Human T-Cell Leukemia/Lymphoma Virus Type 1 p30, but Not p12/p8, Counteracts Toll-Like Receptor 3 (TLR3) and TLR4 Signaling in Human Monocytes and Dendritic Cells

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The human T-cell leukemia/lymphoma virus type 1 (HTLV-1) p30 protein, essential for virus infectivity *in vivo*, is required for efficient infection of human dendritic cells (DCs) but not B and T cells *in vitro*. We used a human monocytic cell line, THP-1, and dendritic cells to study the mechanism of p30 and p12/p8 requirements in these cell types. p30 inhibited the expression of interferon (IFN)-responsive genes (ISG) following stimulation by lipopolysaccharide (LPS) of Toll-like receptor 4 (TLR4) and by poly(I-C) of TLR3 but not of TLR7/8 with imiquimod. Results with THP-1 mirrored those for *ex vivo* human primary monocytes and monocyte-derived dendritic cells (Mo-mDC). The effect of p30 on TLR signaling was also demonstrated by ablating its expression within a molecular clone of HTLV-1. HTLV-1 infection of monocytes inhibited TLR3- and TLR4-induced ISG expression by 50 to 90% depending on the genes, whereas the isogenic clone p30 knockout virus was less effective at inhibiting TLR3 and TLR4 signaling and displayed lower infectivity. Viral expression and inhibition of ISG transcription was, however, rescued by restoration of p30 expression. A chromatin immunoprecipitation assay demonstrated that p30 inhibits initiation and elongation of PU.1-dependent transcription of IFN- α 1, IFN- β , and TLR4 genes upon TLR stimulation. In contrast, experiments conducted with p12/p8 did not demonstrate an effect on ISG expression. These results provide a mechanistic explanation of the requirement of p30 for HTLV-1 infectivity *in vivo*, suggest that dampening interferon responses in monocytes and DCs is specific for p30, and represent an essential early step for permissive HTLV-1 infection and persistence.

Human T-cell leukemia/lymphoma virus type-1 (HTLV-1) is the etiologic agent of adult T-cell leukemia/lymphoma (ATL), a malignant proliferation of CD4⁺ T lymphocytes, and of a neurodegenerative disease (1, 2) designated tropic spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM). It is currently estimated that 20 million people worldwide are HTLV-1 infected, but only a small percentage of them progress to disease, usually after long clinical latency. In fact, 3 to 5% of HTLV-1-infected individuals develop ATL and 0.3 to 2% develop TSP/HAM, whereas the majority remain asymptomatic (3–10). T cells have been considered the key target cells for HTLV-1 infection, as the virus is almost always present in the ATL cells and is found in the T cells of TSP/HAM patients. However, whether cells other than T cells also contribute to the pathogenesis of HTLV-1 infection remains unclear. HTLV-1 also infects monocytes/macrophages and dendritic cells (DCs) (11–15) that are professional antigen-presenting cells and shape the host response to viruses, as well as to other pathogens. DCs isolated from HTLV-1-positive patients are defective in alpha interferon (IFN- α) response upon *in vitro* stimulation, and their ability to activate T cells is impaired (16). In addition, in HTLV-1 infection, alteration in monocyte differentiation and activation has been reported (17, 18). Although the majority of HTLV-1 DNA is found in both CD4⁺ and CD8⁺ T cells, up to 20% of the total virus burden is found in monocytes (our unpublished data). In the macaque model, but not in the rabbit model, the ablation of p30 expression (p30 knockout [p30-KO]) or of p12/p8 (p12-KO) within a biologically active HTLV-1 molecular clone severely affects its infectivity.

When infection occurs in the case of p30-KO, it is associated with early reversion of the virus to the wild-type genotype, and in the case of p12-KO, neither infection nor nongenetic reversion is observed. These data underscore the importance of these viral genes (19). Furthermore, the infectivity of p30-KO and p12-KO in human primary monocyte-derived dendritic cells (Mo-mDCs) is also severely impaired. In contrast, the lack of expression of p30 or p12/p8 in human B cells (19) or primary human CD4⁺ T cells (our unpublished data) does not affect viral replication *in vitro*. Prior data demonstrated that p30, by interacting with the cellular transcription factor PU.1 in the monocytic cell line THP-1 (20), affects Toll-like receptor 4 (TLR4) signaling and, by modulating the CREB-responsive promoters, affects the expression of several genes involved in apoptosis, cell cycle, and transcription, pointing to p30 as a regulator of innate response to HTLV-1 (21).

