T cell dynamics, and intestinal histopathology

The viral loads in plasma, colon, and jejenum from all SIV-infected macaques are shown in Table I. Intestinal viral loads were substantial and in the colon ranged from \( 0.06 \times 10^6 \) to \( 10.228 \times 10^6 \) copies/mg total RNA with a median of \( 2 \times 10^6 \) copies/ mg total RNA. In the jejenum, viral loads ranged from \( 0.04 \times 10^6 \) to \( 1482 \times 10^6 \) copies/mg total RNA with a median of \( 3.5 \times 10^5 \) copies/mg total RNA. Similar to tissue viral loads, plasma viral loads from 7 dpi postinfection (DPI) to terminal disease ranged from \( 0.006 \times 10^6 \) to \( 500 \times 10^5 \) copies/ml with a median of \( 10 \times 10^6 \) copies/ml. Plasma viral loads were not available for six animals (IA85, HT44, HV39, HV61, CG32, and CT16).

SIV-infected animals at the 13–14 DPI, 21 DPI and 90 DPI time points (Table I) had significant mucosal CD4+ T cell depletion (Fig. 1). Analysis of CD4+ T cell percentages using Kruskal–Wallis (nonparametric method) test revealed statistically significant differences among groups (\( p = 0.0005 \)). Post hoc analysis using Dunn’s multiple comparison test identified all postinfection time points with the exception of 7–10 DPI to be significantly different (\( p < 0.05 \) to \( p < 0.01 \)) from the uninfected control group (Fig. 1). Data on CD4+ T cell status in the intestine were not available for seven animals. This included two animals at 29 DPI (CG32 and CT16) and five macaques that progressed to AIDS (FT11, HL01, AT56, H405, and L441) (Table I).

Histologic evaluation of H&E stained sections of colon and jejenum from all non–SIV-infected animals with diarrhea revealed the presence of moderate to severe colitis including other intestinal lesions such as crypt dilatation/abscess and diverticulosis (Fig. 2D, 2E, Table I). No bacterial pathogens were detected in seven of the eight non–SIV-infected macaques with diarrhea and colitis. In contrast, the colonic lamina propria of the SIV-infected macaques (7 and 14 DPI) showed minimal to no histological signs of inflammation (Fig. 2A–C) and appeared similar to the uninfected control macaque (Fig. 2F) (Table I).

Acute SIV infection of the intestinal immune system is characterized by marked alterations in miRNA expression

To determine whether SIV infection of the GI tract/immune system was associated with alterations in miRNA expression we performed global miRNA profiling of colon tissue during acute SIV infection using the human microRNA TLDA cards. As shown in Table I, one animal each was at 7, 8, and 10 DPI, three each at 13 and 21 DPI, and one at 29 DPI. After applying multiple comparisons correction (Benjamini–Hochberg adjusted \( p \) values for false-discovery rate) simultaneously to all miRNA probes (cards A and B combined), nine miRNAs (four up and five downregulated) were identified as statistically significant (adjusted \( p < 0.05 \)) and differentially expressed following analysis using DataAssist software version 3.01 (Table V). Raw Ct values shown in Table V provide additional information on the cellular abundance (high, medium, or low) of each differentially expressed miRNA and the extent of variation across samples.

Among the four upregulated miRNAs, the expression of one miRNA, namely, miR-190b exceeded 5-fold (\( \sim 6 \)-fold) and in terms of magnitude showed the highest increase in expression following SIV infection (Table V). The expression levels of the remaining three miRNAs (miR-222, miR-22*, and miR-223*) ranged from 1.5- to 3.0-fold compared with the uninfected control group. The expression of five miRNAs (hsa-miR-425*, -199a-5p, -221, -324-3p, and -361-5p) decreased \( \sim 1.5- \) to 1.7-fold during acute SIV infection (Table V). These findings suggest that early SIV infection is characterized by significant changes in miRNA expression in the colon and that the profile is slightly dominated by downregulated miRNAs.

miR-190b upregulation occurs in both colon and jejenum at all stages of SIV infection and its expression follows a trend observed with peripheral blood viral loads during SIV infection

miR-190b was selected for further characterization as the magnitude of increase was the highest among the four upregulated miRNAs (Table V) and ranked second based on \( p \) values (adjusted \( p = 0.0032 \)) making it an ideal target for further characterization. As shown in Fig. 3A and 3B, miR-190b expression significantly increased in the colon and jejenum of SIV-infected compared with uninfected controls and non-SIV-infected macaques with diarrhea and colitis (labeled as colitis in Fig. 3A, 3B). With the exception

