Differential Regulation of the Let-7 Family of MicroRNAs in CD4$^+$ T Cells Alters IL-10 Expression

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MicroRNAs (miRNAs) are ~22-nt small RNAs that are important regulators of mRNA turnover and translation. Recent studies have shown the importance of the miRNA pathway in HIV-1 infection, particularly in maintaining latency. Our initial in vitro studies demonstrated that HIV-1–infected HUT78 cells expressed significantly higher IL-10 levels compared with uninfected cultures. IL-10 plays an important role in the dysregulated cytotoxic T cell response to HIV-1, and in silico algorithms suggested that let-7 miRNAs target *IL10* mRNA. In a time course experiment, we demonstrated that let-7 miRNAs fall rapidly following HIV-1 infection in HUT78 cells with concomitant rises in IL-10. To show a direct link between let-7 and IL-10, forced overexpression of let-7 miRNAs resulted in significantly reduced IL-10 levels, whereas inhibition of the function of these miRNAs increased IL-10. To demonstrate the relevance of these results, we focused our attention on CD4$^+$ T cells from uninfected healthy controls, chronic HIV-1–infected patients, and long-term nonprogressors. We characterized miRNA changes in CD4$^+$ T cells from these three groups and demonstrated that let-7 miRNAs were highly expressed in CD4$^+$ T cells from healthy controls and let-7 miRNAs were significantly decreased in chronic HIV-1 infected compared with both healthy controls and long-term nonprogressors. We describe a novel mechanism whereby IL-10 levels can be potentially modulated by changes to let-7 miRNAs. In HIV-1 infection, the decrease in let-7 miRNAs may result in an increase in IL-10 from CD4$^+$ T cells and provide the virus with an important survival advantage by manipulating the host immune response. ***The Journal of Immunology*****, 2012, 188: 6238–6246.**

MicroRNAs (miRNAs) are ~22-nt small RNAs that are critical regulators of protein expression within cells at a posttranscriptional level. They are mostly transcribed from noncoding regions of the genome and are initially transcribed by RNA polymerase II into a long primary transcript (1) and subsequently processed by *Drosha* and DGCR8 into a precursor miRNA (pre-miRNA), a shorter stem-loop structure (2, 3) with a 2-nt overhang at the 3′ end. Pre-miRNAs are transported to the cytoplasm where they are further processed by *Dicer*, another RNase III endonuclease (4), into a RNA duplex consisting of a guide strand (antisense) and a passenger strand, each with a 2-nt overhang at the 3′ end. The guide strand is incorporated into the RNA-induced silencing complex, whereas the passenger strand is degraded. Binding of miRNAs to relevant mRNA targets at the 3′ untranslated region (3′UTR) region of mRNA via RNA-induced silencing complex leads to mRNA degradation or translational inhibition (5).

IL-10 is a pleiotropic cytokine expressed by a large number of immune cells, including dendritic cells, monocytes/macrophages, neutrophils, mast cells, basophils, NK cells, and a number of T cell subsets, including Th1, Th2, Th9, Th17, and regulatory T cells (Tregs) (6). Plasma IL-10 levels are increased in patients with HIV-1 infection and directly contribute to the abnormal cytotoxic T cell response to this viral infection (7). The regulation of IL-10 occurs at a number of levels within the cell, including at the epigenetic level, via various signal transduction pathways, by a number of transcription factors, and may also be modulated at a posttranscriptional level by miR-106a (8).

There is a growing literature that shows that cellular miRNAs play an important role in HIV-1 pathogenesis (9). To demonstrate the importance of miRNAs in HIV-1 infection, *Dicer* and *Drosha*, key components of the miRNA pathway outlined above, have been previously knocked down using small interfering RNAs in HIV-1–infected PBMCs with a marked increase in viral replication noted (10). Likewise, certain miRNAs have been shown to play an important role in maintaining latency in HIV-1–infected resting CD4$^+$ T cells, and these particular miRNAs can also modulate HIV-1 replication kinetics (11).

In this study, we present data clearly demonstrating that let-7 miRNAs are able to modulate the expression of IL-10 at a posttranscriptional level. We show that HUT78 cells (a T cell line)

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Abbreviations used in this article: CHI, chronic HIV-1 infected; Ct, cycle threshold; HC, healthy control; LTNP, long-term nonprogressor; miRNA, microRNA; pre-miRNA, precursor miRNA; qPCR, quantitative PCR; Treg, regulatory T cell; UTR, untranslated region.

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express high levels of IL-10 and that overexpression of let-7 miRNAs results in a significant decrease in IL-10 levels, whereas blocking these same miRNAs results in a marked and significant rise in IL-10 levels. HIV-1 infection of HUT78 cells results in a fall in let-7 miRNAs with a reciprocal rise in IL-10, suggesting that the decreases in let-7 may play a role in the rise in IL-10 noted with infection. We demonstrate that primary $CD4^+$ T cells from HIV-1–infected individuals have significantly lower let-7 miRNAs than healthy controls (HC), and we postulate that these changes in miRNA levels may be associated with the increased IL-10 seen in HIV-1 infection. The altered let-7/IL-10 axis observed in HIV-1 infection may play an important role in the dysfunctional cytotoxic T cell response that is known to occur in HIV-1 infection.

Materials and Methods

Ethics statement

Ethics approval to carry out the work presented in this article was approved by the St. Vincent's Hospital Human Research Ethics Committee. All study participants provided informed written consent prior to blood being drawn.

Patient selection

All HIV-1–infected patients (long-term nonprogressor [LTNP] or chronic HIV-1 infected [CHI]) were antiretroviral treatment naive prior to the samples being taken. To reduce variability in results due to sex differences, all participants studied were male. LTNP samples were obtained from the Australian LTNP cohort that was established in 1994 and is being followed up by the Kirby Institute (formerly the National Centre for HIV Epidemiology and Clinical Research), Sydney, Australia. The LTNPs enrolled in this cohort met the following criteria: asymptomatic HIV-1 disease who had been infected with HIV-1 for at least 8 y prior to study entry and had a $CD4^+$ T cell count $\geq$500 cells/μl in the absence of any antiretroviral treatment (12). For this study we excluded LTNPs who had defective virus (*nef* deletion) or HIV-1 coreceptor polymorphisms (homozygous or heterozygous to the delta 32 CCR5 receptor mutation), which may have explained their LTNP status. It is important to note that many of the LTNPs used in the study had detectable viraemia and are not elite controllers. This was deliberate, as we were looking for other factors related to nonprogression apart from those already described. Several elite controllers were excluded from the study, as they had other factors that explained their nonprogressor status. Demographics for LTNPs are listed in Table I. Patients with progressive HIV-1 infection (CHI) were chosen based on HIV-1 plasma viral loads of >20,000 copies/ml, declining $CD4^+$ T cell counts, and not meeting LTNP criteria (see Table II). HC were recruited from St. Vincent's Centre for Applied Medical Research and were all HIV-1 negative on serology.

