The HTLV-I p30 Interferes with TLR4 Signaling and Modulates the Release of Pro- and Anti-inflammatory Cytokines from Human Macrophages*

Received for publication, January 23, 2006, and in revised form, June 13, 2006 Published, JBC Papers in Press, June 19, 2006, DOI 10.1074/jbc.M600684200

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Whereas adaptive immunity has been extensively studied, very little is known about the innate immunity of the host to HTLV-I infection. HTLV-I-infected ATL patients have pronounced immunodeficiency associated with frequent opportunistic infections, and in these patients, concurrent infections with bacteria and/or parasites are known to increase risks of progression to ATL. The Toll-like receptor-4 (TLR4) activation in response to bacterial infection is essential for dendritic cell maturation and links the innate and adaptive immune responses. Recent reports indicate that TLR4 is targeted by viruses such as RSV, HCV, and MMTV. Here we report that HTLV-I has also evolved a protein that interferes with TLR4 signaling; p30 interacts with and inhibits the DNA binding and transcription activity of PU.1 resulting in the down-regulation of the TLR4 expression from the cell surface. Expression of p30 hampers the release of pro-inflammatory cytokines MCP-1, TNF-α, and IL-8 and stimulates release of anti-inflammatory IL-10 following stimulation of TLR4 in human macrophage. Finally, we found that p30 increases phosphorylation and inactivation of GSK3-β a key step for IL-10 production. Our study suggests a novel function of p30, which may instigate immune tolerance by reducing activation of adaptive immunity in ATL patients.

Human T cell leukemia virus type I (HTLV-I),2 is the etiologic agent of lymphoproliferative diseases known as adult T cell leukemia (ATL) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (1–3). Adaptive immunity in patients with HTLV-I-associated ATL or HAM/TSP has been extensively studied. HTLV-I-associated neurological disorders HAM/TSP are characterized by polyclonal expansion of infected cells, high proviral loads, and virus-specific immune responses, including increased pro-inflammatory cytokine production and HTLV-I-specific CTL (4, 5). Studies have demonstrated that virus-expressing cells are rapidly and continuously eliminated by the immune system in HAM/TSP patients (6, 7). It has also been shown that HTLV-I infected dendritic cells from HAM/TSP patients exhibit an enhanced capacity to stimulate antigen-specific CD4+ and CD8+ T-cell activation, which leads to autoimmune (8). In sharp contrast ATL pathogenesis is characterized by monoclonal expansion of infected cells, very low proviral loads, absence of pro-inflammatory cytokines (9), and very low frequency of virus-specific CTL (10, 11). HTLV-I-infected monocytes produce dysfunctional dendritic cells because of improper differentiation, which do not stimulate autologous T-cells (12). HTLV-I-infected ATL patients have pronounced immunodeficiency associated with frequent opportunistic infections by various pathogens, including Pneumocystis carinii, Toxoplasma gondii, Cryptococcus neoformans, Candida albicans, Mycobacterium avium, and Aspergillus, Cytomegalovirus, and Strongyloides (13, 14). HTLV-I regulatory protein p30 has been shown to suppress virus expression at both transcriptional and post-transcriptional levels. Hence, it is anticipated, though not yet demonstrated, that p30 expression and or its functions are increased in ATL patients.

The host immune system detects and responds to microbial infection mainly through a family of pattern recognition receptors called Toll-like receptors (TLRs). Signaling by these receptors induces antimicrobial genes, inflammatory cytokines, and dendritic cell maturation, which are necessary for initiating an adaptive immunity (15). Among the 10 TLRs identified in humans, TLR4 is the major lipopolysaccharide (LPS) receptor and elicits innate immune response against Gram-negative bacteria. TLR4 is expressed by B cells, myeloid dendritic cells, monocytes, macrophages, granulocytes, and T-cells. Several recent reports indicate that TLR4 is targeted by viruses such as the respiratory syncytial virus (RSV) (16), hepatitis C virus (HCV) (17), and mouse mammary tumor virus (MMTV) (18). Previous studies have established that TLR4 expression from its promoter is mainly regulated by the transcription factor PU.1 (19).

