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Human Immunodeficiency Virus Type 1 Nef in Human Monocyte-Like Cell Line THP-1 Expands Treg Cells via Toll-Like Receptor 2

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ABSTRACT

$CD4^+CD25^+$ regulatory T cells (Tregs) represent a unique T-cell lineage that is endowed with the ability to actively suppress immune responses in order to inhibit pathogenic damage resulting from over activation of the immune system. In human immunodeficiency virus-1 (HIV-1) infection, suppression of the immune response by Tregs appears to play an opposing role that promotes chronic viral infection. Treg expansion is known as a marker of the severity of HIV infection and as a potential prognostic marker of disease progression. HIV-1 Nef is one of the earliest expressed viral regulatory genes whose expression may play an important role in regulating Treg cells. We established a THP-1 cell line stably expressing HIV-1 Nef and showed that Nef protein was a potent factor for increasing Treg numbers in vitro. We further found that TLR2 plays a critical role in the increase in Treg cells induced by Nef using TLR2-specific siRNA. Our results suggest new strategies for therapeutic and preventive interventions of HIV infection. J. Cell. Biochem. 112: 3515–3524, 2011. © 2011 Wiley Periodicals, Inc.

KEY WORDS: HIV-1 Nef; TLR2; TREG CELLS; SiRNA

Human immunodeficiency virus type 1 (HIV-1) infection is characterized by a slow, progressive depletion of $CD4^+$ T cells and a lack of HIV-1 specific T-cell responses, resulting in progression to AIDS in the absence of antiretroviral combination therapy. HIV-1-specific $CD4^+$ T cell proliferation is undetectable soon after primary infection (Deeks et al., 2002; Quaranta et al., 2009). Host-mediated immunosuppressive activity in the face of persistent HIV replication has not been well delineated. Also the molecular basis for such impaired responses has not been fully elucidated.

Phenotypically, regulatory T cells (Tregs), a subset of $CD4^+$ lymphocytes, have been characterized as expressing CD25 and CTLA-4, in addition to the most specific Treg marker forkhead-winged-helix transcription factor 3 (FOXP3) (Mills, 2004; Roncarolo and Battagalia, 2007; Curotto de Lafaille et al., 2009). $CD4^+CD25^+FOXP3^+$ Treg cells have constitutive immunosuppressive activity (Sakaguchi et al., 1995). Tregs are recruited and expanded during host immune responses to modulate over reactive immunity, and have been implicated in a number of pathologic processes during infectious diseases, as well as autoimmune diseases (Sakaguchi et al., 2001; Shevach, 2009). Tregs have been implicated in the potential suppression of HIV-specific $CD4^+$ and $CD8^+$ T cell responses in HIV-infected individuals (Aandahl et al., 2004; Weiss et al., 2004; Estes et al., 2006). Both animal and human studies demonstrate that Treg numbers are elevated in the acute stage of virus infection and could dampen the virus-specific adaptive T-cell response, which may promote chronic viral infection (Tobias and Vito, 2007; Maureen et al., 2011). In addition, chronic HIV infection changes the tissue distribution of Tregs with a marked increase of these cells in peripheral lymph nodes (LNs) and mucosal lymphoid tissues, areas with high levels of HIV replication (Kinter et al., 2007; Ji and Cloyd, 2009). Taken together, these studies suggest that Tregs could play a major role in the induction/maintenance of an environment that would favor HIV replication and persistence by impairing protective immune responses (Cao et al., 2009; Suchard et al., 2010; Xing et al., 2010). On the other hand, Tregs may themselves be infected by HIV. Oswald-Richter et al. (2004) showed that both natural $CD4^+CD25^+$ Tregs and conventional $CD4^+$ T cells transduced with FOXP3 to generate functional Tregs were easily infected by HIV.

Conflict of interest: None.

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The HIV-1 accessory protein Nef (27 kDa), which is the regulatory protein expressed earliest and most abundantly in the viral infection cycle, is expressed in the cytoplasm and membrane of infected cells (Guatelli et al., 1990). Nef is involved in multiple processes that favor viral replication, immune evasion of infected cells, and alteration of immunological functions (Fackler and Baur, 2002; Greenway et al., 2003; Quaranta et al., 2006). However, whether Nef is associated with increased numbers of Tregs during HIV infection remains debatable. The aim of this study was to analyze the relationship between Nef and the expansion of Tregs in peripheral blood mononuclear cells (PBMCs), and to investigate potential mechanisms involved in the increase in Tregs induced by Nef.

MATERIALS AND METHODS

CELL CULTURE

The human monocytic cell line THP-1 transfected with pcDNA3.1(+) vector (THP-1-3.1), THP-1 cell lines selected for stable expression of HIV-1 Nef after transfection (THP-1-Nef), and PBMCs were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% streptomycin/penicillin (Invitrogen Life Technologies, Carlsbad, CA). Transfected cell strains were pulsed with 200 μg/ml G418 at monthly intervals for 1 week to prevent loss of stable-transfected Nef protein expression. All cells were incubated at 37°C and 5% CO_2.

CELL TRANSFECTION

Purified pcDNA3.1(+) and pcDNA3.1(+)-HIV-1 Nef plasmids were obtained from the Institute of Molecular Biology of Three Gorges University (Yichang, P.R. China). THP-1 cells were plated at a concentration of $0.5–2 \times 10^5$ cells/well in 24-well plates and cultured in conventional culture medium as described previously. Cells were transfected the following day with cationic lipid Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Briefly, complexes of diluted DNA and Lipofectamine™ 2000 (total volume = 100 μl) were prepared and added to each well containing THP-1 cells. The cells were incubated at 37°C in a CO_2 incubator for 4–6 h and then passaged 1:3 in fresh growth medium 24 h after transfection. Different concentrations of G418 (100–500 μg/ml) selective medium were added the following day. Clones were selected on the basis of their resistance to G418 and expression of Nef proteins assessed by reverse transcriptase-polymerase chain reaction (RT-PCR), western blot, and cell immunofluorescence as described below.