PU.1, also known as Spi-1, is a transcriptional factor in the ETS family. Its expression is cell specific, and it has been found in macrophages, dendritic cells, B cells, and neutrophils (22, 23). PU.1 plays a critical role in cell activation and differentiation (24). Interestingly, PU.1 can interact with a variety of factors, including

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IRF4, ICSBP/IRF8, JUN, and NF- κ B, and promotes transcription of type 1 interferons (7, 14, 15, 17, 23–26). Therefore, PU.1 is involved in the signal transduction upon activation of different TLRs. In fact, TLR3 and TLR4 can trigger NF- κ B, TLR4 activates IRF4, and TLR7, TLR8, and TLR9 activate JUN (22, 22–24, 27, 28). Type 1 IFNs then are able to stimulate the production of a wide variety of antiviral interferon-stimulated genes (ISGs), such as those producing myxovirus resistance GTPase protein A (MxA); apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC-3G or A3G); and 2',5'-oligoadenylate synthetase (OAS) (29–31).

The interaction of p30 and PU.1 has been demonstrated to inhibit the transcriptional activity of PU.1, resulting in a decreased expression of TLR4 at the cell surface and decreased secretion of proinflammatory cytokines, such as MCP-1, tumor necrosis factor alpha (TNF- α), and interleukin-8 (IL-8), and increased production of the anti-inflammatory cytokine IL-10 (20). Type 1 IFNs are the most potent innate antiviral factors, and both DNA and RNA viruses, as well as other pathogens, have evolved a sophisticated and varied strategy to counteract these innate protective responses (31).

While TLR3 and TLR4 receptors are expressed on the cell surface and the TLR7/8 receptors are expressed in the endosomes (32), they share common downstream pathways for the induction of expression of type 1 IFN-responsive genes. TLR3 and TLR7/8 are involved in detection of viruses, since they recognize single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) structures that can be present during the replication of retroviruses, such as HTLV-1. We hypothesized that p30 affects not only TLR4 but also TLR3 and TLR7/8 signaling in virus-infected monocytes and in dendritic cells, and that by affecting the overall type 1 IFN response, p30 affects the activation and differentiation of these cells and ultimately the host response to the virus. We also hypothesized that p12/p8 also affects TLR7/8 significantly, since these proteins are localized in the endoplasmic reticulum (ER) and Golgi complex (25, 33). Indeed, the other known human-pathogenic retrovirus, HIV, which replicates at a high level in the host and causes, if untreated, AIDS in nearly all infected individuals (34–37), has evolved several genes that are able to counteract restriction factors. An example is the *vif* gene that counteracts the ability of APOBEC-3G, an interferon-inducible gene to deaminate the base composition of the viral RNA genome, rendering it non-infectious (38). Thus, understanding how HTLV-1 evades the innate host response and affects immune activation/inflammation is of importance to gain more understanding of its ability to persist and to induce autoimmune manifestations.

MATERIALS AND METHODS

Cell lines and primary human cells. The 729-6 B-cell lines infected with the pACH wild-type (WT) virus and the p30-KO and p12-KO viral mutants were maintained in RPMI 1640, 10% fetal bovine serum (FBS). In the case of the leukemic monocyte-like THP-1 human cell line, the same medium was supplemented with 50 μ M β -mercaptoethanol. Primary monocyte-derived dendritic cells (Mo-mDC) were obtained from heparinized human peripheral blood from healthy donors and were treated with Ficoll-Paque plus (GE Healthcare, Chalfont St. Giles, United Kingdom) according to the manufacturer's instructions, and monocytes then were separated by elutriation, checked for purity (>98% CD14 positive), and differentiated after approximately 7 days of culture in RPMI 1640, 20% BIT (Stem Cell Technologies, Vancouver, Canada) with 50 ng/ml interleukin-4 (IL-4; Peprotech, Rock Hill, NJ), 50 ng/ml granulocyte-

macrophage colony-stimulating fibroblast (GM-CSF) (Peprotech, Rock Hill, NJ), and 10 ng/ml transforming growth factor beta (TGF- β) (R&D Systems, Minneapolis, MN). Mo-mDC purity was checked by phenotype as illustrated by CD14⁺, CD3[−] CD19[−], CD1A⁺, and CD11C⁺ cells. CD4⁺ or CD8⁺ T lymphocytes were separated from peripheral blood mononuclear cells (PBMCs) by positive-selection magnetic beads (Invitrogen, Carlsbad, CA) and cultured in RPMI 1640, 10% FBS, 250 U/ml IL-2. Fifty ng/ml phorbol myristyl acetate (PMA) (Sigma, St. Louis, MO), 20 ng/ml lipopolysaccharide (LPS) (List Biological Laboratories Inc., Campbell, CA), 10 μ g/ml poly(I:C) HMW (InvivoGen, San Diego, CA), and imiquimod (InvivoGen, San Diego, CA) were utilized for stimulation of TLRs.