PU.1 is a member of the ets family of transcription factors with a restricted expression to B lymphocytes, macrophages and all hematopoietic lineages except T-cell lines and mature
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T-lymphocytes. PU.1 levels increase during granulocytic/monocytic differentiation of immature hematopoietic progenitor cells and decline during erythroid differentiation (20). The human PU.1 protein consists of glutamine-rich and acidic residues toward the N-terminal necessary for transactivation, and DNA binding (ets) domain at the C-terminal (21). PU.1 regulates the expression of lymphoid as well as myeloid genes. Among some of the human myeloid genes regulated by PU.1 are M-CSF receptor, G-CSF receptor, GM-CSF receptor, IL-1β, macrophage inflammatory protein 1α (MIP-1α), and tumor necrosis factor α (TNF-α). Although CD4+ T lymphocytes represent the primary target for HTLV-I infection, the virus can infect other cell types including CD8+ T lymphocytes, dendritic cells, B lymphocytes, and central nervous system (CNS) astrocytes, and monocyte lineage cells such as tissue macrophages (22–25). In vitro infection of HTLV-I in human monocytes, macrophages and microglial cells has also been demonstrated (26). Of note spliced viral mRNAs encoding for p40tax, p27rex, p12, and p30 were also identified in human macrophages infected with HTLV-I (25).

Here, we report that HTLV-I p30 protein targets the TLR4 signaling pathway. We found that virus-encoded protein p30 binds to and inhibits PU.1 DNA binding activity and PU.1-dependent transcription, leading to the down-regulation of TLR4 cell surface expression. As a result LPS-stimulated macrophages expressing p30 have a marked decrease in pro-inflammatory and an increase in anti-inflammatory cytokines released. This strategy may help the virus to evade the host innate immune responses and could make innate immune cells tolerant to opportunistic infections in ATL patients.

EXPERIMENTAL PROCEDURES

Cell Lines—The human 293T and COS-7 cell lines were maintained in Dulbecco modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT). The human monocytic cell line, THP-1, was maintained in RPMI (Invitrogen), 10% fetal bovine serum, (Hyclone, Logan, UT). The human monocytic cell line, THP-1 supplemented with 10% heat-inactivated fetal bovine serum was maintained in Dulbecco modified Eagle’s medium (Invitrogen).

Expression of these mutants was confirmed by Western blot

Pu.1 deletion mutants with Myc tag were constructed by cloning the truncated ΔE and ΔA fragments of Pu.1 into the Sall/NotI sites of pCMV/myc/cyto vector. The expression of these mutants was confirmed by Western blot with Myc antibody (A-14, Santa Cruz Biotechnology).

In Vitro and in Vivo Binding Assay—p30-HA was in vitro transcribed and translated by using TnT Quick-coupled Transcription/Translation kit (Promega). For in vitro binding, the in vitro translated p30-HA was mixed with anti-GST antibody (Amersham Biosciences) and 100 ng of purified hPu.1-GST or truncated hPu.1-GST mutants in binding buffer (50 mM Tris-Cl, pH 7.6; 50 mM NaCl; 0.5 mM EDTA; 5 mM MgCl2, 0.1% Triton X-100, and 5% glycerol) containing 2.5 mg/ml bovine serum albumin and Complete protease inhibitor. After overnight binding at 4 °C, protein G-agarose slurry was added, and the mixture incubated for 2 h with rotation at 4 °C. The immunoprecipitated complex was washed three times to wash out the beads in SDS-sample loading buffer, resolved on 12% SDS-PAGE gel, and detected by Western blot with anti-Myc (A-14) antibody.