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of the 21 and 90 DPI time points in both colon and jejunum, miR-190b expression at all other time points showed statistical significance ($p$, 0.05) (Fig. 3A, 3B). However, comparison of both (21 and 90 DPI) time points separately with uninfected controls using the nonparametric Wilcoxon’s rank-sum test or Mann–Whitney U test revealed statistical significance ($p$, 0.05) (data not shown) in colon and jejunum. Furthermore, miR-190b expression in the colon and jejunum of non–SIV-infected macaques with diarrhea and colitis did not differ from normal uninfected controls (Fig. 3A, 3B). Although not a longitudinal study, some interesting trends in miR-190b expression were observed in the current study. From Fig. 3A and 3B, it is clearly evident that enhanced expression of miR-190b occurred as early as 7 DPI ($p$, 0.05) and peaked between 13 and 14 DPI (coincident with peak viremia) ($p$, 0.01). The initial elevation in miR-190b at 7 DPI occurred even before there was a significant loss of mucosal CD4+ T cells (Fig. 1). This indicates that the increase in miR-190b expression occurs before peak viral loads and the nadir of CD4+ T cell depletion. Subsequent to the peak of viremia between 13 and 14 DPI, average miR-190b expression dropped by $\sim 2$-fold in colon and $\sim 3$-fold in jejunum at 21–29 DPI. Interestingly, this abrupt drop in expression at this time point (21–29DPI) coincides with the nadir of CD4+ T cell depletion, which may account for the sudden drop in miR-190b expression. At 90 DPI and in animals with terminal disease, miR-190b expression showed an upward trend in both colon and jejunum (Fig. 3A, 3B). Because intestinal tissues from the non–SIV-uninfected macaques with diarrhea and colitis were collected over a period of 6–7 y, there was always the possibility that technical batch

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Duration of Infection (DPI)</th>
<th>Inoculum</th>
<th>Plasma Viral Loads Copies/ml ($10^6$)</th>
<th>Viral Load - Colon Copies/mg RNA ($10^6$)</th>
<th>Viral Load - Jejunum Copies/mg RNA ($10^6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA85a</td>
<td>7</td>
<td>SIVmac251</td>
<td>NA</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>HI52a</td>
<td>8</td>
<td>SIVmac251</td>
<td>4</td>
<td>900</td>
<td>200</td>
</tr>
<tr>
<td>AV91a</td>
<td>10</td>
<td>SIVmac251</td>
<td>157</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>M992a</td>
<td>13</td>
<td>SIVmac251</td>
<td>35</td>
<td>200</td>
<td>9</td>
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<tr>
<td>HI63a</td>
<td>13</td>
<td>SIVmac251</td>
<td>24</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>H958a</td>
<td>13</td>
<td>SIVmac251</td>
<td>9</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>HC36a</td>
<td>21</td>
<td>SIVmac251</td>
<td>20</td>
<td>1</td>
<td>2</td>
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<tr>
<td>HB31a</td>
<td>21</td>
<td>SIVmac251</td>
<td>10</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>GA19a</td>
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<td>SIVmac251</td>
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<td>10</td>
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</tr>
<tr>
<td>CT16a</td>
<td>29</td>
<td>SIVmac259</td>
<td>NA</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>HT44</td>
<td>7</td>
<td>SIVmac251</td>
<td>NA</td>
<td>0.4</td>
<td>3</td>
</tr>
<tr>
<td>T108</td>
<td>8</td>
<td>SIVmac251</td>
<td>0.06</td>
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<td>ND</td>
</tr>
<tr>
<td>HV61</td>
<td>14</td>
<td>SIVmac251</td>
<td>NA</td>
<td>0.8</td>
<td>3</td>
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<tr>
<td>HV39</td>
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<td>SIVmac251</td>
<td>NA</td>
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<td>0.9</td>
</tr>
<tr>
<td>HF27</td>
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<td>SIVmac251</td>
<td>9</td>
<td>0.06</td>
<td>5</td>
</tr>
<tr>
<td>HB48</td>
<td>21</td>
<td>SIVmac251</td>
<td>40</td>
<td>300</td>
<td>6</td>
</tr>
<tr>
<td>GK31</td>
<td>21</td>
<td>SIVmac251</td>
<td>2</td>
<td>2</td>
<td>0.04</td>
</tr>
<tr>
<td>HR57</td>
<td>21</td>
<td>SIVmac251</td>
<td>6</td>
<td>0.4</td>
<td>1</td>
</tr>
<tr>
<td>HV95</td>
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<td>SIVmac251</td>
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<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>CG32</td>
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<td>SIVmac239</td>
<td>NA</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HC36</td>
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<td>SIVmac251</td>
<td>0.1</td>
<td>0.2</td>
<td>ND</td>
</tr>
<tr>
<td>HB31</td>
<td>90</td>
<td>SIVmac251</td>
<td>70</td>
<td>7</td>
<td>0.97</td>
</tr>
<tr>
<td>HF27</td>
<td>90</td>
<td>SIVmac251</td>
<td>10</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td>HB48</td>
<td>90</td>
<td>SIVmac251</td>
<td>20</td>
<td>0.1</td>
<td>100</td>
</tr>
<tr>
<td>GK31</td>
<td>90</td>
<td>SIVmac251</td>
<td>30</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>GA19</td>
<td>90</td>
<td>SIVmac251</td>
<td>100</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>HR57</td>
<td>90</td>
<td>SIVmac251</td>
<td>80</td>
<td>2</td>
<td>0.04</td>
</tr>
<tr>
<td>HV95</td>
<td>90</td>
<td>SIVmac251</td>
<td>7</td>
<td>0.5</td>
<td>0.08</td>
</tr>
<tr>
<td>FT11</td>
<td>145</td>
<td>SIVmac251</td>
<td>500</td>
<td>2,075</td>
<td>1,176</td>
</tr>
<tr>
<td>HL01</td>
<td>180</td>
<td>SIVmac251</td>
<td>7</td>
<td>0.8</td>
<td>30</td>
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<tr>
<td>L441</td>
<td>170</td>
<td>SIVmac239</td>
<td>1.3</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>H405</td>
<td>232</td>
<td>SIVmac239</td>
<td>71.4</td>
<td>542</td>
<td>758</td>
</tr>
<tr>
<td>AT56</td>
<td>1460</td>
<td>SIVmac239</td>
<td>360</td>
<td>10,228</td>
<td>1,482</td>
</tr>
</tbody>
</table>