Virus infection, transfection, detection of virus productions, and protein expression. The HTLV-1-WT or the HTLV-1-p30-KO or HTLV-1-p12-KO producer 729-6 B-cell line was used to harvest HTLV-1 virions (19). The supernatants of such cell lines were collected and ultracentrifuged at 23,000 rpm for 150 min to isolate the virions, which then were resuspended in phosphate-buffered saline (PBS). In order to enhance the infectivity, THP-1 cells were spin infected at 3,000 rpm for 1 h in the presence of 8 μ g/ml Polybrene (Sigma, St. Louis, MO). The production of HTLV-1 in the supernatant of the infected cell cultures was assessed by measuring the amount of MA (p19 Gag) protein by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Zeptometrix, Buffalo, NY). HTLV-1 proviral load was measured in the various cell lines as previously described (19). Briefly, real-time PCR was performed on genomic DNA extracted with the DNeasy tissue kit according to the manufacturer's protocol. The TaqMan probe and PCR primers for the real-time PCR were designed within the integrase gene of HTLV-1. The sequence of the TaqMan probe was 5'-TGTCACCTGCCATTAAGCCCGA-3', and the DNA primers sequences were the following: forward, 5'-GCAGAGGAGGAAATTACCCAGTAC-3'; reverse, 5'-CAATTTTACCCAGGCATTTAATGT-3'. The normalized value of the HTLV-1 proviral DNA load was calculated as HTLV-1 DNA copy number/albumin gene copy number and expressed as the number of HTLV-1 proviral DNA copies per 10⁶ cells. In order to determine the surface expansion of specific markers on the THP-1 cell lines, phenotype was assessed by flow cytometry. Cells were incubated with the proper antibodies for 15 min at room temperature and then fixed in 1% paraformaldehyde, run on an LSR II (BD Bioscience, San Jose, CA), and analyzed with FlowJo (Tree Star Inc., Ashland, OR). In the case of intracellular cytokine staining, 1 μ g/ml of the protein transport inhibitor brefeldin A (BD, San Jose, CA) was added to the culture 6 h before the analysis. The antibodies used were CD14 (BioLegend, San Diego, CA); CD80, CD83, CD86, TNF- α , and CCR7 (BD Bioscience, San Jose, CA); IL-12 (eBioscience, San Diego, CA); and TLR4. In order to efficiently express the p30 protein, THP-1 cells were transduced with lentiviral vectors based on Naldini's system. In order to produce lentiviruses, pSDM101 or pSDM101-p30, pDM2.G, and pSAX were transduced into 293T cells with LipoD293 (Signagen, Rockville, MD). The supernatant was harvested, filtered (pore size, 0.22 μ m), and used during spin infection as described above. The efficiency of transduction was checked by flow cytometry and ranged between 80 and 95%.

Quantification by real-time PCR of ISGs. For real-time PCR, total RNA was extracted from cells and retrotranscribed (Qiagen, Valencia, CA). Reactions were performed using a SYBR fast quantitative PCR (qPCR) mix (KapaBiosystems, Woburn, MA). The primer sequences were the following (5' to 3'): 18s forward, GCCGAAGCGTTTACTT TGA; reverse, TCCATTATTCCTAGCTGCGGTATC; MxA forward, AG GAGTTGCCCTTCCAGA; reverse, TCGTTCACAAGTTTCTTCAGTT TCA; APOBEC3G forward, CCGTCTGGGTGTGCTACGAA; reverse, GCTTCCTCCACTTGCTGAACCA; OAS forward, CAGTCCTGGTGAG TTTGCAGT; reverse, GCCAGTGCTTTATCAAGAGGAT. Results are expressed as delta delta threshold cycles ($\Delta\Delta C_T$) and are presented as ratios between the target gene and the 18S housekeeping mRNA.