In Vivo Binding—p30-HA was in vivo transcribed and translated by using TnT Quick-coupled Transcription/Translation kit (Promega). For in vivo binding, the in vivo translated p30-HA was mixed with anti-GST antibody (Amersham Biosciences) and 100 ng of purified hPu.1-GST or truncated hPu.1-GST mutants in binding buffer (50 mM Tris-Cl, pH 7.6; 50 mM NaCl; 0.5 mM EDTA; 5 mM MgCl2, 0.1% Triton X-100, and 5% glycerol) containing 2.5 mg/ml bovine serum albumin and Complete protease inhibitor. After overnight binding at 4 °C, protein G-agarose slurry was added, and the mixture incubated for 2 h with rotation at 4 °C. The immunoprecipitated complex was washed two times for 15 min with the binding buffer without bovine serum albumin. The beads were boiled in loading dye, resolved on 15% SDS-PAGE, and detected by Western blot with anti-Myc (A-14) antibody.
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Luciferase Assays and Western Blots—Luciferase assays to detect the effect of p30 on PU.1 expression was carried out in COS-7 cells with a trimerized PU.1 responsive-luciferase element, pTKPU.1 × 3 Luc, a gift by Dr. T. Oikawa. COS-7 cells were seeded at a density of 1 × 10⁶ cells/60-mm dish and transfected using Effectene reagent (Qiagen). The amounts of different constructs transfected are as indicated in Fig. 4. 36-h post-transfection, cells were lysed in 1× luciferase lysis buffer (Promega) and analyzed using the Luciferase Reporter Assay system (Promega) according to the manufacturer’s protocol. Luciferase activity was normalized with protein concentration. Data represent results obtained from two independent experiments. To study the effect of p30 on TLR4 expression, transient transfection of human monocytic THP-1 cells was carried out using Effectene (Qiagen) with TLR4-P-Luc reporter construct (gift of Dr. M Rehli). THP-1 cells were diluted with RPMI-10% fetal bovine serum the day before transfection. Next day the cells were centrifuged, washed twice with 1× PBS and seeded at a density of 5 × 10⁵ cells/60-mm dish. Transfection was carried out at a ratio of 8 (enhancer):25 (effectene) for 1 µg of transfected DNA. The cells were lysed 36-h post-transfection and analyzed as described above. Data (Fig. 5B) represent results from two independent experiments. To show that similar effects could be seen with physiological expression of p30, luciferase assay was carried out with TLR4 reporter construct in THP-1 cells with wild type (pHTLV-X1MT) and p30/p12-deleted (pHTLV-ΔPSX) HTLV-1 molecular clones. Western blots were carried out with equal amounts of protein extracts to confirm the expression of transfected constructs. As a negative control, we used pGL3-MRE (myb-responsive element) luciferase plasmid (27) and repeated the same experiment with a dose-dependent increase of p30. PU.1 was detected with T-21 antibody, c-Myb with C-19, p30 with 3F10, actin with C-11 (Santa Cruz Biotechnology), and β-tubulin with H-235 (Santa Cruz Biotechnology). To show that the activation of TLR4 promoter was specific, as a negative control (interferon regulatory factor-1) IRF-1 was cloned between Sall-NotI sites of pCMV/myc/cyto and the IRF-1 Myc-tagged expression plasmid was used in increasing doses along with a TLR4-luciferase reporter promoter construct. Western blots to detect phospho-GSK3 (Ser⁹) and phosphoglucogen synthase (Ser⁶¹) were carried out using phosphospecific antibodies from Cell Signaling.

Biotin-labeled DNA Pull-down Assay—To examine the effects of p30 on the DNA binding activity of PU.1 and to elucidate if increasing amounts of p300 restore this activity, 40 ng of biotinylated trimerized PU.1 responsive element (PU.1 × 3) was added to 100 µl of nuclear extract from 293T cells transfected with either 5 µg of PU.1 and increasing amounts (2, 4, 6 µg, respectively) of p30-HA expression plasmids (Fig. 4C) or 2 µg of PU.1, 5 µg of p30-HA and increasing amounts of p30 (1, 3, 5 µg, respectively) (Fig. 4D) in a total volume of 200 µl of binding buffer (25 mM HEPES pH 7.9, 5 mM KCl, 0.5 mM MgCl₂, 0.5 mM EDTA, 1 mg/ml bovine serum albumin, 10% (v/v) glycerol, and 0.25 mM dithiothreitol). The nuclear mixtures with biotin-labeled probe were preincubated on ice for 2 h with gentle agitation. 60 µl of 50% slurry of streptavidin magnetic beads (Roche) was added to the nuclear lysates and kept on ice for 1 h. The beads were collected by a magnet, washed two times with binding buffer and resuspended in 2× SDS-PAGE loading buffer. The samples were heated for 5 min at 95 °C and loaded on 12% SDS-polyacrylamide gel, blotted, and detected for PU.1 using T-21 antibody (Fig. 4, C and D).