Colon and jejunum tissues from all 35 animals were used for miR-190b qRT-PCR characterization studies. NA, not available; ND, not detectable.

+Colon tissue from 10 animals (IA85-CT16) was used for genome-wide miRNA expression profiling using the TLDA platform.

**FIGURE 1.** Percentage of CD4+ T cells among LPLs isolated at different time points after SIV infection. The cells were first gated on singlets, followed by lymphocytes, CD3 and then on CD3+CD4+ T cell subsets. Data analysis using Kruskal–Wallis test identified significant differences among the different groups ($p$, 0.0004). Post hoc analysis using Dunn’s multiple groups comparison identified 13–14, 21, and 90 DPI time points to be significantly different from uninfected controls. The error bars represent SE of mean CD4+ T cell percentage within each group. *$p$, < 0.05, **$p$, < 0.01.
effects or RNA degradation could have contributed to the lack of miR-190b upregulation in this group. To specifically address this issue, we collected colon tissues from a second batch of six non–SIV-infected macaques necropsied within the past 6 mo for chronic diarrhea nonresponsive to treatment (colitis). Similar to the findings shown in Fig. 3A, miR-190b expression did not increase (Supplemental Fig. 2C) and was no different from the uninfected control group. As shown in Supplemental Table I, average miR-190b C_T values in this group were identical to the uninfected controls (24.2 versus 24.3). Furthermore, the colonic lamina propria of the SIV-infected macaques (7 and 13 DPI) did not show any histological signs of inflammation (Fig. 2A–C) and appeared no different from the uninfected control macaque (Fig. 2F). In contrast, both non–SIV-infected macaques with diarrhea (Fig. 2D, 2E) have moderate to severe colitis (marked inflammatory cell infiltration of the colonic lamina propria along with other lesions such as crypt abscesses), providing strong evidence that inflammation/immune activation is not driving miR-190b upregulation.

The correlation of miR-190b with increased viral replication in the intestine is supported by the finding that miR-190b expression

---

**Table II. List of non–SIV-infected macaques with diarrhea and uninfected control macaques used for TLDA and miR-190b qRT-PCR studies**

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Colon</th>
<th>Jejunum</th>
<th>Bacterial Pathogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIV-uninfected with diarrhea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DJ15</td>
<td>3 and CD</td>
<td>1</td>
<td>None</td>
</tr>
<tr>
<td>DV87</td>
<td>1 and CD</td>
<td>NA</td>
<td>None</td>
</tr>
<tr>
<td>DV98</td>
<td>3 and CD</td>
<td>NA</td>
<td>None</td>
</tr>
<tr>
<td>EB12</td>
<td>3 and CD</td>
<td>NA</td>
<td>None</td>
</tr>
<tr>
<td>J053</td>
<td>2</td>
<td>1</td>
<td>None</td>
</tr>
<tr>
<td>EJ54</td>
<td>3</td>
<td>1</td>
<td>None</td>
</tr>
<tr>
<td>EL71</td>
<td>3 and CD, DV</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>EB27</td>
<td>3 and CD, DV</td>
<td>1</td>
<td>Shigella flexneri, Campylobacter coli</td>
</tr>
<tr>
<td>Uninfected controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FF15</td>
<td>0</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>FT23</td>
<td>0</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>HT22</td>
<td>0</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>EL66^a</td>
<td>0</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>EH70^a</td>
<td>0</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>EH80^a</td>
<td>0</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>GB2^a</td>
<td>0</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>FX25^a</td>
<td>0</td>
<td>0</td>
<td>None</td>
</tr>
</tbody>
</table>

Sections of jejunum and colon were examined and inflammation was scored semiquantitatively on a scale of 0–3 as follows: 0, within normal limits; 1, mild; 2, moderate; and 3, severe. In addition, the presence of crypt dilatation (CD), diverticulosis (DV), villus blunting, and amyloidosis were recorded. NA, not applicable.