TABLE I. Primer Sequences Used for RT-PCR

Primer	Orientation	(Sequence 5′-3′)
β-actin	Forward	TGGCACCCAGCACAATGAA
	Reverse	GTCATAGTCCGCCTAGAAGCA
HIV-1 Nef	Forward	ATGGGTGGCAAGTGGTCA
	Reverse	TCAGCAGTTCTTGAAGTACTC
TLR2	Forward	CAGGAGCTCTTAGTGACCAAGTGAA
	Reverse	CACAAAGTATGTGGCATTGTCCAG

RNA ISOLATION AND REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

RNA from cell lines was extracted using Trizol reagent (Invitrogen) and cDNA was synthesized using SuperScript™ II Reverse Transcriptase according to the manufacturer's protocol. RNA/primer mixtures were incubated at 65°C for 5 min and then placed on ice for at least 1 min. Reaction mixtures were then incubated at 42°C for 50 min. We used β-actin as the normalization control. PCR amplification of HIV-1 Nef and TLR2 cDNAs was performed using specific oligonucleotide primers selected within the coding regions of the genes. The sequences of the primers were designed through DNAMAN 5.0 software and listed in table I. PCR reactions contained 25 ng cDNA template, 100 ng each of sense and antisense oligonucleotide primers, 2.5 μl of optimized *Taq* PCR buffer (Promega, Madison, WI), 0.4 mM dNTP mixture, and 1 U of *Taq* polymerase in a total reaction volume of 25 μl. Following an initial 3 min incubation at 94°C, PCRs were performed using a 45 s denaturation step at 94°C and a 45 s annealing step at 55°C followed by a 1 min elongation step at 72°C. A total number of 30 PCR cycles were conducted for amplification. PCR products were separated by electrophoresis at 100 V for 30 min through a 1% agarose gel and were detected by ethidium bromide staining. Expected sizes of specific PCR products were verified by reference to a 1 kb DNA ladder.

WESTERN BLOT ANALYSIS

Cell lysates were prepared from THP-1-3.1 and THP-1-Nef cells cultured for 16 h. The protein samples were subjected to SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween20 (PBST) and incubated with primary antibodies (Nef polyclonal antibody diluted 1:5000) and then with anti-rabbit horseradish peroxidase(HRP)-conjugated secondary antibody according to the manufacturer's instructions. Nef polyclonal antibody was prepared by Institute of Molecular Biology of Three Gorges University (Yichang, P. R. China). The immunoblots were visualized by a 3,3′-diaminobenzidine (DAB) kit obtained from Sigma.

CELL IMMUNOFLUORESCENCE

Cells were grown for 16 h on gelatin-coated, glutaraldehyde-cross-linked coverslips. For immunofluorescence analysis, the cells were fixed with ice cold 4% paraformaldehyde in PBS for 20 min at room temperature, and then incubated with 10% goat serum and 1% BSA blocking solution for 90 min at room temperature with gentle rocking. After an overnight incubation with primary antibody (Nef polyclonal antibody diluted 1:1000), the cells were washed three times with PBS, and incubated with secondary antibody (rhodamine-conjugated goat anti-rabbit antibody). Plates were protected from light and incubated 60 min at room temperature with gentle rocking. Cells were then washed five times for 5 min each with PBS. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (100 ng/ml). Photography was performed with the NIKON TE2000 microscope.

LUCIFERASE ASSAY

Cells were transiently transfected with the nuclear factor-kappa B (NF-κB)-firefly luciferase reporter gene obtained from the Institute of Molecular Biology of Three Gorges University (Yichang, P.R.China). After 48 h, the luciferase activity was measured with the Luciferase Assay System (E4030 Promega). Luminescence was read from a 96-well plate on an Infinite^{TM} 200 luminometer. Cell viability was determined and samples were normalized to equal numbers of viable cells prior to lysis.

siRNA KNOCKDOWN

Small interfering RNA (siRNA) duplex oligoribonucleotides for siRNA-targeted disruption of Homo sapiens TLR2 (GeneBank no.NM_003264.3) were designed using the ambion RNA interference (RNAi) designer program (http://www.ambion.com/techlib/misc/siRNA_finder.html). The sequence of target mRNAs used in this study was: TLR2, 5′-AAUCCUCCAAUCAGGCUUCUC-3′. The target sequence was submitted to a BLAST search to ensure that only the TLR2 gene was targeted. Scrambled siRNA, 5′-AACUAUCCAUUCUCGCCACGU-3′ did not target any genes. Sense and antisense hairpin oligonucleotides were separated by an eight-nucleotide spacer, and a restriction enzyme cutting site was composed to facilitate cloning into the pSilencer vector. Sense and antisense sequences were synthesized by Sangon Biotech Co. (Shanghai) and annealed in 1× annealing buffer, then ligated into pSilencer-U6-2.1-hygro (Ambion, Inc.). The pSilencer-U6-2.1-TLR2-siRNA was identified by sequencing.

THP-1-Nef cells were stably transfected with plasmid pSilencer-U6-2.1-TLR2-siRNA (named THP-1-Nef-T2si) or a scrambled siRNA plasmid (named THP-1-Nef-Ctlsi) by using cationic lipid Lipofectamine 2000 according to the manufacturer's instructions as described previously. Clones were selected on the basis of their resistance to G418 and hygromycin. Knockdown of expression of TLR2 was verified by flow cytometry (FACS)(EPICS XL-MCL; Beckman Coulter) and RT-PCR. The cells were then subjected to further experiments.