Flow Cytometry for TLR4 Cell Surface Expression—Human monocytic THP-1 cells were transfected with p30 (0.5 µg) using Nucleofector Kit V (Amaxa Biosystems) according to the manufacturer’s protocol. For this study, untransfected and p30 transfected THP-1 cells were unstimulated or stimulated with 5 μM phorbol 12-myristate 13-acetate (PMA) (CalBiochem) for 3 h after 15 h post-transfection. Cells were harvested by centrifugation at 600 rpm, washed twice with 1× PBS and incubated with anti-human TLR4 monoclonal antibody HTA125 (BD Biosciences) or isotype control at a final concentration of 10 μg/ml for 1 h on ice. Cells were then washed twice and incubated with FITC-conjugated mouse IgG (BD Biosciences) at a final concentration of 5 μg/ml for 1 h on ice. Cells were then washed twice and analyzed by a flow cytometer equipped with the manufacturer’s software (FACS Diva, Becton Dickinson) for data acquisition and analysis. As a negative control, the surface expression of CD14 was analyzed as described above with CD14-FITC-conjugated antibody by flow cytometry in THP-1-untransfected or p30-transfected cells by Nucleofector kit V. To demonstrate physiological relevance of p30’ effects, the TLR4 surface expression was measured as described above by flow cytometry in THP-1 cells by nucleofection of wild type (pHTLV-X1MT) and p30/p12-deleted (pHTLV-ΔPSX) HTLV-1 molecular clones.
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**RESULTS**

**Interaction between p30 and Transcription Factor PU.1 in a Yeast Two-hybrid Assay**—To identify binding partners of the HTLV-I p30, we performed a yeast two-hybrid screen using the full-length p30 protein as bait. L40 yeast cells were co-transformed by a plasmid encoding a chimera between p30 and the Lex A transcription factor DNA binding domain, along with a normal spleen-derived cDNA library fused in-frame to the Gal-4 activation domain (Invitrogen). The fusion protein LexA-p30 was expressed in L40 yeast strain as demonstrated by Western blot analysis (Fig. 1A). Following screening on selective media, β-galactosidase-positive clones were retrieved by PCR, cloned, and sequenced. One of the isolated clones was found to correspond to a fragment of the PU.1 transcription factor.

This clone was selected for further analysis on the basis of its hematopoietic restricted expression and also because of the fact that p30 has been reported to act as a transcriptional and post-transcriptional regulator of gene expression. We next determined if HTLV-1 p30, without being fused to Lex A, could associate with PU.1 in mammalian cells. 293T cells, which do not express endogenous PU.1, were transfected with an untagged PU.1 expression vector with or without an HA-tagged p30 vector. **In vivo** interaction was detected by co-immunoprecipitation using an anti-HA antibody followed by Western blot analysis (Fig. 1C). When both p30 and PU.1 were coexpressed, a complex was precipitated that was not observed in the absence of either protein. When PU.1 was immunoprecipitated, a band corresponding to p30 was readily detected by Western blotting (Fig. 1D). These results confirm the yeast two-hybrid data and show that p30 binds to PU.1 in mammalian cells.

**Cytokine Profile Analysis**—THP-1 cells (2 × 10⁶) were plated into 24-well dishes and were transfected with p30 by Nucleofector kit. After 12 h of post-transfection, cells were stimulated with LPS (Sigma). Supernatants were collected at intervals of 3 h and were analyzed for the levels of tumor necrosis factor-α, IL-8, MCP-1 (monocyte chemoattractant protein-1), and IL-10 using the Beadlyte™ human multicytokine detection system from Superarray BioScience. The authenticity of the PCR products was verified by melting curve analysis.

**Real-time RT-PCR**—Total RNA was extracted from untransfected and p30 transfected THP-1 cells by TRIzol (Invitrogen) and treated with DNaseI (Invitrogen). The total RNA was reverse-transcribed, and the resulting cDNA was analyzed by real-time PCR using human TLR4 and GAPDH primer sets (Superarray BioScience). The total RNA was treated with DNaseI (Invitrogen). The total RNA was reverse-transcribed, and the resulting cDNA was analyzed by real-time PCR using human TLR4 and GAPDH primer sets (Superarray BioScience). The authenticity of the PCR products was verified by melting curve analysis.
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FIGURE 3. PU.1 ets domain is necessary and sufficient for in vivo binding to p30. A, schematic of human PU.1 protein and its functional domains. PU.1 deletion mutants with Myc tag are designated according to amino acid residues. B, 293T cells were transfected with 5 μg each of Myc-tagged PU.1 deletion constructs with or without 5 μg of p30-HA construct. Co-immunoprecipitation with anti-HA antibody (12CA5) followed by immunoblot by anti-Myc antibody (A-14) showed that the ets-domain of human PU.1 physically interacts with p30. C, Myc-tagged PU.1 deletion mutants were detected by anti-Myc antibody (3F10) and β-tubulin by H-235.