Immunoblot analysis. Protein samples (20 μ g each) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4 to 12%)

FIG 1 p30 affects TLR4 signaling in THP-1 cells. (A) Flow-cytometric analysis of THP-1 cells transduced with p30 (black line) or control (dotted line) lentivirus. TLR4 expression on the cell surface was assessed following 3 h of stimulation with PMA (+PMA) or after no stimulation (–PMA). The CD14 surface marker was used as a control (right). (B) Real-time PCR was performed on the TLR4 mRNA in THP-1 cells transduced with p30 or the control with or without PMA stimulation. The results are shown as a percentage of relative expression of the PMA-stimulated mock-transduced cell. The statistically significant differences are marked with an asterisk, which indicates a *P* value of less than 0.0001. Statistics were determined from three independent experiments (*n* = 3). (C) Flow-cytometric analysis of THP-1 cells transduced with p30 (black line) or mock transduced (dotted line). Twelve hours following LPS stimulation, cells were collected and analyzed for intracellular production of IL-12 and TNF- α .

in morpholineethanesulfonic acid (MES) SDS running buffer (Invitrogen, Carlsbad, CA) and transferred to Immobilon P membranes (Millipore, Billerica, MA). The membranes were blocked with PBS, 0.1% Tween, and 4% nonfat dried milk and then probed with primary antibody directed to the hemagglutinin (HA) tag or tubulin, and the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody was used for detection. SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) was used as the HRP substrate.

ChIP assay. Chromatin immunoprecipitations (ChIPs) were performed as follows. Briefly, for each chromatin preparation, 5×10^7 cells were resuspended in 50 ml of medium without FBS and cross-linked in 1% formaldehyde for 10 min at room temperature. Cross-linking was quenched by 125 mM glycine for 5 min at room temperature. Cross-linked cells were then washed with ice-cold PBS and resuspended in ice-cold radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl pH 8, 140 mM NaCl, 1 mM EDTA pH 8, 0.5 mM EGTA, 1% Triton X-100, 0.3% SDS, and 0.1% sodium deoxycholate) to a final concentration of 5×10^6 cell/ml. DNA was sheared with a Misonix XL sonicator by performing 12 30-s sonication cycles at power setting 5. For each single immunoprecipitation, 5×10^6 chromatin cell equivalents in the 200- to 500-bp range were immunoprecipitated with 5 μ g of the following antibodies: anti-HA, anti-RNA-polymerase II (Pol II), anti-RNA-polymerase II phosphoserine5 (Pol II-S5) (Abcam), anti-RNA-polymerase II phosphoserine2 (Pol

II-S2) (Covance), and anti-PU.1 (Cell Signaling). Parallel ChIPs were run with a Flag M2 antibody as the negative control (Sigma). Immunoprecipitation-enriched DNAs were used to perform real-time PCR. Oligonucleotides were designed to specifically target and quantify the promoter region of IFN- α 1 (forward, 5'-GGAACAAGATGGGGAAGACA-3'; reverse, 5'-GCAGATACTTCTGGGCTTGC-3'), IFN- α 2 (forward, 5'-AAG GCTCTGGGGTAAAAGA-3'; reverse, 5'-GACCTTGCTTTGTGCCCTAG C-3'), IFN- β (forward, 5'-AGGACCATCTCATATAAATAGGCCATAC CC-3'; reverse, 5'-ACTGAAAATTGCTGCTTCTTTGTAGGAATC-3'), TLR4 (forward, 5'-GCCAACTAGCTTCTCTTGTCTG-3'; reverse, 5'-C ACCGTCTGACCGAGCAGTT-3'), and UbiquitinB (forward, 5'-GAAG GAAGAGAAGCGCATAGAGGAGAA-3'; reverse, 5'-CTCATAGCCGT AAGAAAGGCTCCTAAA-3'). The $\Delta\Delta C_T$ method was used to calculate ChIP qPCR enrichments.

Statistical analysis. Analysis of variance (ANOVA) tests with Bonferroni posttests were performed with the informatics support of Graphpad Prism 5.

RESULTS

HTLV-1 p30 decreases TLR4 expression. HTLV-1-p30 protein interacts in the nucleus with PU.1 and is able to repress TLR4 expression on the cell surface (20). Thus, at first we verified that in

FIG 2 p30 decreases TLR3 and TLR4 but not TLR7/8 signaling. (A to C) Real-time quantitative PCR analysis of type 1 ISGs. THP-1 cells were transduced with p30 or control lentivirus, kept in culture for 72 h, and stimulated for 6 h with poly(I-C) (A), LPS (B), or imiquimod (C). The results are presented as the percentage of inhibition of the expression of the MxA, A3G, and OAS mRNAs versus the mock-transfected control. The statistically significant differences are marked with one or two asterisks, which indicate a *P* value of less than 0.01 or 0.0001, respectively. Statistics were determined from four independent experiments. (D) Immunoblot analysis of tubulin and p30 expression of the experiments presented in panels A to C.