COCULTURE AND LYMPHOCYTE PROLIFERATION

PBMCs from healthy blood donors were isolated from buffy coats by density gradient centrifugation over Ficoll/Hypaque according to manufacturer's protocol. PBMCs resuspended at 1×10^6 ml in PBS with 0.1% FBS were stained with 5 μmol/L 5-(-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes, Inc., Eugene, OR) for 10 min at 37°C. The staining was quenched by a 5 min incubation in PBS containing 10% FBS. Excess CFSE was removed by the addition of PBS and subsequent centrifugation. This washing step was repeated twice. The CFSE-labeled PBMCs were cocultured with THP-1 cells transfected with different genes at a 1:100 ratio. After 3 days, the cells were harvested and subjected to FACS to determine the cell proliferation.

For Tregs analysis, the above cocultured PBMCs (without CFSE prestain) were stained with a phycoerythrin(PE)-conjugated monoclonal antibody (mAb) for cell surface CD25 (Pharmingen, BD Biosciences, San Diego, CA). After fixation and permeabilization, cells were stained with anti-FOXP3-fluorescein isothiocyanate (FITC) mAb (eBiosciences, San Diego, CA) according to manufacturer's protocol, and Tregs production was measured by FACS.

FLOW CYTOMETRY

For analysis of TLR2 expression, cells were stained with anti-TLR2–PE-conjugated mAb (eBiosciences) or isotype control antibody for 30 min at 4°C and then washed twice with FACS buffer (PBS containing 5% FBS). Cells were resuspended in 500 μl of the same buffer, then analyzed by FACS to determine the levels of surface expression of the receptor.

STATISTICAL ANALYSIS

Data were expressed as means ± SD. Differences between two groups were analyzed with the Student's *t*-test, while differences between three or more groups were analyzed with analysis of variance (ANOVA). Differences were considered to be statistically significant when $^{*}P < 0.05$, $^{**}P < 0.01$.

RESULT

EXPRESSION OF HIV-1 Nef ON THP-1 CELLS

To examine the effects of Nef expression on THP-1 cells, we stably transfected THP-1 cells with either an expression vector for Nef (pcDNA3.1(+)-Nef) or the control empty vector pcDNA3.1(+). Two cell clones (THP-1-Nef1 and THP-1-Nef2) were selected, which provided a range of Nef expression levels as confirmed by RT-PCR (Fig. 1A) when compared with a control cell clone not expressing Nef. We also confirmed these results by western blot (Fig. 1B) and cell immunofluorescence (Fig. 1C).

Many studies found that various strains of HIV-1 Nef could reduce CD4 expression on the cell surface of T cells. In order to investigate the biological activity of HIV-1 Nef expressed in THP-1 cells, cell surface CD4 expression was analyzed by flow cytometry. CD4 positive THP-1-Nef and THP-1-3.1 cells were 8.58 ± 0.8% and 55.9 ± 1.2%, respectively, which was a statistically significant difference in cell surface CD4 expression ($^{**}P < 0.01$). This result suggests that Nef, stably expressed in THP-1 cells, possesses biological activity, resulting in a significant loss of cell surface CD4 (Fig. 1D).

HIV-1 Nef INCREASES TREGS PRODUCTION

In order to investigate the immunoregulatory effects of Nef, CFSE-labeled PBMCs were cocultured with THP-1 or THP-1-Nef cells (THP-1-Nef1 and THP-1-Nef2). The results showed that during cocultivation, the proliferation of PBMCs was suppressed to a greater extent by THP-1-Nef cells (41.56 ± 5.19%) when compared with control THP-1-3.1 cells (65.60 ± 9.20%) (Fig. 2A,C). We further investigated Tregs production in the PBMC cocultures. The phenotypic characteristics of Tregs in our cocultures were consistent with the reported intrinsic Treg markers, including CD4, CD25, and the Treg-specific transcription factor FOXP3 (Mills, 2004; Roncarolo and Battagalia, 2007; Curotto de Lafaille et al., 2009). We found that coculturing PBMCs with THP-1-Nef cells increased the number of Tregs over twofold as compared to cocultures with THP-1 cells (Fig. 2 B,D). These data show that HIV-1 Nef could inhibit the