FIGURE 4. HTLV-I-encoded p30 represses PU.1 transactivation, which is rescued by dose-dependent increase in p300 but not CBP, because restoration of PU.1 DNA binding activity. A, COS-7 cells were transfected with 1 μg each of trimerized PU.1-responsive luciferase construct (pTKPU1.1 × 3 Luc), hPU.1, and p30-HA wherever indicated. Expression of PU.1 and p30 were confirmed by Western blot. B, transfection of increasing amounts of p30-HA (0.5 and 1 μg) causes dose-dependent repression of PU.1 transactivation. The repression is rescued by increasing amounts (1 and 2 μg for both p300 and CBP) of p300 but not CBP. Luciferase readings are representative of two independent experiments. C, p30 binding to PU.1 ets domain leads to dose-dependent decrease in PU.1 DNA binding activity. 293T cells were transfected using 5 μg of PU.1 and increasing amounts (2, 4, 6 μg, respectively) of p300 expression plasmids. 48-h post-transfection, nuclear lysates of transfected cells were incubated with biotin-labeled PU.1 × 3 DNA probe. The bound proteins were isolated by streptavidin magnetic beads, washed extensively, resolved using 12% SDS-PAGE, and detected by immunoblot using PU.1 antibody (T-21, upper panel). Equal amounts of nuclear extracts used as input were examined by Western blot to determine expression levels of each component per transfection (lower panel). D, dose-dependent increase in p300 restores PU.1 DNA binding activity. 293T cells were transfected using 2 μg of PU.1, 5 μg p30-HA and increasing amounts (1, 3, 5 μg) of p300 expression plasmids. 48-h post-transfection, nuclear lysates of transfected cells were incubated with biotin-labeled PU.1 × 3 DNA probe. The bound proteins were isolated by streptavidin magnetic beads, washed extensively, resolved using 12% SDS-PAGE, and detected by immunoblot using PU.1 antibody (T-21, upper panel). Expression levels of PU.1 and p30 in nuclear extracts used as input were examined by Western blot (lower panel).

P30 Suppresses PU.1 DNA Binding and PU.1-mediated Transcription in a CBP-independent, p300-dependent Manner—To test if the activity of the PU.1 protein was affected by the pres-
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As mentioned above HTLV-I has been found to infect and replicate in human macrophages in vitro as well as in vivo. To confirm that p30 represses PU.1 transcription in cells expressing endogenous PU.1, the human macrophage cell line THP-1 was transfected with a PU.1 reporter construct along with increasing amounts of p30. Consistent with results presented above, p30 was able to inhibit PU.1-mediated transcription in a dose-dependent manner (Fig. 4B) and p300 but not CBP was able to rescue p30-mediated repression (Fig. 4B). Because previous studies have demonstrated that PU.1 ets domain is involved in DNA binding activity (32, 33), and this domain interacted with p30, we tested whether such interaction may impair ability of PU.1 to bind DNA. The binding assays were performed using biotinylated DNA probe containing 3× PU.1 consensus binding sites incubated with nuclear protein extracts from cells transfected with PU.1 in the absence or presence of an increasing amounts of p30 as described under “Experimental Procedures.” (Fig. 4C). Results from these experiments demonstrated that p30 inhibits the PU.1 DNA binding activity in a dose-dependent manner (Fig. 4C) thereby preventing PU.1-dependent transcription. Consistent with luciferase results presented above increasing amounts of p30 efficiently competes with p30 and restore DNA binding activity of PU.1 (Fig. 4D).

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expression of endogenous PU.1 protein can be observed. This is in contrast with PU.1 expressed from a CMV-driven promoter (Figs. 3 and 4) and is explained by the fact that PU.1 autoregulates expression from its own promoter, therefore decreased PU.1 transcriptional activity will effect in a decrease in endogenous PU.1 expression (34).