T cell cultures and growth conditions

The T cell line, HUT78, was obtained through the AIDS Research and Reference Reagent Program (catalog 89), Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, from R. Gallo (13). HeLa cells were obtained through the AIDS Research and Reference Reagent Program (catalog 153), Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, from R. Axel (14). HUT78 cells were cultured in RPMI 1640 and 10% FBS, whereas HeLa cells were cultured in 90% DMEM and 10% FBS. For the time course experiment, 80 μl HIV-1_{IIIb} virus stock (3.1 pg reverse-transcriptase activity/μl) was added to 1.2×10^6 cells, placed on a rotator, and incubated at 37°C with 5% CO_2 for 2 h. These cells were then washed twice in PBS and then split into wells with 1 ml culture medium representing each time point. p24 levels in culture supernatant were determined using the Genetics Systems HIV-1 p24 Ag kit (Bio-Rad, Hercules, CA).

Pre-miRNA and anti-miRNA experiments

Pre-miRNA precursor molecules for let-7b, let-7c, let-7f, and the Negative Control #1 were obtained from Applied Biosystems (Foster City, CA). A total of 1.5×10^6 HUT78 cells was electroporated using the Amaxa Nucleofector system (Kit R) with 10 nM respective pre-miRNA. Cells were cultured for 3 d, and supernatants were collected for IL-10 measurement using Human IL-10 Ultrasensitive Immunoassay Kit (Invitrogen). Samples were diluted 1:50 in PBS prior to running on the IL-10 ELISA. Anti-miRNA molecules were obtained from Applied Biosystems for let-7b, let-7c, and let-7f, and the Anti-miR Negative Control #1.

Synthesis of IL10 3′UTR reporter construct

A 765-bp region of the 1021-bp *IL10* 3′UTR was first amplified using PCR and then cloned into the pMIR-REPORT miRNA Expression Reporter Vector System (Applied Biosystems). This region contained the binding site for the let-7 miRNA seed (nt 139–145 of *IL10* 3′UTR).

The forward primer for the *IL10* 3′UTR PCR contained a restriction enzyme cloning site for the restriction enzyme, SpeI (underlined), as follows: 5′-ATATCGACTAGTAGAGGTCTCCAAAATCGGATCT-3′.

The reverse primer for the *IL10* 3′UTR had a restriction site for MluI (underlined), as follows: 5′-ATATCGACGCGTAGCAATTCTCTTGCCTCAGCCTC-3′.

The *IL10* 3′UTR was ligated (Rapid DNA Ligation Kit; Roche) into enzymatically double-digested (MluI and SpeI) pMIR-REPORT, transformed in One Shot TOP10 Chemically Competent *Escherichia coli* (Invitrogen), and plasmid DNA extracted following a maxi-prep using the PowerPrep HP Plasmid Purification Kits (Marligen Biosciences). Sequencing was performed to confirm that the *IL10* 3′UTR contained the appropriate sequence with the let-7 miRNA binding site.

Pre-miRNA and anti-miRNA tranfection into IL-10 3′UTR reporter construct

One day prior to transfection, 50×10^4 HeLa cells were plated into each well of 24 wells. Twenty-four hours later, cells were cotransfected with the *IL10* 3′UTR reporter and a control plasmid, pRL-CMV (Promega), and appropriate pre-miRNA or anti-miRNA using Lipofectamine 2000 reagent. A total of 4.8 μl pre-miRNA or anti-miRNA at a concentration of 6.25 μmol/l was used for the transfection experiments. Samples were lysed the following day using Passive Lysis Buffer from the Dual-Luciferase Reporter Assay System (Promega). A total of 20 μl lysate was plated and appropriate substrate was added for luciferase, and then read on a luminometer (Fluostar), followed by the substrate for *Renilla* firefly and then rereading for luminescence. The seed sequence refers to nt 2–8 from the 5′ end of the miRNA.

Let-7 mimics

Let-7b seed mutants (either 2 bases or complete scrambled sequence) were synthesized by Life Technologies and cotransfected with the *IL10* 3′UTR reporter described above. Sequences are described from 5′ to 3′, as follows: let-7b mimic,antisense strand, UGA GGU AGU AGG UUG UGU GGTT, and sense strand, CCA CAC AAC CUA CUA CCU CATT; let-7b M2 (2-base mutation highlighted in bold), antisense strand, UGA **UA**U AGU AGG UUG UGU GGTT, and sense strand, CCA CAC AAC **CU**A CUA UAU CATT; let-7b scrambled (scrambled seed sequence highlighted in bold), antisense strand, U**CU ACG GA**U AGG UUG UGU GGTT, and sense strand, CCA CAC AAC CUA UCC GUA GATT.

$CD14^+$ monocyte and $CD4^+$ T cell isolation and RNA extraction

PBMCs were separated from blood using density centrifugation (Ficoll). $CD4^+$ T cells and monocytes were separated from PBMCs using magnetic bead technology (Miltenyi Biotec). Initially, $CD14^+$ cells (monocytes) were positively selected using the magnetically labeled beads and isolated, followed by positive selection and isolation of $CD4^+$ T cells. Flow cytometric examination of a subset of samples confirmed the purity of magnetically sorted $CD4^+$ T cells to be >98%. Following miRNA extraction (Qiagen miRNeasy kit), a stringent three-step quality control procedure was followed to ensure only high-quality RNA was used in the arrays. We performed spectrophotometry on the samples, and only RNA that had 260/280 ratios of ~2 and 260/230 ratios >1 (preferably >1.5) were accepted. Samples were then analyzed on an Agilent Bioanalyzer chip to confirm RNA purity and to ensure RNA quality was adequate (aiming for samples with a RNA integrity number >7). Lastly, RNA was run on a small RNA chip on the Bioanalyzer to confirm small RNA species were present prior to running the array.