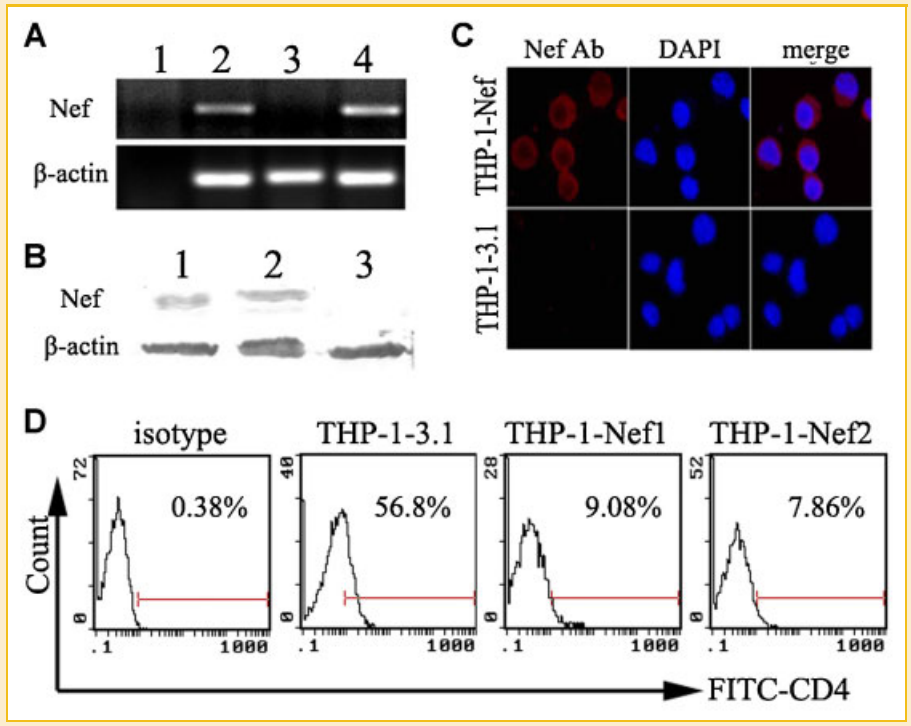

Fig. 1. HIV-1 Nef gene expression in THP-1 cells. Plasmids pcDNA3.1(+)-Nef and pcDNA3.1(+) were stably transfected into THP-1 cell respectively. A: Nef mRNA expression was determined by RT-PCR. Lane 1:negative control using RNA of THP-1-Nef cells. Lane2, 4:the cellular cDNA of two clones of THP-1-Nef cells(THP-1-Nef1 and THP-1-Nef2).Lane3: the cellular cDNA of THP-1-3.1 cells. B: Nef protein expression was determined by western blot. Cell extracts were prepared and analyzed for Nef protein expression. A 27 kD strap was displayed in the Nef positive cell lyses. C: The localization of Nef protein was visualized with a TE2000-S-type fluorescence microscope by indirect immunofluorescence using primary antibody for Nef and secondary antibody of rhodamine labeled anti-IgG. Immunofluorescence demonstrated that Nef protein expression was primarily located in the cytoplasm and partially in the membrane. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

proliferation of PBMCs and increase the number of Tregs in cultured PBMCs, which may be one potential mechanism by which Nef contributes to HIV-specific immunosuppression.

HIV-1 Nef MEDIATES TLR2 EXPRESSION IN THP-1 CELLS

Toll-like receptors (TLRs) play an important role in the induction and regulation of innate and adaptive immunity (Thibault et al., 2007; Shankar et al., 2011). Recent evidence supports the idea that TLR2 signaling regulates the adaptive immune response by acting directly on both effector and regulatory T cells and plays an important role in the expansion and function of Tregs (Liu et al., 2006; Sutmuller et al., 2006, 2007; Layland et al., 2007). In order to investigate the role of TLR2 in Treg expansion mediated by HIV-1 Nef, we analyzed TLR2 expression in THP-1-Nef cells by RT-PCR and FACS. The results showed that TLR2 mRNA (Fig. 3A,C) in THP-1-Nef cells was up-regulated when compared to control cells, and TLR2 protein expression, as analyzed by FACS, was also up-regulated in THP-1-Nef cells ($61.2 \pm 6.5\%$ or $76.8 \pm 8.1\%$) when compared to THP-1-3.1 cells ($10.5 \pm 1.425\%$) (Fig. 3B,D). Based on these data, we conclude that Nef is responsible for TLR2 up-regulation in THP-1 cells.

In several cell types of the innate immune system including macrophages and microglia, NF-κB is an important downstream effector of TLR2 activation. Over-expression of TLR2 has been observed to cause higher basal NF-κB activation and cytokine production (Medzhitov et al., 1997; Kurt-Jones et al., 2002; Equils et al., 2004; Syed et al., 2007). Here we investigated the activity of NF-κB in THP-1-Nef cells which we showed exhibited increased TLR2 expression when compared to THP-1-3.1 cells. Luciferase activity was analyzed in THP-1-Nef and control THP-1-3.1 cells 48 h after transient transfection with NF-κB-luc plasmid. Data showed that NF-κB activation was higher in THP-1-Nef cells when compared with THP-1-3.1 cells (over 30-fold increase). The changes in NF-κB activation were consistent with the expression of TLR2 in these different cell lines.

HIV-1 Nef EXPANSION OF TREGS VIA TLR2 IN THP-1 CELLS

We have demonstrated that coculturing PBMC with THP-1-Nef cells significantly increased the number of Tregs when compared to cocultures of PBMC and THP-1 cells. We also showed that Nef increased the expression of TLR2 in THP-1 cells and TLR2 signaling plays an important role in regulating the expansion and function of regulatory T cells (Liu et al., 2006; Sutmuller et al., 2006). Therefore, we investigated whether HIV-1 Nef contributed to the inhibition of PBMC proliferation and the expansion of Treg numbers via TLR2 signals. THP-1-Nef cells were stably transfected with scrambled control or TLR2-specific siRNA plasmids. We identified two TLR2-specific siRNA clones (THP-1-Nef -T2si1 and THP-1-Nef -T2si2) which exhibited suppressed TLR2 mRNA(Fig. 4A) and protein