**P30-mediated Inhibition of PU.1 Decreases Cell Surface Expression of the TLR4**—Expression of human TLR4, a major component of innate immune response against Gram-negative bacteria, is mainly regulated by the transcription factor PU.1. Therefore, results outlined above would predict a repressive effect of p30 on TLR4 expression. To verify this hypothesis a TLR4 promoter luciferase reporter construct was transfected in THP-1 cells. In agreement with the results presented above, p30 expression efficiently repressed TLR4 promoter activity, in a dose-dependent manner (Fig. 5B). We also confirmed our finding by measuring endogenous TLR4 mRNA expression by quantitative real time RT-PCR. In these assays p30 expression reproducibly resulted in significant ($p = 0.007$) 3.5–4-fold down-regulation of TLR4 mRNA expression in THP-1 cells (Fig. 5C). The specific effect of p30 on the TLR4 promoter was demonstrated by transfection of a Myb responsive luciferase construct in THP-1 cells, which express abundant levels of endogenous c-Myb protein. In this experiment, the dose increase amount of p30 had no effect on Myb-mediated transactivation showing that the effect observed on the TLR4 are specific, and p30 does not generally interfere with transcription (Fig. 5D). To further demonstrate that the inhibition on the TLR4 luciferase vector was caused by p30 expression and not transfection artifacts, we cloned IRF-1, a transcription factor involved in immunity but not in TLR4 expression or activity. In contrast to p30-mediated inhibition, transfection of a dose increase of IRF-1 had no significant effect on the TLR4 promoter reporter construct (Fig. 5E). Together these results demonstrate a specific effect of HTLV-I p30 on the TLR4 promoter.

Because TLR4 signaling is transduced by interactions of the ligands with the TLR4 receptor expressed on the cell surface, we further investigated surface expression of TLR4 by FACS analysis in mock- or p30-transfected THP-1 cells using nucleofector according to the manufacturer’s instructions. Under our experimental conditions, nearly 70% of THP-1 cells were transfected as monitored by transfection of a GFP-expressing vector (data not shown). Previous studies have shown that expression of TLR4 is very low on THP-1 cells but drastically increase following phorbol myristyl acetate (PMA) stimulation (35). In the absence of stimulation by PMA, p30 expression resulted in a small decrease in TLR4 surface expression (5%) in THP-1 cells (Fig. 6A). In contrast, p30 had a more significant effect following PMA stimulation resulting in nearly 70% down-regulation of TLR4 expression from the THP-1 cell surface (Fig. 6A). The presence of two distinct populations in PMA-stimulated THP-1 cells detected by FACS, based on their level of TLR4 expression (medium and high) has been previously reported (35). To ensure that the down-regulation of TLR4 from the cell

**FIGURE 6.** p30 specifically decreases TLR4 surface expression but not the CD14 marker. A, THP-1 cells were transfected with (0.5 $\mu$g) or without p30 by nucleofection and were either PMA stimulated or unstimulated for 3 h. The cells were washed, incubated on ice for 1 h with anti-human TLR4 monoclonal antibody (HTA125), incubated for a similar time period on ice with FITC-mouse IgG secondary antibody, and washed. The cells were analyzed by FACS using software FACS Diva. B, surface expression of CD14 was analyzed with CD14-FITC-conjugated antibody by flow cytometry in THP-1-untransfected or p30- (0.5 $\mu$g) transfected cells by Nucleofector kit V and then stimulated with PMA as indicated in A.
Because the data presented above relied on high levels of p30 expression from a CMV-driven promoter, we decided to investigate whether these effects could be reproduced when p30 was expressed at more physiological levels in the context of an infectious HTLV-I molecular clone. HTLV-I wild-type molecular clone (pHTLV-X1MT) and p30/p12-deleted pHTLV-deltaPSX (kindly provided by D. Derse) (36) were used for these assays. Whereas the wild-type HTLV-I molecular clone suppressed TLR4 promoter activity, pHTLV-deltaPSX deleted for p30/p12 had no effect (Fig. 7A). Although the pHTLV-deltaPSX is deleted for both p30 and p12, the effect observed on the TLR4 promoter (Fig. 7A) can be attributed to p30 because cotransfection of pHTLV-deltaPSX along with a p12 expression vector had no effect (Fig. 7B). To further confirm the effect on endogenous TLR4 surface expression, THP-1 cells were nucleofected with pHTLV or pHTLV-deltaPSX and TLR4 expression was quantified by FACS analysis. Under these experimental conditions, physiological expression of p30 from an HTLV-I molecular clone resulted in 30% down-regulation of TLR4 expression (Fig. 7C) as opposed to 70% when p30 was expressed from a CMV promoter (Fig. 6A). In fact, pHTLV-deltaPSX lacking p30 and p12 had no effect on TLR4 but this could be rescued by coexpression of p30 thereby confirming that the effect is dependent on p30 and not p12. This further excludes the possibility that another viral gene missing from the pHTLV-deltaPSX may be involved (Fig. 7C). Together these data demonstrate that physiological expression of p30 from a molecular clone is sufficient to repress TLR4 expression in a biological setting.