our experimental conditions, p30 affected TLR4 expression. We transduced THP-1 cells with control lentivirus or lentivirus expressing the p30 cDNA (Fig. 1). We confirmed the appropriate nuclear localization of p30 in this cell type (21, 39, 40, and data not shown). TLR4 expression on the cell surface of transduced cells was analyzed by flow cytometry with or without stimulation with phorbol myristyl acetate (PMA). While untreated, p30- or control-transduced THP-1 cells showed similar levels of TLR4 expression on the cell surface; when treated with PMA, an upregulation of TLR4 expression was observed in the control but not in the p30-transduced THP-1 cells (Fig. 1A, left and middle). As previously published (20), the CD14 surface marker expression, used as a control, was not affected by p30 (Fig. 1A, right). The mechanism underlying the effect of p30 was transcriptional, as confirmed by quantitative real-time PCR performed on the TLR4 mRNA (Fig. 1B).

These data are consistent with the findings of Datta and colleagues (20), which demonstrated that p30 decreases the release of proinflammatory cytokines, such as TNF- α , IL-8, and MCP1, upon stimulation with the TLR4-specific agonist lipopolysaccharide (LPS) by decreasing the expression of TLR4 itself. Similarly, we transduced THP-1 cells with p30 lentivirus or with the control lentivirus, and after 12 h of LPS stimulation, cells were collected and analyzed by flow cytometry for intracellular production of IL-12 and TNF- α (Fig. 1C). In both cases, the presence of p30 decreased the expression of such cytokines upon LPS stimulation compared to that with the control.

p30 decreases TLR3 induction of ISG expression in THP-1 cells but not that of TLR7/8. We next studied the effect of p30 on

TLR3 and TLR7/8 because of their relevance in virus immune surveillance, since they recognize the dsRNA or ssRNA, respectively, which are intermediates of HTLV-1 infection. THP-1 cells were p30 or mock transduced, and then they were stimulated with a specific agonist of TLR3, such as poly(I-C), or a specific agonist of TLR7/8, such as imiquimod (Fig. 2). LPS stimulation on TLR4 was used as a control. The magnitude of the stimulation was approximately 4 times in the case of poly(I-C), between 10 and 15 times in the case of LPS, and approximately 3 times for imiquimod. We analyzed by real-time PCR the level of mRNA expression of ISGs and selected the most common ISGs to measure the overall type I interferon response, such as MxA, APOBEC-3G (also called A3G), and OAS. The results are shown in terms of percentage of relative expression of these ISGs in the presence of p30 expression versus the mock-transduced control following 6 h of TLR3 and TLR4 stimulation. Following the poly(I-C) stimulation, MxA and OAS expression was significantly inhibited by p30, and this difference also was significant when the expression of all genes together was analyzed ($P < 0.01$) (Fig. 2A). A similar effect was observed following LPS stimulation (Fig. 2B), as expected. Surprisingly, no difference was found following stimulation with imiquimod (Fig. 2C). The lack of p30-mediated inhibition of imiquimod was not due to differences in the expression of p30, as demonstrated in Fig. 2D. Similar experiments were performed using cDNA encoding p12/p8, since the presence of these proteins is essential for infectivity of monocytes as well (19). In contrast to p30, p12/p8 did not have a significant effect on the

FIG 3 p30 decreases TLR3 and TLR4 signaling in primary human monocytes and primary human monocyte-derived dendritic cells. Real-time PCR quantitative analysis of the expression of ISGs on primary monocytes and Mo-mDC was performed. Primary monocytes were transduced with p30 or control lentivirus. Seventy-two h later, the cells were stimulated with poly(I-C) (A), LPS (B), or imiquimod (C). The results are shown as the percentage of relative expression of ISG mRNAs, such as MxA, A3G, and OAS, following 6 h of TLR stimulation. Primary elutriated monocytes were differentiated into Mo-mDC and transduced with p30 or control lentivirus. Seventy-two h later, the cells were stimulated with poly(I-C) (D) or LPS (E). Immunoblot analysis of tubulin and p30 was performed, and no differences were found in p30 expression (data not shown). The statistically significant differences are marked with one or two asterisks, which indicate a P value of less than 0.01 or 0.0001, respectively. Statistics were determined from two and four independent donors for primary monocytes and Mo-mDC, respectively.