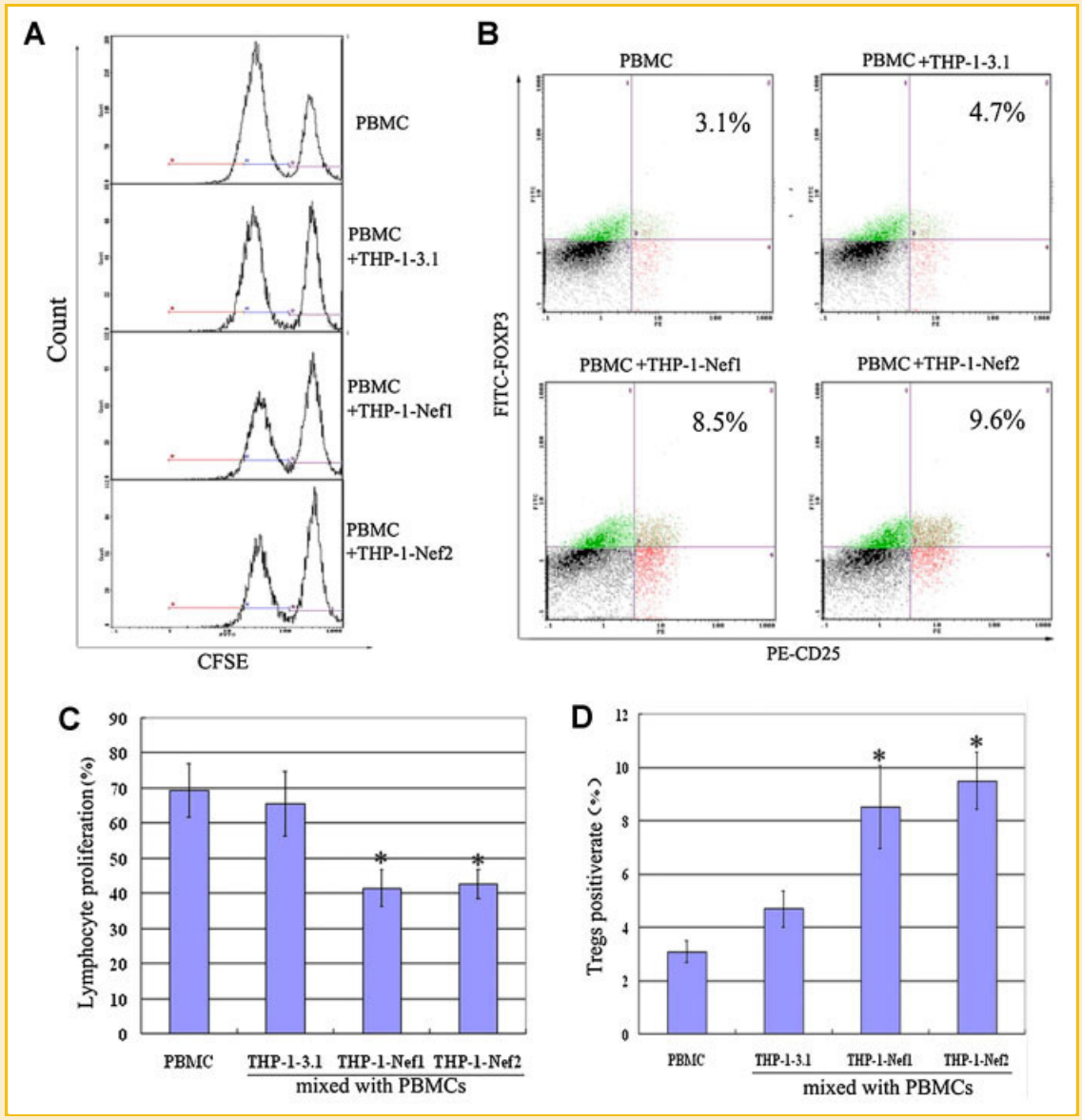

Fig. 2. The immunoregulatory effect of Nef in THP-1 cells. A, C: CFSE-labeled PBMCs were cocultured with two clones of THP-1-Nef and THP-1-3.1 cells for 72 h. Proliferation resulting in a decrease of fluorescent signal in the daughter cells was monitored by FACS. The result showed that two clones of THP-1-Nef cells can significantly inhibit the proliferation of PBMCs compared with THP-1-3.1 cells. B, D: HIV-1 Nef expanded Tregs in PBMCs which were cocultured with THP-1-3.1 or two clones of THP-1-Nef cells. CD25 and FOXP3 as specific markers of Tregs were analyzed by FACS and indicated that Nef can increase the numbers of Tregs compared to the control THP-1-3.1 cells. Representative results from three experiments are shown. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

(Fig. 4B,C). The knockdown of TLR2 in these two clones significantly reduced NF-κB over tenfold when compared to THP-1-Nef cells (Fig. 4D, $^{**}P < 0.01$).

PBMCs were cocultured with THP-1-3.1, THP-1-Nef, THP-1-Nef-T2si1, or THP-1-Nef-Ctlsi cells for 72 h, and then the cells were harvested and assayed for proliferation and number of Tregs. In this study we showed that the proliferation of PBMC can be significantly inhibited upon coculture with THP-1-Nef cells when compared to cocultures with THP-1 cells. Tregs expansion also occurred in PBMC when cocultured with THP-1-Nef cells. With the knock-down of TLR2 expression, the effects of Nef on the proliferation of PBMCs (Fig. 5A,C) and the numbers of Tregs (Fig. 5B,D) was inhibited. Thus, these data suggest that HIV-1 Nef may increase Tregs numbers and inhibit the proliferation of PBMCs via TLR2 signals.

DISCUSSION

HIV Nef is an accessory protein unique to HIVs and SIVs. In humans, several cases of individuals infected with Nef-defective HIV-1 have been reported, and many of these patients maintained low-to-undetectable levels of viremia with vigorous antiviral immunity and delayed disease progression (Jere et al., 2010). Experimental