Microarray analysis

In brief, 100 ng total RNA was dephosphorylated, desalted, and labeled with Cy-3 for each sample and then subsequently hybridized to the Agilent Human miRNA Microarray 2.0 G4470B array that contained probes for 723 human miRNAs, and scanned on an Agilent G2505B scanner at the Ramaciotti Centre for Gene Function Analysis, University of New South Wales, according to the manufacturer's instructions. We used three microarray slides, each containing eight arrays ($n = 24$). Agilent TXT files for each array were imported into GeneSpring GX 10 (Agilent), and two samples (one LTNP and one CHI) were outliers by a principal components analysis, had elevated additive error rates, and were thus excluded from

further analysis. The remaining 22 arrays were reimported into GeneSpring GX 10, and preprocessed using default settings, as follows: threshold raw signals to 1.0, followed by log-base-2 transformation, then percentile shift normalization, setting the 75th percentile on each array to 0. We excluded nonhuman miRNAs (leaving n = 723) and miRNAs detected fewer than five times (leaving n = 224). The two-dimensional hierarchical clustering was done using the XCluster2 module in *GenePattern*, using Pearson correlation as the distance metric. Differential expression analysis was done using GeneSpring GX 10, using an unpaired two-sample t test between relevant groups, using the Benjamini-Hochberg correction for multiple testing (15).

Microarray data

The microarray data have been deposited into Gene Expression Omnibus (16) and are accessible at the following site: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24022.

Quantitative real-time PCR

Reverse transcription of RNA followed by real-time PCR was performed using commercial kits from Applied Biosystems. PCR was performed according to manufacturer's recommendations for patient samples. The Δ cycle threshold (Ct) values were obtained for each patient in each group for each miRNA tested, and p values were generated by performing an unpaired two-tailed Student t test between groups. RNU44 was used as the endogenous control RNA species (considered as a housekeeper control), making comparisons between groups possible. Relative expression (ΔCt) and quantification ($RQ = 2^{-(\Delta\Delta Ct)}$) for each miRNA were calculated using the ΔΔCt method, as suggested by the manufacturer.

Flow cytometry

mAbs that were used included the following: anti-CD3 PerCP-Cy5.5, CD4-PECy7 and FITC, CD25-allophycocyanin, CD62L-allophycocyanin-Cy7 (BD Pharmingen), CD45RO-ECD (Beckman Coulter), CD127-PE (Immunotech), and CD127-Pacific Blue (eBiosciences). Tregs were stained and sorted using standard protocols (17) on a BD FACSAria flow cytometer.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.0a (GraphPad Software, San Diego, CA). Differences between study groups were evaluated using unpaired t tests. The p values <0.05 were considered statistically significant. For array statistics, see below. Error bars in graphs refer to the SEM.

Results

IL-10 levels are significantly increased in HIV-1 infection

It is well established that IL-10, an important anti-inflammatory cytokine, is elevated in the plasma of HIV-1–infected patients compared with HC (7, 18, 19). We wanted to investigate the regulatory mechanisms behind the observed increase in IL-10 in HIV-1 infection. We first chose to investigate the effect of HIV-1 infection in HUT78 cells, a T cell line. These cells were chosen as they support productive HIV-1 infection (20) and produce high levels of IL-10 even in the absence of HIV-1 infection (21). We measured IL-10 production over time in supernatants of uninfected and infected HUT78 cells using a cytokine bead array. By 24 h postinfection, IL-10 levels were markedly elevated in HIV-1–infected HUT78 cells compared with uninfected cells (Fig. 1A). This difference was most prominent at 72 h (p = 0.008). These results confirmed that, in an in vitro setting, HIV-1 infection is associated with increased IL-10 levels, a finding consistent with the elevated IL-10 observed in plasma from patients infected with HIV-1 (7).

To investigate whether miRNAs played a role in regulating IL-10 levels in HIV-1 infection, we used the in silico screening tool TargetScan 5.1. This online tool demonstrated that the let-7 family of miRNAs possessed a highly conserved 7mer seed match to the *IL10* 3′UTR (Fig. 1B).

Let-7b and let-7c levels fall rapidly after HIV-1 infection in a T cell model

Using the same in vitro model, we investigated whether let-7 miRNAs were altered following HIV-infection. After infecting HUT78 cells with HIV-1_{IIIb}, we measured HIV-1 Gag p24 levels in supernatant and found these levels increased significantly at 24 h and were markedly elevated at 72 h (Fig. 2A), consistent with these cells to be infected with the virus. There was downregulation of expression of let-7 family members within 24 h for both let-7b (p = 0.11) (Fig. 2B) and let-7c (p = 0.007) (Fig. 2C) in infected cultures, reaching statistical significance for let-7b at 72 h (p = 0.04). Let-7f levels were not statistically significantly different at any of the time points (Fig. 2D). These results demonstrated that expression levels of let-7 miRNAs fall rapidly following acute infection in an in vitro model, with different let-7 members showing variable expression kinetics. Consistent with our findings, reduced expression of let-7 family members (let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, and let-7g) has been previously demonstrated in HeLa cells infected with the HIV-1 clone, NL4-3 (22). As let-7 miRNAs potentially can target the *IL10* 3′UTR, a fall in let-7 miRNAs is therefore consistent with them playing a role in the increasing levels of IL-10 observed in HIV-1 infection. To explore this hypothesis, however, we needed to manipulate the levels of