^a Denotes uninfected control animals used for TLDA miRNA profiling.
levels in both colon and jejunum of two macaques with undetectable tissue viral load (T108 [7–8 DPI], CG32 [21–29 DPI], Table I and arrows in Fig. 3A, 3B) were no different from uninfected controls. Because miR-190b upregulation in intestine was only detected in macaques with detectable tissue viral load, we next examined the statistical correlation between miR-190b expression levels and intestinal tissue viral load. As shown in Fig. 4A and 4B, miR-190b expression levels in colon (r = 0.48, p = 0.0024) and jejunum (r = 0.50, p = 0.0017) were positively correlated with viral loads. Finally, the absence of miR-190b upregulation in the intestines of non–SIV-infected macaques with diarrhea and colitis suggests that miR-190b is not a general marker of intestinal inflammation or immune activation because these animals had moderate to severe colitis.

The intestinal lamina propria and not the epithelial compartment contributes to miR-190b upregulation in SIV-infected macaques

The failure to detect miR-190b upregulation in the intestines of non–SIV-infected macaques with diarrhea and colitis (Fig. 3A, 3B) suggested a strong link between miR-190b upregulation and viral replication. The association with viral replication also suggested that targets of SIV infection such as CD4+ T cells and macrophages may be necessary to drive miR-190b upregulation. If this hypothesis is true then miR-190b upregulation should occur predominantly in the LPL and not the epithelial compartment as both CD4+ T cells and macrophages reside in the LPL compartment. To confirm that the LPL compartment of the intestine was contributing to the upregulation of miR-190b expression in SIV-infected macaques, we quantified miR-190b expression in isolated intestinal epithelial cells and LPLs using the individual TaqMan primer sequences used for real time SYBR Green Two-step qRT-PCR

miR-190b expression was also quantified in PBMCs isolated from the same four animals shown in Fig. 5A and 5B at the same time points (preinfection, 21 and 90 DPI) as intestinal resection segments. Although miR-190b expression was considerably elevated in the LPL (p < 0.05 at 90 DPI) (Fig. 5A), parallel statistically significant upregulation was not observed in PBMCs at the same time points as shown in Supplemental Fig. 2D, suggesting that PBMCs may not contain a sufficient proportion of SIV-infected cells to result in miR-190b upregulation.

These findings show that under basal conditions, miR-190b is expressed in both the intestinal epithelium and LPLs. However, after SIV infection its expression is significantly increased only in the LPL compartment. This finding along with the correlation between intestinal viral load and miR-190b expression and the absence of miR-190b upregulation in the intestines of non–SIV-infected macaques with diarrhea and colitis (Fig. 3A, 3B) suggests that miR-190b upregulation is linked to viral replication.

miR-190b is expressed in CD4+ T cells and macrophages and its expression is significantly increased in in vitro–cultured CD4+ T cells and primary intestinal macrophages in response to SIV infection

To further explore the linkage between expression of miR-190b and viral replication we examined miR-190b expression in CD4+ T cells and macrophages with and without SIV infection. Accordingly, we isolated peripheral blood CD4+ T cells and primary intestinal macrophages and determined miR-190b expression separately in both cell types using stem loop qRT-PCR assay specific to miR-190b. We preferred this approach to miRNA in situ hybridization because qRT-PCR is highly specific and truly quantitative. As illustrated in Fig. 6, we were able to obtain high-purity macrophage populations that were consistently positive for macrophage specific markers CD68 (Fig. 6A) and CD163 (Fig. 6B) and negative for T lymphocyte markers such as CD3 (Fig. 6C). Furthermore, we successfully infected these cells in vitro with SIVmac251 as demonstrated by the in situ detection of viral RNA 4 DPI (Fig 6D, 6E). As shown in Supplemental Fig. 3, miR-190b was strongly expressed by both CD4+ T cells and macrophages. Furthermore, as shown in Fig. 3A and 3B, miR-190b expression increased as early as 7 DPI, peaked at 13–14 DPI (peak viral replication), and particularly, its abrupt drop in expression at 21–29 DPI in both colon and jejunum that coincided with the nadir of CD4+ T cell depletion strongly suggests that CD4+ T cells are likely to be a major cellular source of elevated miR-190b expression during acute infection. Nevertheless, as