**P30 Expression Reduces Pro-inflammatory Cytokine Production from LPS-stimulated THP-1 Cells**—Interaction of LPS with TLR4 activates downstream signaling pathways and mediates the release of pro-inflammatory cytokines such as MCP-1,
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![Graphs](image)

**FIGURE 8.** p30 down-regulates pro-inflammatory cytokine secretion in response to LPS. THP-1 cells were transfected with (0.5 μg) or without p30-HA and then either treated or untreated with LPS. The supernatants were collected at indicated time points and analyzed for cytokine profile using Luminex. TNF-α (A), MCP-1 (B), and IL-8 (C) were analyzed 6 h after LPS stimulation. Statistical significance from two independent experiments (p values) was calculated. Expression of p30 in THP-1 cells transfected by nucleofactor is shown.

**FIGURE 9.** p30 inhibits GSK-3β and augments anti-inflammatory IL-10 production in response to TLR4 stimulation by LPS. A, augmentation of IL-10 production by p30 in response to LPS stimulation in THP-1 cells. IL-10 production in p30-transfected and untransfected cells following 6 h of LPS stimulation assayed from supernatants by Luminex. B, THP-1 cells were transfected with (0.5 μg) or without p30-HA by nucleoefaction and then either stimulated with 1 μg/ml LPS or treated with 10 μM GSK3-β inhibitor (SB216763) for 1 h, as indicated. Following incubation, cells were lysed in 1× radioimmune precipitation assay buffer and resolved by a 4–15% SDS-PAGE gradient gel. The blots were detected using phospho-GSK-3β (Ser9) and phosphoglycogen synthase (Ser641) antibody. β-Actin (C-11) was used as loading control. Lanes 1, THP-1; lane 2, +LPS; lane 3, +GSK3 inhibitor; lane 4, +LPS and GSK3 inhibitor; lane 5, +p30; lane 6, +p30 and LPS.

TNF-α and IL-8 (37). Because p30 reduces the cell surface expression of TLR4, we investigated whether this effect may be associated with variation on cytokine release following stimulation of TLR4 by LPS. As expected, pro-inflammatory cytokines are produced in very low amounts by THP-1 cells in the absence of LPS stimulation. Our studies showed no significant effect of p30 under these conditions (data not shown). However, the presence of p30 strongly and significantly (p < 0.05) decreased the release of pro-inflammatory cytokines MCP-1, TNF-α, and IL-8 after stimulation with LPS (Fig. 8, A–C).

**P30 Inhibits GSK-3β Activity and Increases Anti-inflammatory Cytokine IL-10 Release by THP-1 Cells Following Stimulation**—Recent studies showed that inhibition of GSK3-β potently suppresses the production of pro-inflammatory cytokines while concurrently augmenting production of anti-inflammatory cytokine, IL-10 in response to multiple agonists of immunity. We found that HTLV-I accessory protein p30 interacts with the transcription factor PU.1. We further delineated the binding domain of PU.1 by in vivo and in vitro binding experiments showing that p30 interacted with the ets domain of PU.1. As a result of this interaction DNA binding activity of PU.1 was greatly impaired and PU.1-dependent transcription inhibited in a dose-dependent manner by p30. Consistent with the fact that both PU.1 and p30 interact with the coactivator p300, exogenous expression of the latter restored both DNA binding and transcriptional activity of PU.1. Previous studies have demonstrated that TLR4 promoter is mainly controlled by PU.1 and TLR4 signaling is targeted by viruses such as RSV, HCV, and MMTV. We found that p30 expression in human macrophage like THP-1 cells resulted in inhibition of PU.1-dependent transcription and decreased expression of TLR4 mRNA by real-time quantitative RT-PCR along with decreased