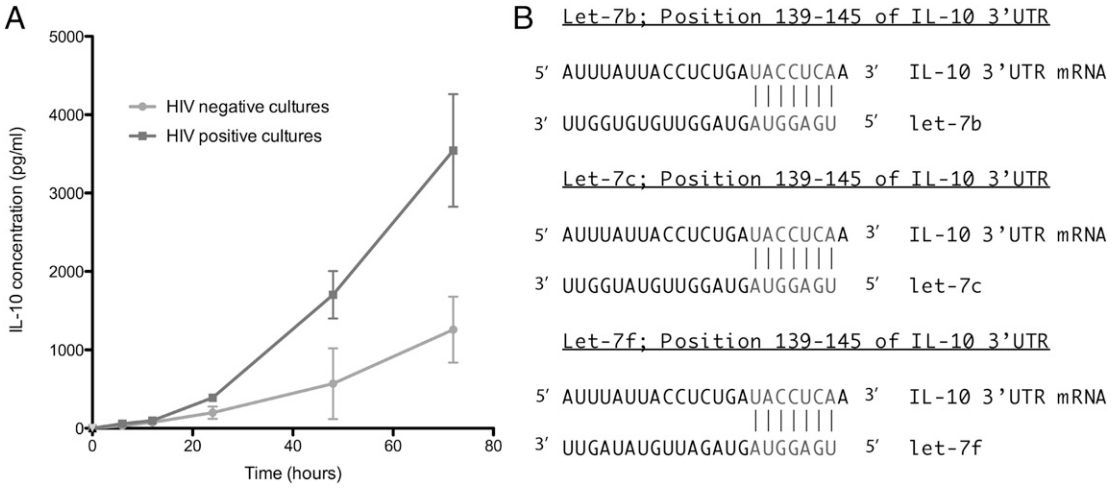

FIGURE 1. IL-10 is significantly increased in HUT78 cells infected with HIV-1 compared with uninfected cultures. (**A**) HUT78 cells were infected with HIV-1 and followed by a time course experiment over 72 h. Within 24 h, there is a divergence in IL-10 levels between infected and uninfected cultures, most noticeable by 72 h (p = 0.008). (**B**) Using TargetScan 5.1, the *IL10* 3′UTR is highly targeted by a number of let-7 family members. Cultures were performed in triplicate and error bars represent +SEM. Unpaired two-tailed t tests were used for statistical analysis.

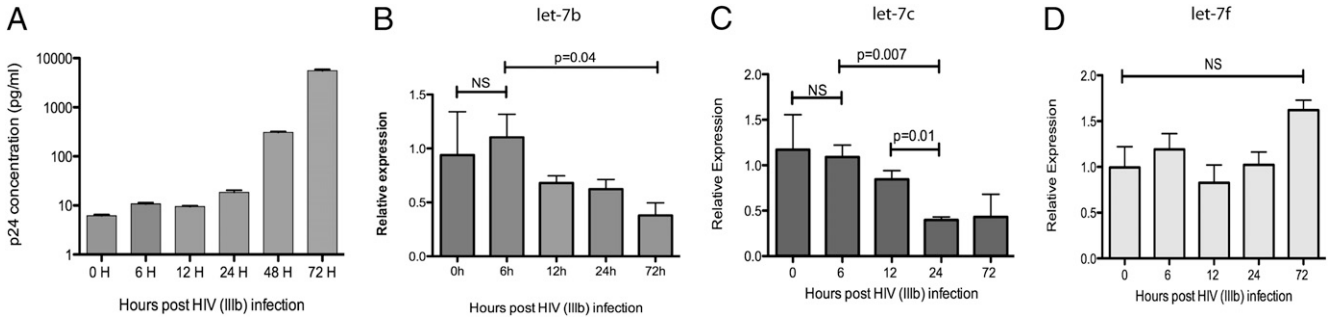

FIGURE 2. Let-7b and let-7c levels fall rapidly following HIV-1 infection. (**A**) p24 levels are measured in the same time course experiment used to measure IL-10 levels as in Fig. 1 (p24 levels rapidly rise after 24 h; note *y*-axis logarithmic scale). (**B**) let-7b levels as measured by qPCR are significantly lower at 72 h compared with baseline, whereas for let-7c (**C**) they are significantly lower by 24 h ($p = 0.007$). In contrast, let-7f levels do not appear to significantly change over time (**D**). Experiments were performed in triplicate, and error bars represent +SEM. Unpaired two-tailed *t* tests were used for statistical analysis.

let-7 miRNAs in HUT78 cells and observe the effect on IL-10 levels.

Modulating let-7 levels directly affects IL-10 levels

Next, we sought direct evidence that manipulation of let-7 levels could impact upon IL-10 expression in HUT78 cells. Overexpression of miRNAs was achieved by transfecting HUT78 cells with let-7 or negative control pre-miRNAs and measuring IL-10 levels in the supernatant. Overexpression of let-7b, c, and f pre-miRNAs (Fig. 3A) alone or in combination reduced IL-10 levels on average by 65% (range 53–83% reduction) compared with negative control pre-miRNA. Overexpression of let-7c did not significantly change IL-10 levels, but there was a strong trend toward reduction ($p = 0.06$). In the converse experiment, the action of let-7 miRNAs was inhibited using anti-miRNAs with an average 300% increase (range 157–438% increase) in IL-10 levels (Fig. 3B) compared with negative anti-miRNA control. Anti-miRNAs to let-7b failed to show a statistically significant rise in IL-10 ($p = 0.06$), although there was a strong trend toward increasing IL-10 in these cultures. It was important to check that modulation of let-7 was not affecting IL-10 by changing HIV-1 viral kinetics. To investigate this, we performed experiments measuring p24 in culture supernatant from HUT78 cells overexpressing let-7b, let-7c, and let-7f and compare this to p24 levels in mock-transfected cells. There was no demonstrable change in p24 levels with overexpression of let-7 miRNAs, suggesting that let-7 was not affecting IL-10 levels by changing viral replication (data not shown).

Finally, to determine the specificity of the interaction between the let-7 family and IL-10, we developed a firefly luciferase reporter construct, which contained the *IL10* 3′UTR. Binding of let-7 miRNAs to the *IL10* 3′UTR should theoretically downregulate luciferase expression in this system. HeLa cells were cotransfected with the reporter and either pre-miRNAs or anti-miRNAs against let-7 family members. These experiments showed significant reduction of luminescence in the pre-miR-let-7–treated cells compared with negative miRNA control and empty vector control (Fig. 4A). Furthermore, using let-7 anti-miRNAs to inhibit the action of endogenous let-7 within HeLa cells was associated with an increase in luminescence compared with empty vector and negative anti-miRNA controls (Fig. 4B). Manipulation of let-7 levels in the HUT78 cell line and the *IL10* 3′UTR reporter system in HeLa cells clearly demonstrates that let-7 directly affects IL-10 and that this is occurring through binding of let-7 miRNAs to the *IL10* 3′UTR. Controls including mutated let-7b seed site by 2