### Table III. Primer sequences used for real time SYBR Green Two-step qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (forward, reverse)</th>
<th>Product Size (bp)</th>
<th>Primer Concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTMR6</td>
<td>5’-CACACAGCCCTGGCAGATAATTCTTT-3’</td>
<td>76</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>5’-TAAAGCTTTGGCAGACAGGTTCTGA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-CACACAGCCCTGGCAGATAATTCTTT-3’</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>5’-GATGCCCTCCACGATAATTCTTT-3’</td>
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<td></td>
</tr>
</tbody>
</table>

### Table IV. Schematic representation of MTMR6 3’-UTR depicting predicted binding site for miR-190b

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Site Conservation</th>
<th>Binding Sites on 3’-UTR</th>
<th>Target Site Sequence</th>
<th>Prediction Algorithm</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTMR6</td>
<td>NM_004685</td>
<td>Human/chimp/macaque/orangutan/mouse</td>
<td>1239–1245</td>
<td>5’...AUUCUGUGUGUACUGUACUGUACU...3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3’...UUGGGUUAUGUUGGUACUGU...5’</td>
<td>TargetScan (48), miRanda (54)</td>
</tr>
</tbody>
</table>
previously published (51), and as illustrated in Fig. 6F, SIV infection of intestinal macrophages in situ can occur as early as 10 DPI.

To conclusively verify that miR-190b was upregulated in response to SIV replication, we infected peripheral blood CD4+ T cells and primary intestinal macrophages with SIV and found significant upregulation of miR-190b in CD4+ T cells (Fig. 7A) and macrophages (Fig. 7B) by days 2 and 4 postinfection, respectively. miR-190b expression in CD4+ T cells was normalized to a combination of RNU44 and RNU48 as it yielded better statistical significance ($p = 0.0121$) compared with RNU44 alone ($p = 0.0367$). These findings clearly demonstrate that SIV-infected

Table V. Differentially expressed miRNAs in colon during acute SIV infection

<table>
<thead>
<tr>
<th>miRNA ID</th>
<th>Acute SIV ($n=10$)</th>
<th>Uninfected Controls ($n=5$)</th>
<th>Fold Change</th>
<th>Adjusted $p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-425*</td>
<td>29.3 28.8 29.3 28.7 29.5 29.1 26.8 28.0 27.7 27.9</td>
<td>28.2 28.6 28.4 29.4 29.0</td>
<td>−1.7</td>
<td>0.0024</td>
</tr>
<tr>
<td>hsa-miR-190b</td>
<td>23.0 23.4 23.0 21.8 22.5 23.1 21.1 22.7 22.7 22.0</td>
<td>25.2 24.7 24.8 26.0 26.4</td>
<td>6.0 0.0032</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-222</td>
<td>15.6 15.4 15.7 15.1 15.2 15.4 14.3 14.7 14.5 14.7</td>
<td>15.7 15.9 15.5 16.4 16.7</td>
<td>1.5 0.0114</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-199a-5p</td>
<td>25.3 24.5 25.6 25.4 25.8 24.4 23.8 24.7 24.7 24.4</td>
<td>24.3 24.3 23.8 24.9 24.6</td>
<td>−1.7 0.0376</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-22*</td>
<td>23.4 22.9 23.0 22.5 23.0 23.3 22.2 23.1 22.8 22.6</td>
<td>23.3 23.4 23.4 24.4 24.5</td>
<td>1.5 0.0376</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-221</td>
<td>21.5 20.9 21.0 21.1 22.0 21.1 20.7 20.7 21.3 21.0</td>
<td>20.4 20.5 20.0 21.3 21.3</td>
<td>−1.6 0.0376</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-223*</td>
<td>26.7 26.1 25.4 25.6 25.0 26.6 24.7 25.0 24.4 24.5</td>
<td>26.5 27.2 26.7 27.0 29.0</td>
<td>2.8 0.0376</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-324-5p</td>
<td>22.8 22.5 23.0 22.5 23.3 22.3 21.5 22.6 22.7 22.7</td>
<td>22.0 22.2 21.6 22.7 22.7</td>
<td>−1.6 0.0376</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-361-5p</td>
<td>22.0 22.2 22.5 22.0 22.4 22.0 21.6 22.2 22.1 22.1</td>
<td>21.5 21.6 21.1 22.6 22.3</td>
<td>−1.5 0.0376</td>
<td></td>
</tr>
</tbody>
</table>

The table shows raw $C_T$ and fold change for all differentially expressed (adjusted $p < 0.05$) miRNAs after applying multiple comparisons correction (Benjamini–Hochberg adjusted $p$ values for false-discovery rate) in the colon of 10 acutely SIV-infected macaques.