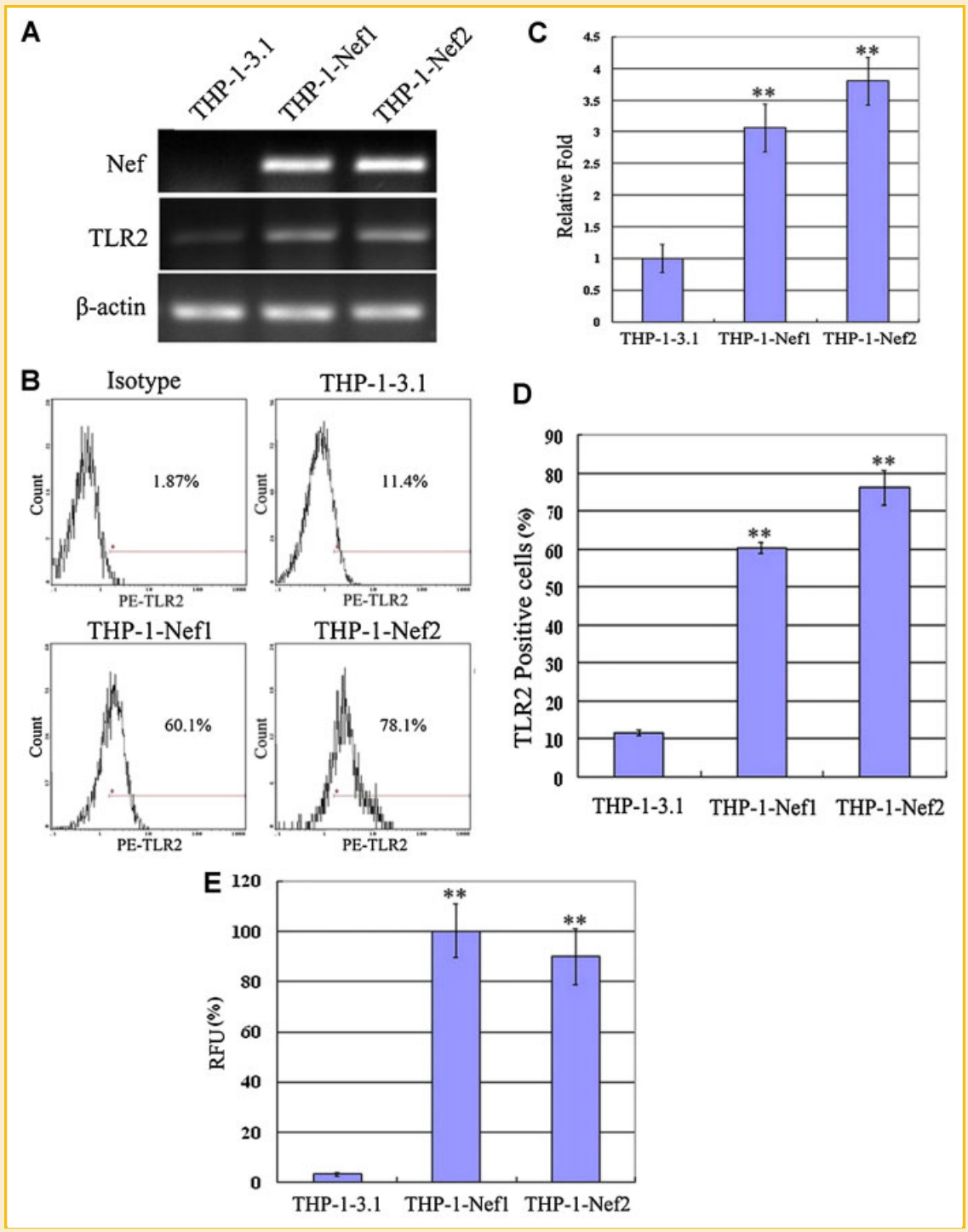

Fig. 3. TLR2 protein was up-regulated in THP-1-Nef and increased activity of NF-κB. A, C: mRNA expression of TLR2 was determined by RT-PCR. RT-PCR products were analyzed with 1% agarose gel electrophoresis. Values were normalized to the endogenous control β-actin within each sample. In each experiment, the results attained in control group were considered as a ratio of 1.0. Values were based on results obtained from multiple experiments. B, D: Protein expression of TLR2 was determined by FACS. Staining cell surface molecule was performed using mAb PE-TLR2, PE-conjugated mouse IgG1 as isotype control. Representative histograms of relative cell number versus PE fluorescence intensity for the TLR2-positive cells, the percentage of positive cells was indicated. Histograms show a single representative experiment. E: The up-regulation of TLR2 expression increased the activity of NF-κB approximately 31.41 ± 3.29 or 28.21 ± 3.53-fold based on the control cells. $^{**}P < 0.01$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