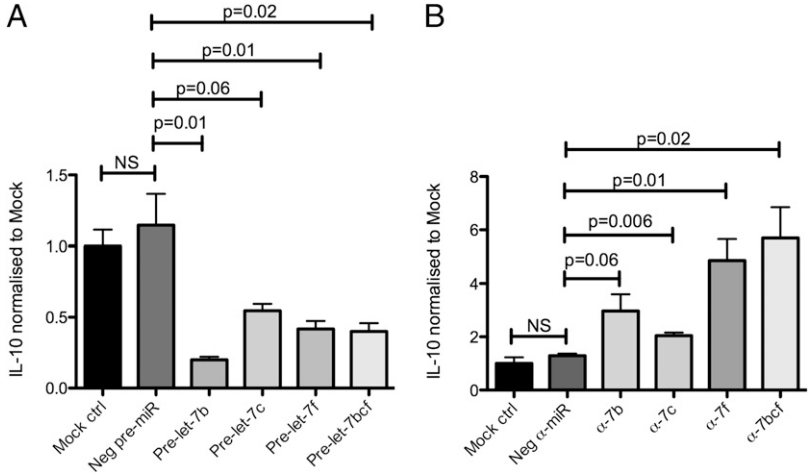

FIGURE 3. Modulation of let-7 miRNAs directly alters IL-10 expression levels. (**A**) HUT78 cells were electroporated with either no pre-miRNAs (mock control), a negative pre-miRNA control (Neg pre-miRNA), pre-let–7b, pre-let–7c, pre-let–7f, or a combination of pre-let–7b, c, and f in equimolar amounts. Supernatants were collected at 72 h, and IL-10 levels were measured. This showed that IL-10 was significantly lowered in HUT78 cells overexpressing let-7 family members (apart from let-7c). (**B**) Inhibition of let 7 family members increased IL-10 expression. HUT78 cells were electroporated with no anti-miRNAs (mock ctrl), a negative anti-miRNA control (neg anti-miR), anti–let-7b, anti–let-7c, anti–let-7f, or a combination of anti–let-7b, c, and f in equimolar amounts. This resulted in a significant increase in IL-10 levels for all let-7 family members, apart from let-7b. All experiments were performed in triplicate. Error bars represent +SEM. Unpaired two-tailed *t* tests were used for statistical analysis.

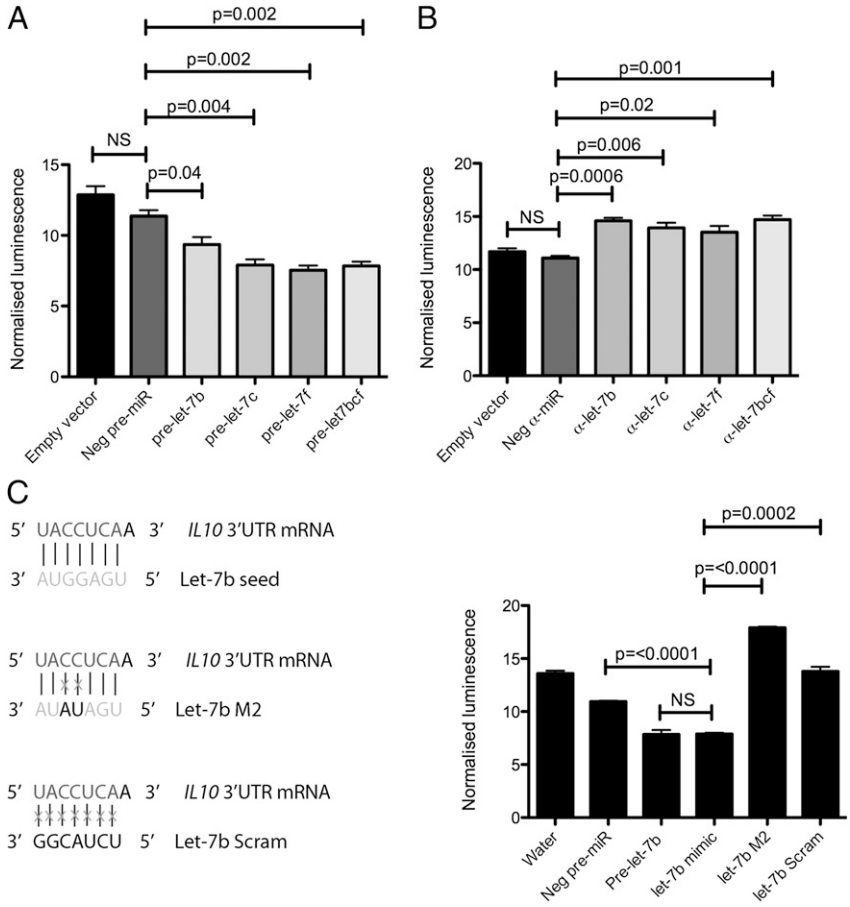

FIGURE 4. Let-7 miRNAs affect IL-10 levels by binding to the *IL10* 3′UTR. The *IL10* 3′UTR was cloned into a luciferase reporter and transiently transfected into HeLa cells, which were then cotransfected with either water alone (empty vector), a negative pre-miRNA, pre-let–7b, pre-let–7c, pre-let–7f, or a combination of pre-let–7b, c, and f (in equimolar amounts). All combinations of pre-let–7 members (alone or in combination) showed a statistically significant decline in luminescence compared with the negative pre-miRNA and water control (**A**). (**B**) HeLa cells were cotransfected with the *IL10* 3′UTR reporter and anti-miRNAs (mock ctrl), a negative anti-miRNA control (neg α-miR), α–let-7b, α–let-7c, α–let-7f, or a combination of α–let-7b, c, and f (in equimolar amounts). All anti-miRNAs against let-7 members had a statistically significant increase in luminescence compared with empty vector and negative control anti-miRNAs. To demonstrate specificity of binding, we created a let-7b mimic that had the exact seed sequence that binds to nt 139–145 of the *IL10* 3′UTR [*top panel* in (**C**)]. We created a let-7b mutant mimic [*middle panel* in (C)] that was identical to let-7b apart from a two-base substitution (bases 5 and 6 from 5′ end). Finally, we created a let-7b scrambled mimic (Let-7b Scram) that was identical to let-7b apart from a completely scrambled seed sequence [positions 2–8 from 5′ end; *lower panel* in (C)]. We transiently transfected HeLa cells with our *IL10* 3′UTR reporter construct and the let-7 mimics. We clearly show that the let-7b mimic bound to the *IL10* 3′UTR to the same extent as pre-let–7b (no statistical difference between the mimic and pre-let–7b). Both the let-7b M2 and Let-7 Scram failed to bind to the *IL10* 3′UTR compared with the let-7b mimic or pre-let–7b, demonstrating specificity of binding of let-7b to the *IL10* 3′UTR. The *p* values refer to unpaired *t* tests between groups. Experiments were carried out independently in triplicate. Error bars indicate +SEM. Unpaired two-tailed *t* tests were used for statistical analysis.

bases (M2) or transfection with a scrambled seed sequence did not impact on luminescence demonstrating specificity (Fig. 4C).