The figure illustrates elevated miR-190b expression in the colon (A) and jejunum (B) during SIV infection compared with uninfected normal controls and non–SIV-infected macaques with diarrhea (indicated as colitis). Note the absence of miR-190b upregulation in non–SIV-infected macaques with diarrhea and colitis, suggesting that upregulation of miR-190b is not inflammation driven but occurs in response to viral replication. Arrows in colon (A) and jejunum (B) point to two animals (T108 [7 DPI] and CG32 [21–29 DPI]) that had no detectable SIV in intestinal tissue and where the levels of miR-190b was no different from controls. The error bars represent SE of mean fold change within each group. Data analysis using nonparametric Kruskal–Wallis test revealed differences among groups in both colon ($p = 0.0002$) and jejunum ($p < 0.0001$). Asterisks (*$p < 0.05$, **$p < 0.01$) indicate groups that showed statistical significance compared with uninfected controls following Dunn’s multiple groups comparison test.
CD4+ T cells and macrophages directly contribute to miR-190b upregulation. In addition, the data also suggest that although SIV-infected CD4+ T cells are likely to be the primary cellular source of miR-190b upregulation during early acute infection (7–10 and 13–14 DPI), SIV-infected macrophages could contribute significantly to miR-190b upregulation at later time points and chronic infection.

miR-190b can directly regulate the expression of MTMR6 and in doing so potentially play an important role in SIV pathogenesis

To investigate the significance of increased miR-190b expression in response to SIV infection, we examined potential targets of miR-190b. TargetScan (version 6.2) (48) lists a total of 186 predicted targets for mml-miR-190b. Among these is MTMR6, which has been shown to negatively regulate CD4+ T cell activation and proliferation by inhibiting Ca2+-dependent activated potassium channel KCa3.1, thereby reducing Ca2+ influx (52). Furthermore, KCa3.1 is also required for macrophage activation (53). Consequently, MTMR6 represented a very interesting miR-190b target from the perspective of CD4+ T cell/macrophage activation and HIV replication. Interestingly, MTMR6 mRNA expression decreased significantly (p = 0.013) in cultured intestinal macrophages 4 d post-SIV infection (Fig. 7C). As shown in Fig. 7D, cotransfection of pmirGLO-WT-MTMR6 with miR-190b mimic resulted in significant reduction in Firefly/Renila ratios (p = 0.0069).

In contrast, cotransfection of pmirGLO-MUT-MTMR6 with miR-190b restored the Firefly/Renila ratios to the level observed with unmanipulated pmirGLO vector (Fig. 7D). These results clearly show that miR-190b can physically interact with the 3’-UTR and potentially regulate the expression of MTMR6. The precise effect of this regulation on target cell function including its direct or indirect impact on viral replication in infected cells needs future investigation.

**Discussion**

HIV/SIV infection is associated with robust viral replication in the intestinal immune system, depletion of mucosal CD4+ T cells and marked immune activation. The dramatic loss of mucosal CD4+ T cells in the intestine has also been associated with intestinal inflammation, damage and microbial translocation. These changes are associated and in some cases preceded by marked changes in gene expression (7–10). Regulation of gene expression associated with the host response to infectious agents is complex and our

![FIGURE 4. Correlation of miR-190b in colon (A) and jejunum (B) with viral loads. A positive statistical (p < 0.05) correlation between miR190b and SIV viral load was found in both colon and jejunum.](http://www.jimmunol.org/)

![FIGURE 5. The LPL and not the epithelial compartment of the intestine is the predominant source of miR-190b upregulation in SIV-infected macaques. Statistically significant increase in miR-190b expression was detected in the intestinal lamina propria at 90 d post-SIV infection (DPI) (A) but not in the intestinal epithelium (Epith) (B). Data were analyzed using nonparametric Kruskal–Wallis test and post hoc multiple groups comparison was performed using Dunn’s test. *p < 0.05 compared with preinfection samples.](http://www.jimmunol.org/)
understanding of this process has for the most part been dominated by the role played by transcription factors, coactivators, corepressors, chromatin modifiers, histone modulators, and so on. The discovery of miRNAs has added another layer of complexity to the standard linear concept of DNA being transcribed to mRNA which is then translated to protein. While the regulatory role of miRNAs in immune cell development including several immunopathological and inflammatory conditions (18, 22, 23) is receiving a lot of attention, their contributions to regulating HIV/SIV replication and host responses, particularly, in the GI tract, an important site of viral replication, CD4⁺ T cell depletion and viral persistence remain unknown and unexplored. In the current study, using the rhesus macaque model of AIDS we investigated genome wide changes in miRNA expression to better understand their regulatory role in the GI tract following SIV infection. We performed the initial high throughput miRNA profiling on the colon for numerous reasons. First, similar to the jejunum, CD4⁺ T cell depletion has been well documented to occur in the colon (2, 55). Second, from our previously published studies (13, 14) it is clear that although SIV affects the jejunum, the colon is more severely impacted. Third, the increased concentration of bacteria in the colon (10¹² bacterial organisms per milliliter of content) (56) makes it an important source of intestinal bacteria/bacterial products that is well documented to translocate into the systemic circulation via a disrupted intestinal epithelial barrier leading to chronic immune activation and AIDS progression (6). To our knowledge, the present study for the first time describes dysregulated miRNA expression in the GI tract in response to SIV infection. We specifically identified miR-190b to be significantly upregulated in both colon and jejunum of acutely SIV-infected macaques. Further, we show that miR-190b upregulation is driven by viral replication and not by the immune/ inflammatory processes occurring in response to viral replication. Furthermore, our results provide new insights into the role of miR-190b in regulating host cellular responses, particularly, sustaining the activated state in SIV-infected cells by inhibiting MTMR6 expression.