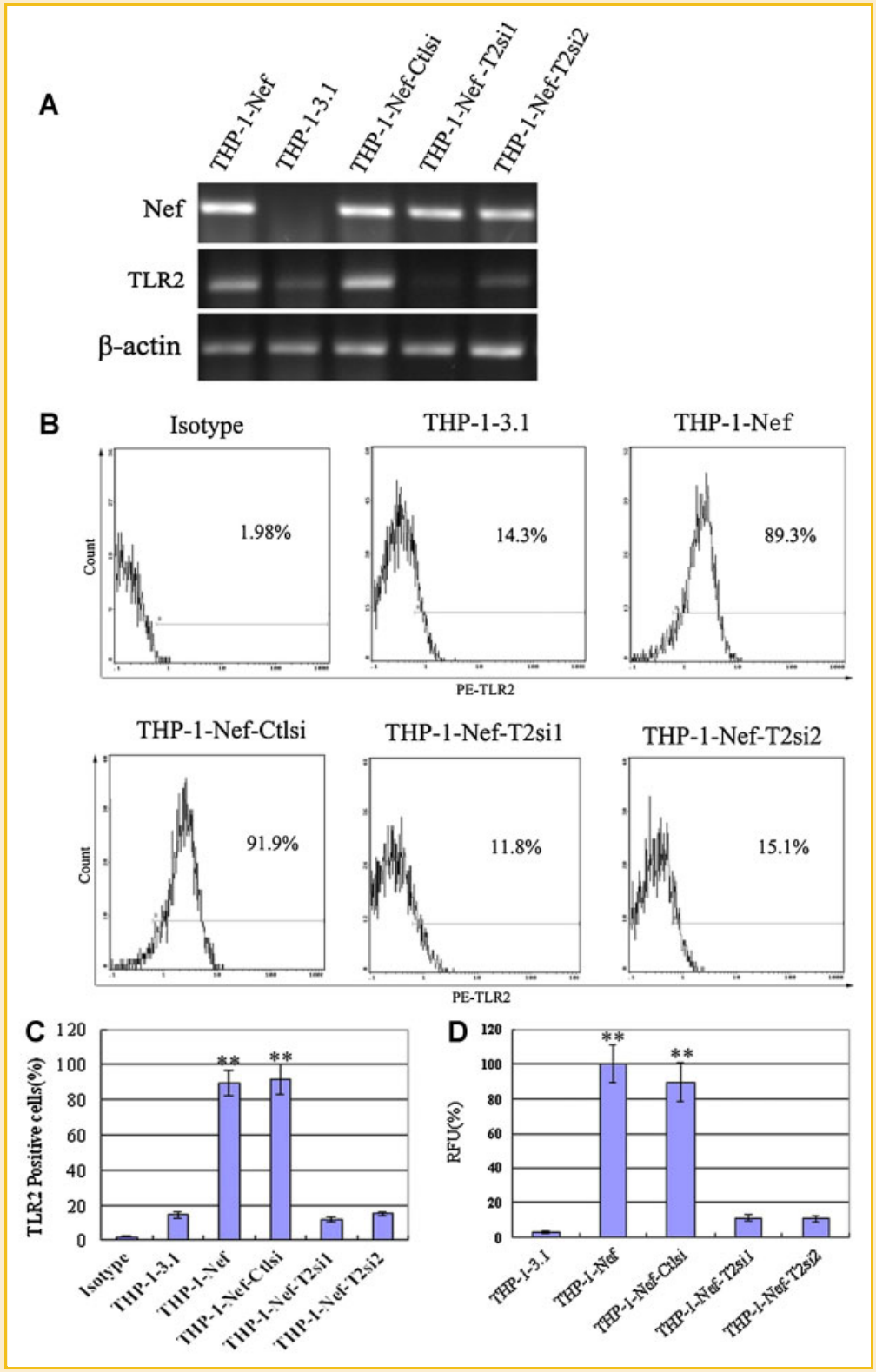

Fig. 4. Effects of TLR2-specific siRNA on the expression of TLR2 and the activity of NF-κB in THP-1 Nef cells. TLR2-silencing was assessed respectively by RT-PCR (A) and FACS (B, C) after stable transfection of TLR2-specific siRNA. THP-1-Nef cells were transfected with control siRNA had readily detectable TLR2, whereas the cells treated with TLR2-specific siRNA had markedly reduced the level of TLR2 transcripts and proteins. D: The activity of NF-κB was evaluated by luciferase assay. The loss of TLR2 expression reduced the activity of NF-κB. ANOVA and the Dunnett multiple comparison tests compared the untreated and control siRNA-treated THP-1-Nef cells with the TLR2 siRNA treated cells. $^{**}P < 0.01$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