Let-7 miRNAs are significantly downregulated in HIV-1 infection

To investigate whether let-7 miRNAs were altered in HIV-1–infected patients, we chose to comprehensively catalog miRNA changes in CD4+ T cells from three distinct patient groups, as follows: eight HIV-1–negative HC, seven patients with chronic HIV-1 infection (CHI), and seven LTNPs (demographics for HIV-1–infected patients are listed in Tables I and II). Total RNA was extracted and run on an Agilent microarray. After normalizing microarray data, we excluded all nonhuman miRNAs, and miRNAs detected fewer than five times, leaving 224 miRNAs de-

Table I. Demographics of LTNPs

Patient	Age (y)	Years after HIV-1 Diagnosis	CD4 Count (/μl)	Viral Load (Copies/ml)	HLA B*57 or B*27 Present
1	40	16	672	<50	B*57
2	49	16	990	84	—
3	48	18	609	293	B*27
4	58	15	703	215	—
5	51	19	552	4,600	B*27
6	60	10	1,014	7,100	B*57
7	52	14	1,073	20,800	—

Table II. Demographics of patients with progressive HIV-1 infection (CHI)

Patient	Age (y)	Years after HIV-1 Diagnosis	CD4 Count (/μl)	Viral Load (Copies/ml)
1	31	2	528	174,500
2	41	6	352	50,400
3	32	3	456	83,800
4	42	1 mo	228	420,140
5	45	1.5	209	24,800
6	33	6	324	144,200
7	41	7	384	976,730

tected. We performed unsupervised hierarchical clustering to determine the relationship between samples in each group. This identified two very distinct groups of samples, with all HC subjects clustering in one group and infected patients (LTNP and CHI) dominating the second group. It was observed that HIV-1–infected patients (both LTNP and CHI) downregulated the expression of a large number of these 224 miRNAs compared with HC. As with the HUT78 cells infected with HIV-1, the let-7 family of miRNAs was uniformly downregulated in the HIV-1–infected group of patients compared with the HC group. The LNTP group also appeared to have an intermediate expression level of let-7 compared with the CHI group, whereas the highest expression was observed in the HC group (Fig. 5).

To find differentially expressed miRNAs among HC, LTNP, and CHI sample groups, an unpaired, two-sample *t* statistic was performed on each miRNA, for each of the three possible comparisons. There were 111 up- and 52 downregulated miRNAs in CHI relative to HC, and 70 up- and 27 downregulated miRNAs in LTNPs relative to HC (false discovery rate <0.05) (see Supplemental Tables I, II for top 45 differentially expressed miRNAs). Using a false discovery rate <0.05, there were no statistically different miRNAs expressed between LTNPs and CHI patients, however. Eight of the top 45 differentially expressed miRNAs in the CHI versus HC group were from the let-7 family, whereas there were nine let-7 family members differentially expressed in the top 45 differentially expressed miRNAs between LTNPs and HC.

To confirm the changes observed on the miRNA microarray, we ran confirmatory quantitative PCR (qPCR) on three representative let-7 family members, let-7b, let-7i, and let-7f (Fig. 6A–C). Using the qPCR assay, we showed highly significant differences between LTNPs and CHI patients for let-7b expression ($p < 0.0001$) (Fig. 6A) and let-7i expression ($p = 0.003$) (Fig. 6B). There was no significant difference in expression levels between HC and LTNPs for let-7b expression ($p = 0.57$) and let-7i levels ($p = 0.70$) by qPCR, indicating similar expression levels in these two groups. For let-7f qPCR failed to find a significant difference between any of the three patient groups, findings that are similar to those observed with HUT78 cells (Fig. 2C). Our results are consistent with previous work showing reduced levels of let-7 family members in whole PBMCs of HIV-1–infected patients compared with HC (23). However, as whole PBMC were used in that study, no conclusions could be drawn about which cell type contributed to the altered miRNA profile.

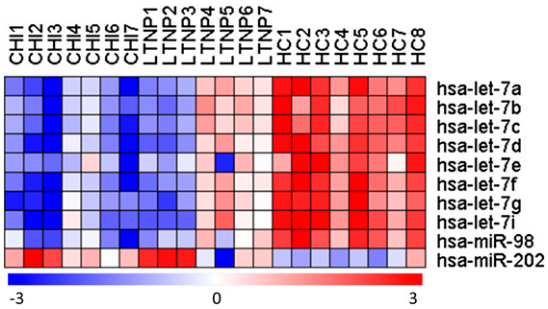

FIGURE 5. Relative expression heat map of let-7 family members from the three patient groups. There are 10 let-7 family members, and, apart from miR-202, they all show significant downregulation in both CHI and LTNP compared with HC. With regard to the relative expression heat map, high expression is denoted by red, and low levels are depicted in blue, as shown by the color legend at the *bottom*.

Differences in miRNA levels between naive and activated $CD4^+$ T cells may also explain differences observed between the various patient groups. We sorted (by flow cytometry) $CD4^+$ T cells from three uninfected donors into naive Tregs and activated Tregs and measured let-7a levels in these cells. In activated Tregs compared with naive Tregs, we observed a strong trend toward a lowering of let-7a levels (~4-fold lower) with activation (see Supplemental Fig. 1). Further work will be required to see whether these differences occur in HIV-1–infected individuals.