In recent years, miRNAs have emerged as potent regulators of immune and inflammatory responses (20–22). Consistent with these findings and after applying multiple comparisons correction we identified a total of 9 miRNAs (Table V) to be markedly altered during acute SIV infection. Among these, eNOS suppressing miR-222 has been linked to regulating vascular inflammation (57) and differentiation of dendritic cells (58). Downregulation of miR-199a-5p promoted wound angiogenesis via derepression of the Ets-MMP1 pathway (59) suggesting that its reduced expression in the intestine may be part of the normal host response to repair tissue damage associated with early viral replication. As observed in the current study, miR-221 expression reduced significantly in bronchial epithelial cells in response to respiratory syncytial virus infection (60). Surprisingly, miR-190b exhibited the highest increase in expression following SIV infection and although reported to exhibit altered expression in several cancer studies (61, 62) has not been previously linked to an infectious disease. However, miR-190/190a, a closely related miRNA originating from a different chromosome with distinct primary and precursor sequences has been well characterized (63, 64). Both miR-190 and miR-190b share identical seed regions and hence have the same predicted targets. Taken together, acute SIV infection of the GI tract results in altered miRNA expression and the markedly elevated miR-190b expression as early as 7 d post-SIV infection suggests an important role for this miRNA in SIV pathogenesis.

As mentioned above, the lack of previously published studies linking miR-190b to an infectious disease combined with the fact that it showed the highest fold increase among the nine differentially expressed miRNAs prompted us to further investigate miR-190b expression at different stages of SIV infection. Surprisingly, miR-190b expression significantly increased in both colon and jejunum as early as 7 DPI. Furthermore, its expression peaked at
13–14 DPI (time of peak viral replication), dropped abruptly at 21 DPI (coinciding with nadir of CD4⁺ cell depletion) and then gradually increased as disease progressed (90 DPI and AIDS). These findings are certainly noteworthy and a longitudinal study is definitely needed in the future to firmly associate alterations in miR-190b expression level with key pathogenic events such as peak viral replication and nadir of CD4⁺ T cell loss. Subsequently, qRT-PCR quantification of miR-190b expression separately in the intestinal epithelium and LPLs helped us to decisively localize the source of miR-190b upregulation to the immune cells residing in the lamina propria. Since viral replication in the intestinal lamina propria elicits an immune/inflammatory response there was definitely the possibility that inflammatory cell infiltration in response to SIV replication may be contributing to miR-190b upregulation. Accordingly, we included a group of macaques with GI disease (non–SIV-infected with diarrhea and colitis) to determine whether inflammatory cell infiltration or SIV replication was driving miR-190b expression as this group of animals has moderate to severe colitis, localized immune activation and consequently massive disruption of the intestinal epithelial barrier. Interestingly, the complete absence of miR-190b upregulation in the colon and jejunum of non–SIV-infected macaques with diarrhea and colitis strongly suggested that SIV replication in the lamina propria target cells likely provides the stimulus to drive miR-190b up-regulation. More importantly, the latter finding undoubtedly means that shifts in immune cell composition (infiltration by inflammatory cells) occurring in response to SIV replication does not account for the observed increase in miR-190b expression. Finally, infection of in vitro cultured peripheral blood CD4⁺ T cells and primary intestinal macrophages with SIV conclusively