ISGs upon specific TLR3, TLR4, and TLR7/8 stimulation (data not shown).

p30 decreases TLR3 and TLR4 induction of interferon-stimulated gene expression in Mo-mDCs. To validate the results obtained in the human tumor THP-1 cell line with p30, we performed the same experiments on *ex vivo* primary human monocytes and *in vitro* primary human Mo-mDCs. PBMCs were isolated by Ficoll gradient from a LeukoPak, and primary monocytes were separated by adherence for 6 h. Cells were transduced with p30 or were mock transduced, and then they were stimulated with poly(I-C), LPS, or imiquimod. The expression of the indicated ISGs was significantly inhibited by the presence of p30 upon stimulation with poly(I-C) (Fig. 3A) or LPS (Fig. 3B) but not with imiquimod (Fig. 3C), consistent with our observations in THP-1 cells. In the case of Mo-mDCs, after isolation of PBMCs by Ficoll gradient, primary monocytes were separated by elutriation to keep them inactivated and left undisturbed. After 7 days of differentiation with IL-4, GM-CSF, and TGF- β (12, 19), the cell phenotype was verified and cells were transduced with p30 or were mock transduced, and then they were stimulated with poly(I-C) or LPS. Expression of p30 inhibited significantly the mRNA expression of all ISGs studied following stimulation with poly(I-C) and LPS, as demonstrated in Fig. 3D and E, respectively. The level of expression of p30 protein was comparable in all of the conditions tested (data not shown). These data demonstrate that in *ex vivo* primary monocytes and *in vitro* primary human dendritic cells, p30 expression inhibits TLR3 and TLR4 signaling and corroborates the THP-1 cell model.

Ablation of p30 from an HTLV-1 molecular clone results in decreased inhibition of TLR3- and TLR4-induced ISG expression in THP-1 cells that is partly restored by p30 overexpression.

To test the contribution of p30 without using overexpression systems, we infected THP-1 cells with the WT clone or the p30-KO HTLV-1 molecular clones (19) and tested the response of the cells to TLR3 and TLR4 stimulation. p30-KO HTLV-1, in which the expression of p30 was ablated, produced a lower level of virus, as measured by the p19 Gag levels released at the peak into the supernatant compared to that of the WT (Fig. 4A) (19). Accordingly, a lower level of viral DNA was found by quantitative real-time PCR in the cells exposed to p30-KO than to the WT, with approximately 1 copy per cell versus 4 copies per cell, respectively (data not shown). However, even under conditions of low levels of virus production, the infected cells continued to express high levels of the CD80/83 and CD86 immune activation markers and the CCR7 homing marker (Fig. 4B). In addition, the replication rate of infected THP-1 cells was approximately doubled compared to that of uninfected cells (15 versus 30 h). At week 19, when viral expression was equivalently low in both cell cultures (Fig. 4A), we stimulated the cells for 6 h with poly(I-C) and LPS for TLR3 and TLR4, respectively. After stimulation, RNA was extracted and tested for ISG expression. The results are shown as a percentage of relative expression of the ISGs in uninfected THP-1 cells versus the WT- or p30-KO-infected THP-1 cells. The response to TLR stimulation was significantly inhibited by infection with the WT virus but much less so in cells infected with the p30-KO virus (Fig. 4C and D).

FIG 4 Chronically infected monocyte responses to TLR signaling. (A) THP-1 cells were infected with WT (dashed line) or p30-KO HTLV-1 (solid line) virus. Productive infection was monitored by p19 Gag ELISA in the cell supernatant. Uninfected controls were negative for p19 Gag and are not represented in the graph. (B) At week 18 postinfection, the phenotypes of the THP-1 cells infected with WT or p30-KO HTLV-1 or left uninfected were analyzed for activation markers, such as HLA-DR, CD80/83, CD86, and the CCR7 homing marker. (C and D) ISGs were measured at week 10 postinfection by real-time PCR on the RNA from the WT or p30-KO HTLV-1 chronically infected or uninfected THP-1 cells following stimulation with poly(I:C) (C) or LPS (D) for 6 h. The statistically significant differences are marked with one or two asterisks, which indicate a P value of less than 0.05 or 0.01, respectively. Statistics were determined from three independent experiments ($n = 3$).