IL-10 levels in patient groups

IL-10 levels in our patient groups were measured from plasma, and there was a significant increase in IL-10 between HC and CHI ($p = 0.04$), but not between HC and LTNP or LTNP and CHI (Supplemental Fig. 2). However, which cells are contributing to the raised IL-10 is not possible to discern from these experiments.

As monocytes are thought to be a major source of IL-10, we performed miRNA profiling experiments on monocytes isolated from the same patients outlined above. There was no differential regulation of let-7 miRNAs between any of the three patient groups, suggesting that the let-7 family of miRNAs does not play a differential role in IL-10 expression in monocytes in the context of HIV-1 infection.

Discussion

This study has demonstrated that, in an in vitro setting, HIV-1 infection of a T cell line is associated with increased IL-10 levels compared with uninfected cultures. Let-7 miRNAs are predicted to target the *IL10* 3′UTR, and we demonstrated that let-7 miRNAs fell rapidly following HIV-1 infection of HUT78 cells, suggesting that they may play a role in regulating IL-10 levels. To demonstrate this more directly, overexpression of let-7 miRNAs significantly lowered IL-10 levels in these cells, whereas blocking these same miRNAs significantly increased IL-10. Changing the let-7 seed sequence by just 2 bases totally abrogated binding to a reporter construct expressing the *IL10* 3′UTR. To investigate the role of let-7 miRNAs in $CD4^+$ T cells from patients infected with HIV-1, we systematically profiled the presence of miRNAs in $CD4^+$ T cells from three distinct patient groups, as follows: HC, CHI, and LTNPs. The microarray revealed 111 up- and 52 downregulated miRNAs in CHI relative to HC, and 70 up- and 27 downregulated miRNAs in LTNPs relative to HC. As with our in vitro model, the let-7 family of miRNAs was significantly downregulated in HIV-1 infection. To our knowledge, these results comprise one of the first major profiling experiments to systematically characterize miRNAs between these three patient groups and also highlight a new role of differentially expressed let-7 miRNAs in cytokine secretion by $CD4^+$ T cells in HIV-1 infection.

IL-10 has an important role in HIV-1 infection, and a number of studies have demonstrated elevated levels occurring in HIV-1 infection (7, 19, 24, 25). However, there remains some debate as to whether elevated levels of IL-10 lead to viral persistence and a poor immune response or whether they are associated with a better outcome. A number of polymorphisms in the IL-10 or IL-10R genes have been recognized, and there have been efforts to

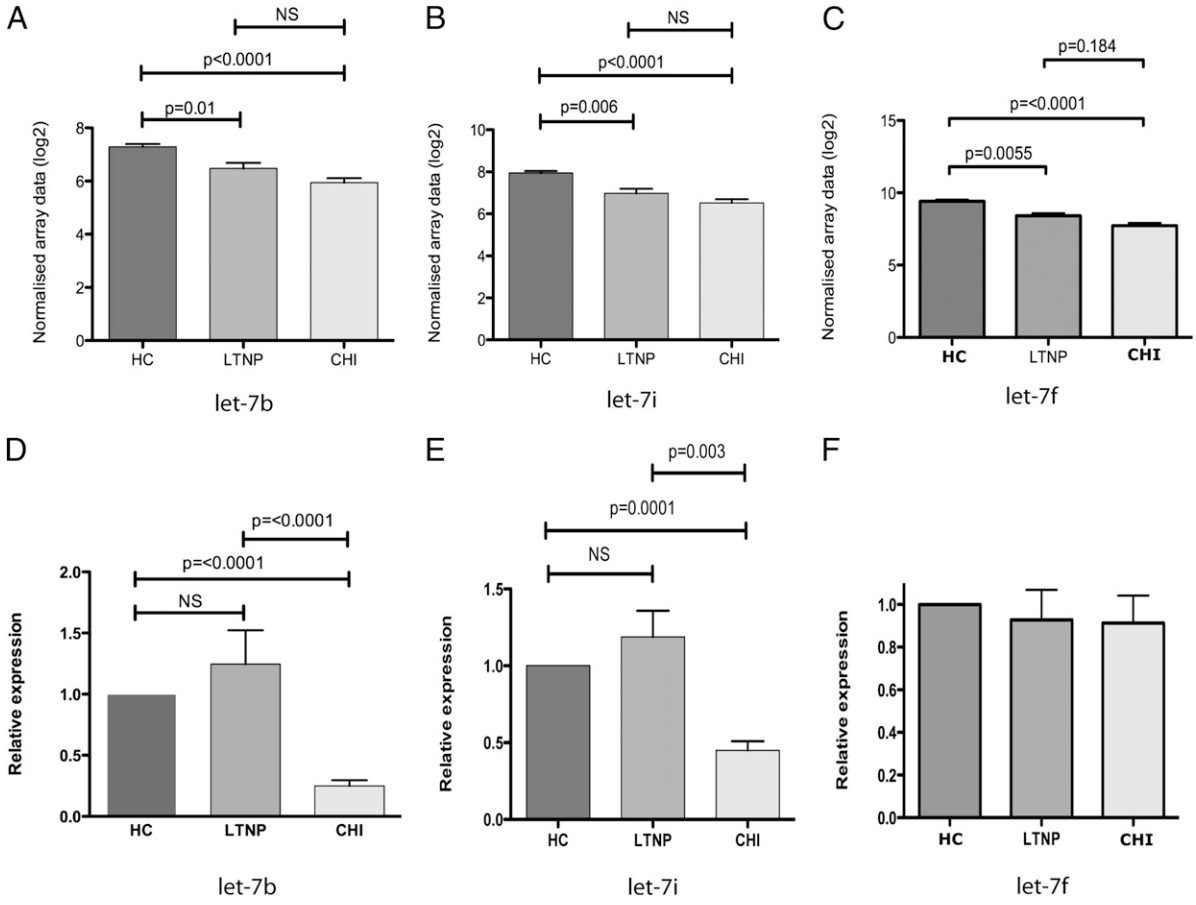

FIGURE 6. Microarray and qPCR results for three representative let-7 family members. Array data are presented in the *top row* (**A**–**C**) for three let-7 family members, let-7b (A), let-7i (B), and let-7f (C). Array data for these three miRNAs demonstrated a highly significant difference ($p < 0.0001$) between HC and CHI, but no statistical difference between CHI and LTNPs. Note expression on the *y*-axis is logarithmic scale. The *bottom row* (**D**–**F**) shows corresponding qPCR results for the same three miRNAs, as follows: let-7b (D), let-7c (E), and let-7f (F). Trends are similar to the array results apart from statistical significance noted between LTNPs and CHI for let-7b and let-7i. Also, let-7f levels are not significantly different using qPCR, which is similar to the in vitro findings for this miRNA. For the qPCR, RNA was available from six of seven LTNPs and six of seven CHI patients, whereas RNA was available for all HC patients. Error bars indicate +SEM. Unpaired two-tailed *t* tests were used for statistical analysis.