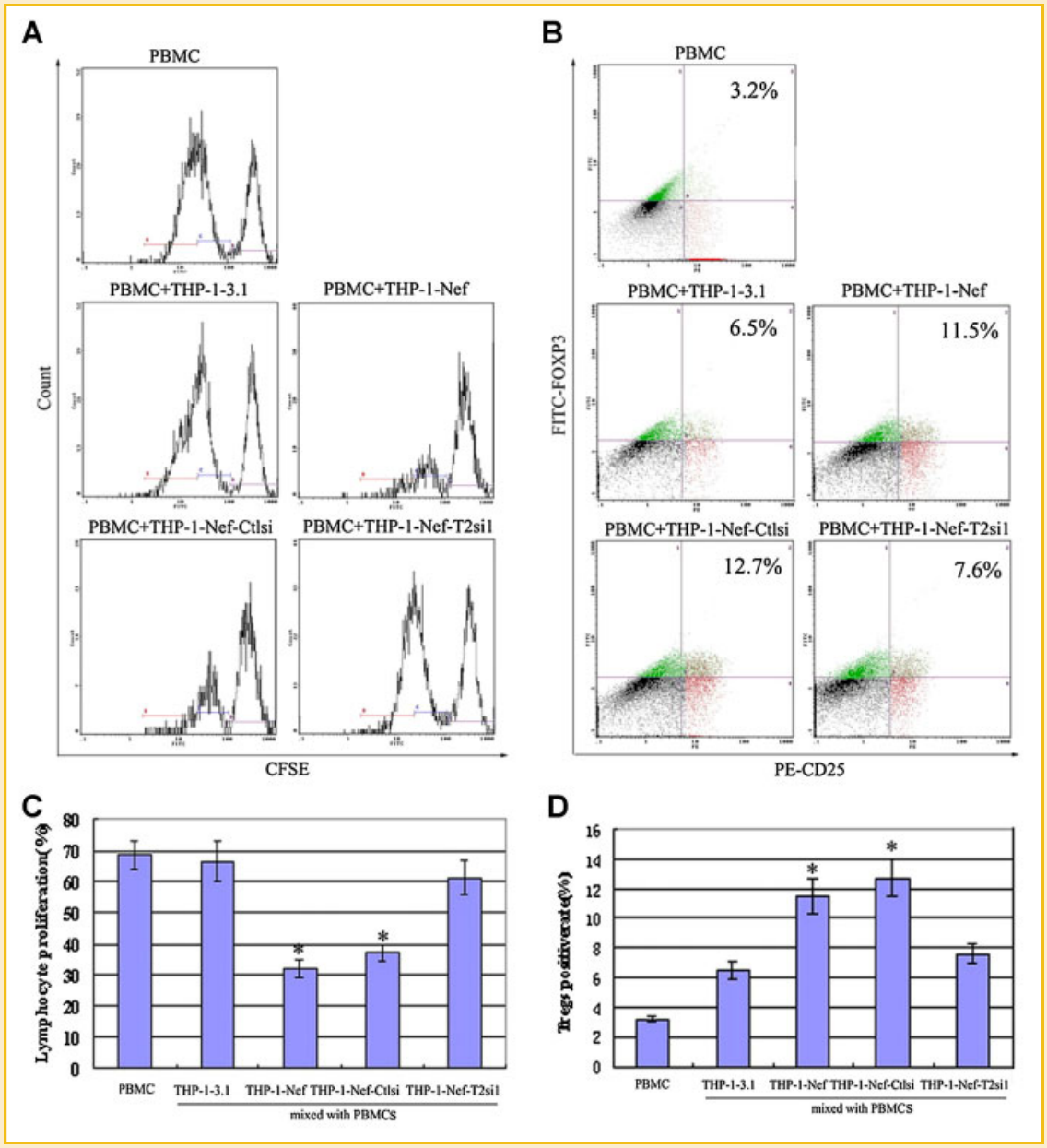

Fig. 5. TLR2 signals mediate PBMCs proliferation and increase the numbers of Tregs. A, C: Proliferation of CFSE-labeled PBMCs was assayed when stable cell lines transfected with different genes were cocultured with CFSE-staining PBMCs for 72 h. The result showed that THP-1-Nef-T2si cells certainly recovery the proliferation of PBMCs compared with THP-1-Nef cells. *P*-values indicate differences on the proliferation of PBMCs of coculture system between control cells and TLR2-specific RNAi cells. B, D: PBMCs were cocultured with the same cells above for 72 h and analyzed by FACS for Tregs number. Data indicated mean percentage of CD25 + FOXP3 +T cell (Tregs) numbers of PBMCs ± SD. Representative results of three experiments are shown. $^{*}P < 0.05$ with controls. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

infection of rhesus macaques with SIV in which Nef has been deleted (SIV239Δnef) results in low-to-undetectable levels of viremia, asymptomatic infection, and protection from subsequent challenge with wild type virus (Kestler et al., 1991). To evaluate the contributions of the viral Nef protein to HIV-associated immunoregulation, a stable Nef-expressed cell line THP-1-Nef was obtained. THP-1 cells were used to investigate the role of Nef in macrophages, which are one of the initial targets of HIV-1 infection and also the main mediator of innate immunity. Our results demonstrated for the first time that HIV-1 Nef could up-regulate expression of the immune molecule TLR2 in THP-1 cells (Fig. 3).

TLRs are one of the key receptor families forming an initial line of defense against invading microbes and viruses. They are widely expressed in the immune system and work cooperatively with associated proteins to generate diverse signaling responses as part of the innate immune response (Xagorari and Chlichlia, 2008; An

et al., 2010). Sutmuller et al. (2006) investigated whether TLR2,4,5,7, and 9 triggering can modulate the ability of IM-MDDCs to capture, internalize, replicate, and transfer HIV-1. They found that only TLR2-mediated augmentation in HIV-1 virus production was detected when using both a synthetic (i.e., Pam3Csk4) and a more natural TLR2 agonist (i.e., LTA). The TLR2-dependent up-regulatory effect on HIV-1 propagation was seen with both R5 and X4 virions (Sutmuller et al., 2006). These results suggested that the absence of Nef regulation of TLR2 expression could contribute to the low level viremia of Nef-defective HIV-1 compared with wild type HIV-1.

TLR2 signaling plays a crucial role in innate immune activation, but its role in T cell biology has long been unappreciated (Thibault et al., 2007). Using a coculture system we found that Nef can expand the numbers of Tregs and attenuate the proliferation of PBMCs when THP-1-Nef cells were cocultured with PBMCs (Fig. 2). Using a coculture system, Shankar et al. (2010) found that the priming of T-cells by DCs pulsed with HIV caused an upregulation of an array of negative costimulatory molecules on T-cells and increased expression of CTLA-4 and FOXP3 when compared to priming with mock-pulsed DCs. Wang et al. (2010) studied 31 chronic hepatitis C patients and 20 healthy controls, and found significant correlations between TLR2 and Treg numbers. These data suggested that Nef induced TLR2 up-regulation could mediate Tregs expansion.

$FOXP3^+$ Tregs are important target cells for HIV infection and replication, particularly in lymphoid tissues during acute infection when most human $CD4^+$T cells are quiescent. As a consequence, accumulation of $CD4^+FOXP3^+$ Treg cells in lymphoid organs during HIV-1 acute infection may contribute to increased amounts of HIV target cells, as well as to suppressed anti-HIV immune responses in lymphoid tissues (Montes et al., 2006; Thorborn et al., 2010). The Tregs support high levels of HIV-1 infection and are preferentially depleted by pathogenic HIV-1 isolates in HIV-infected DKO-hu HSC mice. And depletion of Treg cells in DKO-hu mice leads to reduced HIV infection and replication, which underscores the importance of Tregs in HIV-1 infection (Jiang et al., 2008). These data suggest that Treg expansion by HIV-1 Nef is an important factor in HIV-1 infection and replication.

In order to investigate the relationship between Nef, TLR2, and Tregs, we used siRNA to knock-down the expression of TLR2 in THP-1 Nef cells (Fig. 4). The results showed that along with knockdown of TLR2, the effects of Nef on Tregs expansion and PBMC proliferation were reversed in a coculture assay (Fig. 5). The Sutmuller group (Sutmuller et al., 2006) reported that $TLR2^{-/-}$ mice infected with *Schistosoma mansoni* exhibited associated elevated immunopathology, including the failure of the Treg population to expand. They also functionally inactivated $CD4^+CD25^+$ T cells in wildtype mice and observed that the resulting immunopathology after infection mirrored that found in $TLR2^{-/-}$ mice. These results support our data that Tregs are expanded through TLR2 upregulation which is induced by Nef in THP-1 cells.

In our study, this is the first time that HIV-1 Nef has been shown to induce Treg expansion and inhibit PBMC proliferation via TLR2 signals. Together these results underscore the importance of Nef in immunosuppression and HIV replication. These observations may be important in understanding the role of HIV-1 Nef in increased HIV replication and subsequent immunosuppression and HIV disease progression.

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