p30-KO HTLV-1 virus production and ISG inhibition is rescued by overexpression of p30. We next investigated whether we could rescue viral expression and IFN responses by overexpressing p30 in cells obtained at week 10 postinfection (Fig. 4A). WT- or p30-KO-infected THP-1 cells were transduced with p30 or mock lentivirus. Untransduced cells were used as a control. After 2 days, the supernatants were collected and p19 Gag was analyzed by ELISA. Interestingly, p30 expression restored virus production in cells infected with the p30-KO virus (Fig. 5A). Consistent with p30 reducing ISG expression, reverse transcription-PCR analysis of cellular mRNA demonstrated that expression of MxA and A3G genes was higher in cells infected with p30-KO virus than in cells infected with WT virus (Fig. 5B). Transduction of p30-KO-infected cells with lentivirus expressing p30 only partially rescued

MxA and A3G mRNA expression, suggesting that another viral gene participates in inhibition of ISG expression (Fig. 5B). Proteins were extracted and tested by Western blotting to demonstrate comparable expression of p30 and green fluorescent protein (GFP) (Fig. 5C).

ChIP. To explore the mechanism by which p30 affects TLR3/4 function, we performed a chromatin immunoprecipitation assay. THP-1 cells were p30 or mock transduced with lentivirus. Cells were stimulated for 1 h with the specific agonists poly(I:C) and LPS as a control and then cross-linked. The pull-downs were performed with antibodies against PU.1, p30-HA, and total RNA Pol II, as well as with antibodies specific for the phosphorylated form of RNA Pol II, CTD-Ser5 and RNA Pol II CTD-Ser2, which identify the initiating and the elongating form of active RNA Pol II,

FIG 5 p30 rescues viral production and IFN responses in THP-1 cells infected with the p30-KO mutant virus. (A) Level of p19 Gag in THP-1 cells chronically infected with WT or p30-KO HTLV-1 virus transduced with the lentivirus expressing p30 or the control lentivirus. (B) Real-time PCR for the MxA and A3G ISGs from the RNAs of the infected/transduced cells. (C) Western blot analysis for tubulin, p30, or GFP on transduced cells. The statistically significant differences are marked with one or two asterisks, which indicate a *P* value of less than 0.05 or 0.005, respectively. Statistics were determined from three independent experiments (*n* = 3).

respectively. Real-time PCR was performed on the ChIP-enriched DNA to quantify the amount of PU.1 containing transcriptional complexes bound to the promoters of the IFN- α 1, IFN- β , TLR4, and UbiquitinB genes. We observed that upon TLR4 stimulation with LPS, lentiviral expression of p30 decreased the amount of PU.1 recruited to the IFN- α 1 (Fig. 6A), IFN- β (Fig. 6D), and TLR4 promoters (Fig. 6G). Moreover, the reduced PU.1 binding was paralleled by similar decreases of both initiating and elongating RNA Pol II, as assessed by Pol II-S5 and Pol II-S2 ChIP (Fig. 6B, C, E, F, H, and I). Similarly, after poly(I:C) stimulation of TLR3, the presence of p30 resulted in decreased binding of PU.1, Pol II-S5, and Pol II-S2 to the promoters of IFN- α , IFN- β , and TLR4 genes (Fig. 6A to I). The real-time PCR performed on the UbiquitinB promoter, used as a control, showed no significant differences, as expected (data not shown). All of these data are consistent with the reduced expression of IFN-responsive genes following TLR3 and TLR4 stimulation being mediated by p30 at the transcriptional level, as was previously suggested for TLR4 (20).

DISCUSSION

Interferons (IFNs) are key molecules that mediate antiviral innate immune responses and are potent regulators of adaptive immune responses, since they affect immune cell activation, cell growth, and apoptosis. Type I IFNs (α and β) are produced mainly by dendritic cells, macrophages, and fibroblasts following activation of the pattern recognition receptor (PPR) by pathogens, including

viruses (41). Once the IFN response is triggered, the expression of more than 300 genes (ISGs) is upregulated. These genes encode proteins that have antiviral and immunoregulatory activity and are able in some cases to suppress viral replication and clear virus (42). Among them, MxA, OAS, and A3G exert a particularly important role in the immune intracellular response and are considered excellent markers for IFN action. Viruses, and often pathogens, have evolved a wide variety of strategies to counteract and escape the IFN system by a mechanism called inverse interference (41). The molecular mechanisms involved span from the shutdown of the entire cell metabolism to specifically targeting IFN at different levels, such as to inhibit the type-I IFN production itself, to inactivate secreted IFN molecules, and to block the signaling pathways or the functionality of IFN-induced protein. A variety of viruses, such as influenza virus (43), Sendai virus (44), measles virus (45), Ebola virus (46), herpes simplex virus (41), human papillomavirus (41), adenovirus (41), and hepatitis C virus (47), have evolved a way to interfere with specific components of the IFN induction pathway, mainly by blocking the IFN regulatory factors (IRFs). On the other hand, Dengue virus (41), human papillomavirus (26), hepatitis C virus (47), and paramyxoviruses (41) target the JAK-STAT pathway. HIV interferes with IFN-induced PKR and A3G through Tat and Vif, respectively (48, 49).