correlate these polymorphisms with disease outcome in both infectious and autoimmune diseases (26). The three best-described polymorphisms found within the IL-10 promoter are as follows: 1) −1082 (A to G transition), 2) −819 (C to T transition), and 3) −592 (C to A transversion) (27, 28). The −592 (C to A transversion) is associated with lower IL-10 production and faster disease progression in late stages of HIV-1 infection (29), whereas the −1082 (A to G transition) is associated with higher levels of IL-10 and slower CD4+ T cell decline and longer survival in HIV-1–infected individuals (30). These studies suggest that IL-10 may have a protective effect in HIV-1 infection. The haplotype −1082A/−592A was associated with faster progression to AIDS in Caucasians but not in African-Americans (31). A South-African cohort study found that individuals with genotypes associated with high IL-10 production (−1082G and −592C) had lower rates of HIV-1 infection, but had higher viral loads during the initial stages of HIV-1 infection (32). As the infection became established, the correlation between genotype and viral load reversed. This implies that the effects of IL-10 on HIV-1 pathogenesis are complex and may be different depending on the length of time the individual is infected. It is very clear that there are multiple ways in which IL-10 is regulated (from promoter polymorphisms to posttranscriptional regulation via miRNAs). Our results suggest a new posttranscriptional mechanism that might further modulate IL-10 levels and that requires consideration when designing studies that look to map the effect of IL-10 on outcomes.

Monocytes are an important source of IL-10, but a recent paper has demonstrated that multiple other cell types contribute to the increased IL-10 in HIV-1 infection, including T cells, B cells, and NK cells (7). This group also demonstrated that elevated IL-10 levels in HIV-1 infection impair HIV-1–specific CD4+ T cell responses. Other studies have suggested that the excess IL-10 may be secreted from a subset of CD4+ HIV-1–specific T cells in HIV-1–infected individuals. The IL-10 from these HIV-1–specific T cells is thought to inhibit the proliferation of other T cells in an Ag-nonspecific manner (33). The elevation of IL-10 in plasma from HIV-1–infected individuals does not allow one to conclude which cells have differentially contributed to the IL-10. Hence, we chose to investigate the effects of modulating let-7 levels on IL-10 expression in a T cell model amenable to manipulation of the miRNA levels without activating the cell. Ideally, we would have liked to manipulate miRNA levels in primary T cells infected with HIV-1, but one needs to activate these cells using anti-CD3 and anti-CD28 to stimulate the cells to allow productive infection. Recent research has demonstrated that T cell activation greatly alters endogenous miRNA levels (34) and hence would bias our results. Furthermore, although CD4+ T cells may contribute less IL-10 compared with other cell types such as

macrophages, increased production of IL-10 by $CD4^+$ T cells may be an important source of the additional IL-10 observed in HIV-1–infected patients compared with uninfected controls, which contributes to HIV pathogenesis.

IL-10 is regulated at a number of different levels within the cell. These mechanisms include signaling pathways (35, 36), binding of a number of transcription factors to the *IL10* promoter (37, 38), and, more recently, epigenetic changes that affect chromatin structure at the *IL10* locus (39, 40). All these have been described as potentially important transcriptional regulators of IL-10. There also appear to be several levels of posttranscriptional regulation with one miRNA to date described, miR-106a, binding to the *IL10* 3′UTR (8). Additionally, mRNA-destabilizing motifs in the 3′UTR have also been previously described (41). This highlights the complexity of regulation of this important anti-inflammatory cytokine. In our study, miR-106a was not shown to be differentially regulated between patient groups, and therefore was not a factor that may lead to differential expression of IL-10 between patient groups. However, our results strongly suggest let-7 family members can also regulate IL-10 expression in $CD4^+$ T cells, and their differential expression in $CD4^+$ T cells may thus be associated with altered disease progression. Furthermore, our results demonstrate HIV-1 infection leads to a decrease in let-7 levels and that these changes were associated with a concomitant rise in IL-10 levels. It should be noted that the vast majority of $CD4^+$ T cells from HIV-1–infected patients are not infected with the virus. Hence, the global downregulation in let-7 miRNAs in these cells is likely to be a bystander effect caused possibly by the activating stimulus of an ongoing viral infection. Another possibility is that there is a skewing in the various T cell subsets in HIV-1–infected patients, and differences in miRNA content within these subsets explain differences in miRNA profiles observed between uninfected and infected patients in our study. Recently, it has been demonstrated that T cell subsets display unique miRNA signatures (42), and further work in HIV-1–infected patients will be required to see whether this turns out to be the case in HIV-1 infection.

Let-7b has been recently linked to regulating another cytokine, IFN-β (43) (along with miR-26a, miR-34a, and miR-145). This regulation was demonstrated in macrophages, and there appeared to be a negative loop feedback mechanism between expression of this cytokine and miRNA levels. IL-10 levels have been shown to decrease in the plasma of HIV-1–infected patients who have been treated with highly active antiretroviral treatment (7). Whether or not let-7 miRNAs increase in this population of treated patients is not known, but if let-7 miRNAs normalize with treatment, this would point to a regulatory mechanism directly related to the magnitude of viral load.

This study highlights new areas of research into pathogenic mechanisms of HIV-1 infection. Raised IL-10 in chronic viral infections is believed to impair host immune responses and contribute to viral persistence (44). Targeted alteration of specific miRNAs may also play an important adjuvant role to future vaccine strategies to augment immune responses. Our work shows let-7 miRNAs have the potential to regulate IL-10 at the posttranscriptional level, and this mechanism plays one part in a complex regulatory network controlling IL-10 expression.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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Differential Regulation of the Let-7 Family of MicroRNAs in CD4 $^{+}$ T Cells Alters IL-10 Expression

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