FIGURE 7. miR-190b expression was markedly elevated in in vitro cultured peripheral blood CD4⁺ T cells (A) (p = 0.0121) and primary jejunal macrophages (B) 2 and 4 d post-SIV infection, respectively, suggesting that its upregulation occurs in response to SIV replication. Data were analyzed using nonparametric Wilcoxon’s rank-sum test for independent samples. The error bars represent SE of mean fold change within each group. *p < 0.05 compared with uninfected samples. MTMR6 is a direct target of miR-190b (C and D). miR-190b physically associates with the 3'UTR of MTMR6 mRNA. mRNA expression of MTMR6 is significantly decreased (p = 0.013) in intestinal macrophages 4 d post-SIV infection (C). The error bars represent SE of mean fold change within each group. Data were analyzed using nonparametric Wilcoxon’s rank-sum test for independent samples. *p < 0.05 compared with uninfected macrophages. miR-190b physically associates with the 3'UTR of MTMR6 mRNA (D). The MTMR6 3'UTR sequences, WT or MUT, were inserted into the multiple cloning sites situated in the 3'-end of firefly luciferase gene in the pmirGLO vector. HEK293 cells were cotransfected with 100 nM miR-190b mimic and 100 ng luciferase reporter constructs containing WT- or MUT-MTMR6 3'UTR sequences. Firefly and Renilla luciferase activities were detected using the Dual-Glo luciferase assay system 48 h after transfection. The ratio of luciferase activities (Firefly/Renilla) was calculated and normalized to the wells transfected with only unmanipulated pmirGLO vector. **p < 0.01.
revealed the SIV-infected cell as the primary cellular source of miR-190b upregulation. On the basis of these findings, we can confidently conclude that miR-190b upregulation occurs primarily in response to SIV replication and is not simply a nonspecific result of immune activation and inflammation.

To gain further insight into the biological significance of miR-190b upregulation we next focused on MTMR6, a predicted mRNA target of miR-190b. We focused on MTMR6 as a direct miR-190b target because of its central importance to CD4+ T cell/macrophage activation/proliferation (52, 53). MTMR6 is a phosphatidylinositol-3-phosphate that has been well demonstrated to negatively regulate Ca2+ influx by inhibiting the calcium-dependent activated potassium channel KCa3.1, thereby, preventing CD4+ T cell activation and proliferation (52). In addition to T cells, KCa3.1 channel activity is also critical for macrophage activation (53). KCa3.1 channel activation requires phosphatidylinositol-3-phosphate PI(3)P and MTMR6 downregulates its activity by de-phosphorylating PI(3)P (52). This is evident from studies where siRNA mediated silencing of MTMR6 in CD4+ T cells resulted in increased KCa3.1 channel activity, enhanced calcium influx and facilitated T cell activation with 10-fold less Ag concentration compared with untreated cells (53). In the current study, MTMR6 mRNA expression decreased significantly in in vitro cultured primary intestinal macrophages 4 d post-SIV infection. Additionally, the luciferase reporter assay clearly demonstrated the existence of a physical interaction between miR-190b and the 3′UTR of MTMR6 and provided strong evidence for a miR-190b–mediated MTMR6 gene silencing in SIV-infected macrophages. The latter possibility is strongly supported by the recent study that demonstrated siRNA mediated destabilization of mRNAs as a major mechanism that accounted for >84% of the reduced protein output (65). It has been proposed that MTMR6 functions constitutively to tonically inhibit KCa3.1 channel activity and in doing so sets a threshold stimulus for T cell activation (52). Such a mechanism can be expected to be operational in activated colonic CD4+ T cells and macrophages of non–SIV-infected macaques with diarrhea and colitis maintaining basal miR-190b expression levels. Nevertheless, in SIV-infected intestinal CD4+ T cells and macrophages, miR-190b levels were markedly elevated, which in turn provides an additional layer of regulation to augment KCa3.1 activity by reducing MTMR6 levels so that the constitutive Ca2+ influx required for increased cytokine production and subsequent immune/inflammatory responses are sustained. Although this represents a protective host response to curtail the spread of the virus, paradoxically, miR-190b–mediated silencing of MTMR6 can invariably secure the infected cell in an activated state ultimately promoting viral replication. Collectively, these findings suggest that miR-190b upregulation may be part of the normal cellular stress response to viral replication and its targeting of MTMR6 may be to promote cellular survival, which eventually benefits the virus.

To our knowledge, this is the first report describing genome wide changes in miRNA expression in the GI tract in response to HIV/SIV infection. The fact that miR-190b is markedly elevated predominantly in response to SIV infection suggests important roles for this miRNA in regulating host cell responses to SIV replication. Although we have identified and confirmed MTMR6 as a direct target of miR-190b, additional studies employing high throughput approaches are needed to validate and identify the roles of the remaining 185 predicted targets in regulating virus-host cell interactions. It is also intriguing to know whether miR-190b induction is directly triggered by viral proteins or indirectly by host cellular factors responding to viral replication. To gain detailed insight miR-190b knockdown studies to evaluate its role in viral replication and host cell response are also needed. Finally, although miR-190b expression levels increased in response to SIV replication it cannot be concluded that its upregulation is specific to SIV. Future studies are also needed to determine whether miR-190b expression levels increase in response to infection other than SIV and other viruses.

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Disclosures

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