HTLV-1 has evolved its own strategy to counteract the IFN system. Although the mechanism is not fully understood, Hishizawa et al. have shown that dendritic cells isolated from

FIG 6 p30 displaces PU.1 from the transcription and elongation complex. ChIP assay was performed on THP-1 cells transduced with p30 lentivirus (white bars) or mock transduced (black bars). Cells were stimulated for 1 h with LPS or poly(I:C) (I:C) before the ChIP assay or were left unstimulated. Depicted here is the relative amount of DNA precipitated with the different antibodies normalized to the amount found in the input of the promoters for IFN- α 1, IFN- β , and TLR4 genes. The immunoprecipitations were performed with anti-HA and detected HA-tagged p30, anti-PU.1, anti-PolII α , anti-PolII β , and anti-PolII γ . The data are expressed in terms of fold change (the unstimulated result is 1) and represent the amount of promoter DNA for the various genes immunoprecipitated in cells stimulated in the presence or absence of p30. Statistically significant differences are marked with one or two asterisks, which indicate a *P* value of less than 0.01 or 0.0001, respectively. Statistics were determined from two independent experiments (*n* = 2).

HTLV-1-infected individuals have an impaired ability to secrete type 1 IFN (16). In addition, it has been reported that the cellular protein SOCS, which inhibits STAT1 phosphorylation, inhibits intracellular signal transduction downstream of the IFN receptor, IFNAR1/2, in both *ex vivo* CD4⁺ cells from HTLV-1-infected individuals and *in vitro* in HTLV-1-infected cells (50–52). Moreover, in HTLV-1-infected cells *in vitro*, the level of phosphorylation of Tyk2 and STAT2, two key molecules in the activation cascade of the IFN pathway, is decreased (53). Tax also prevents the binding of CBP/p300 with STAT2 in a competitive manner (54).

Despite all of the above-mentioned anti-IFN mechanisms evolved by HTLV-1, type 1 IFN maintains a potent antiviral function and is used in the clinic as anti-HTLV-1 therapy (42) for ATL. While IFN- α treatment alone results in a modest and transient, although appreciable, beneficial effect on ATL patients, when

combined with zidovudine (AZT) and arsenic trioxide it causes long-lasting remission (42). The mechanisms underlying this therapeutic effect remain unclear. In fact, while some groups demonstrated *in vitro* that the treatment with IFN- α decreases HTLV-1 expression, Kannagi's group showed that the level of p19 Gag released in the supernatant is decreased, but not the expression of other viral genes (55). However, the addition of AZT decreases viral transcription and p19 Gag and Tax production, leaving the p53-dependent apoptotic pathways unaffected and restoring the PKR antiviral activity (55).

Here, we demonstrate that HTLV-1 has evolved additional mechanisms to counteract the IFN system through the interaction of the viral regulatory protein p30 and the myeloid transcription factor PU.1. Because p30 regulates the expression of other viral RNAs, it is still debatable whether p30 acts directly or if other viral proteins are involved in decreasing ISGs. However, the ChIP assay

we performed and the rescued inhibition of the ISGs upon p30 overexpression suggest a direct interaction between p30 and PU.1. This is consistent with the observations reported by Datta and colleagues (20). To address the role of other viral proteins, detailed analysis of the viral expression pattern in the p30-KO mutant-versus WT-infected cells needs to be done. We are currently investigating the expression level of viral transcripts and the differential expression of these in monocytes versus CD4⁺ T lymphocytes. Indeed, we found that after the initial peak of p19 Gag in the supernatant around day 10, virus production drops to a low level of expression in THP-1 (Fig. 4A). Preliminary data, not included in this paper, show that in THP-1 cells the total mRNA level of *hzb* is higher than that of *tax/rex* message during chronic infection. These findings suggest that in monocytes the regulation of viral transcription follows a different pattern. The effect of p30 is pivotal to allow infectivity and persistence, as demonstrated by *in vivo* experiments in macaques (19). Our data demonstrate that in the presence of p30, the interferon response is abrogated during viral replication. Thus, strategies aimed at silencing the expression of p30 with siRNA in conjunction with other approaches may further curtail viral replication and possibly contribute to the eradication of infected cells through innate IFN-mediated immune responses.

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