**DISCUSSION**

This study reports for the first time an effect of the HTLV-I-encoded protein on the Toll-like receptor 4 signaling pathway and suggests that HTLV-I subverts components of the host innate
expression of TLR4 expression from the cell surface by FACS analysis. Importantly, inhibition of TLR4 by p30 was confirmed in a biological relevant system with physiological expression of p30 from an infectious molecular clone. We further demonstrated that, following LPS stimulation in THP-1 cells, p30 expression usurps TLR4 signaling and concurrently decreases the release of pro-inflammatory cytokines TNF-α, MCP-1, IL-8 and increases the release of the anti-inflammatory cytokine IL-10. These findings are relevant to ATL pathogenesis because it has been reported that 80% of ATL have elevated serum IL-10 levels that correlated with disease progression. Moreover, treatment with IL-10 protects tumor cells from lysis by tumor-specific CTLs (42). In addition, the combination of IL-10 and TNF-α stimulate AP1 and NF-κB DNA binding both of which are constitutively activated in ATL patient samples (43, 44).

HTLV-I induces a lifelong infection despite a very low antigenic variability and consequently has evolved multiple strategies to evade host immune clearance including down-regulation of MHC class I by HTLV-I p12 (45), suppression of viral expression by HTLV-I p30 (46, 47), and inhibition of Tax-mediated transcription by HTLV-I HBZ (48). The results presented here suggest an additional role of p30 in targeting TLR4 signaling and inducing immunosuppressive signals. IL-10 inhibits a broad spectrum of activated macrophage and monocyte functions including the production of IL-12, nitric oxide, expression of MHC class II and co-stimulatory molecules (49), and down-regulate immunity to various pathogens including retroviruses (50).

The dendritic (DC) cells located in the blood and periphery are functionally immature in that they are effective in the uptake and processing of antigens but not in stimulating CD4+ or CD8+ lymphocytes. After exposure to inflammatory cytokines and bacterial products, DCs undergo maturation and are able to present antigens for priming and stimulating T cells. Therefore, interference of TLR4 signaling by p30 and reduced pro-inflammatory cytokines released along with increased IL-10 may impair the ability of dendritic cells to activate adaptive immunity in ATL patients and thereby explaining the limited proliferation of virus-specific CTL reported in ATL patients. In fact, HTLV-I-infected DCs obtained from the peripheral blood of ATL patients have been found to be defective in stimulating proliferation of CD4 and CD8 T-cells (51).

Consistent with the recent finding that inhibition of GSK3-β stimulates IL-10 production (38), we found that p30 increased the phosphorylation of GSK3-β on serine 9, a response known to inactivate kinase activity. In fact, we also demonstrated reduced GSK3-β-dependent kinase activity by reduced phosphorylation of glycogen synthase a direct target specifically phosphorylated by GSK3-β. Taken together our results demonstrate novel functions of HTLV-I in exploiting host innate immunity by targeting the TLR4 signaling pathway, inducing decrease in pro-inflammatory cytokines production and by inhibiting GSK3β kinase activity leading to increase in anti-inflammatory IL-10 secretion. The mechanism by which p30 induces phosphorylation and inactivation of GSK3 remain to be uncovered and is currently under investigation. Previous studies have also shown that GSK3 inhibition selectively increases the amount of nuclear CREB (Ser133) DNA binding activity and its association with the coactivators CBP/p300 without any discernible effects on the amount of nuclear NF-κB p65 (RelA) associated with the coactivator (38). Therefore, p30-mediated inhibition of GSK3β may, in part, explain the suppressive effect of p30 on Tax-mediated viral LTR transactivation.

Finally, recent evidence indicates that graded reduction in the expression of PU.1 in CD34+ derived bone marrow stem cell leads to an intermediate stage of poorly differentiated pre-leukemic population, which, with the accumulation of additional genetic mutations results in an aggressive form of acute myeloid leukemia (AML) (41). Along these lines it has been shown that HTLV-I can infect and replicate in bone marrow derived undifferentiated CD34+ stem cells (52). As the origin of leukemic ATL cells is unknown; it is possible that ATL cells are derived from infection of undifferentiated cells such as CD34+, which later differentiate into CD4 T-cell. Combined with our data, p30-mediated reduction of PU.1 expression, these studies allow us to speculate that p30 could play an important role in setting an appropriate cellular environment for further Tax-mediated genetic alterations and transformation which warrant further studies.

Acknowledgments—We thank Dr. Rehli and Dr. T Oikawa for providing the TLR4-P-Luc and pTKPL1x3 Luc constructs, respectively. The flow cytometry core facility is supported by P20 RR016443 (COBRE Program of the National Center for Research Resources).

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