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**References**

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HIV-1 Tat Protein Modulates the Generation of Cytotoxic T Cell Epitopes by Modifying Proteasome Composition and Enzymatic Activity

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Tat, the trans activation protein of HIV, is produced early upon infection to promote and expand HIV replication and transmission. However, Tat appears to also have effects on target cells, which may affect Ag recognition both during infection and after vaccination. In particular, Tat targets dendritic cells and induces their maturation and Ag-presenting functions, increasing Th1 T cell responses. We show in this work that Tat modifies the catalytic subunit composition of immunoproteasomes in B and T cells either expressing Tat or treated with exogenous biological active Tat protein. In particular, Tat up-regulates latent membrane protein 7 and multicatalytic endopeptidase complex like-1 subunits and down-modulates the latent membrane protein 2 subunit. These changes correlate with the increase of all three major proteolytic activities of the proteasome and result in a more efficient generation and presentation of subdominant MHC-I-binding CTL epitopes of heterologous Ags. Thus, Tat modifies the Ag processing and modulates the generation of CTL epitopes. This may have an impact on both the control of virally infected cells during HIV-1 infection and the use of Tat for vaccination strategies. The Journal of Immunology, 2004, 173: 3838–3843.

Materials and Methods

Cells
Lymphoblastoid cell lines (LCL) were established by in vitro infection of B lymphocytes from healthy donors (MIN and MON) with the B95.8 strain of EBV. Transduction of HIV tat (BH10 clone) into MIN and MON LCLs was performed by infection with the retroviral vector pBabeP carrying the puromycin resistance gene (17). Puromycin-selected cells were tested for the presence and expression of tat by DNA-PCR, RT-PCR, and Northern blot analysis. Jurkat T cells expressing Tat have been previously described.
Cells were cultured in medium supplemented with 800 μg/ml neomycin (Sigma-Aldrich, St. Louis, MO). Control cells and cells expressing Tat presented similar amounts of HLA class I molecules and adhesion molecules as judged by immunofluorescence analysis using mAbs specific for HLA-A,-B,-C, HLA-A2, HLA-DR, CD40, CD11a, CD80, and CD86 molecules (BD Pharmingen, San Diego, CA).

**HIV-1 Tat protein**

HIV-1 Tat protein Tat from the human T lymphotropic virus type IIIb isolate (BH10 clone) was expressed in *Escherichia coli* and purified by heparin-affinity chromatography and HPLC, as described previously (6). The lyophilized Tat protein was stored at −80°C to prevent oxidation, reconstituted in degassed buffer before use, and handled, as described (3). Different lots of Tat were used with reproducible results, and, in all cases, endotoxin concentration was undetectable (detection threshold: 0.05 EU/μg).

**Purification of proteasomes**

Cells were lysed with glass beads, as previously described (19). Supernatants were ultracentrifuged for 1 h at 100,000 × g and loaded into an affinity column containing a matrix derivatized with the MCP21 mAb specific for the α2 subunit of the proteasome (Affinity Technology, Exeter, U.K.). Proteasomes were eluted with 25 mM Tris-HCl, pH 7.5, containing 2 M NaCl and 0.5-ml fractions were collected. Homogeneity of the eluted material was confirmed by analysis of an aliquot by 12% SDS-PAGE and Coomassie blue staining of the gel. Fractions containing proteasomes were combined and dialyzed against 25 mM Tris-HCl, pH 7.5. Protein concentration was determined using the bicinchoninic acid method (Pierce, Rockford, IL).

**Western blot assay**

Equal amounts of proteins or equal amounts of purified proteasomes were loaded on a 12% SDS-PAGE and electrophobted onto Protran nitrocellulose membranes (Schleicher & Schuell Microscience, Keene, NH). Blots were probed with Abs specific for α2, LMP2, LMP7, MECL1, and PA28α subunits (Affinity) and developed by ECL (Amersham Biosciences, Uppsala, Sweden).

**Enzymatic assays**

The chymotryptsin-like, trypsin-like, and postacidic activities of purified proteasomes were tested using the fluorogenic substrates Suc-LLVY-AMC, Boc-LRR-AMC, and Ac-YVAD-AMC, respectively, as previously described (19). Fluorescence was determined by a fluorometer (Spectrofluorplus; Tecxan, Salzburg, Austria). Proteasome activity is expressed as arbitrary fluorescence units. The in vitro degradation of CLG, ILMV, GAVV (CLG + 5) was performed using 500 μg of peptide and 127 μg of purified proteasomes in 300 μl of activity buffer at 37°C. At different time points, 60 μl of sample was collected, and the reaction was stopped by adding 2 vol of ethanol at 0°C. Digestion mixtures were centrifuged, and 80 μl of supernatants was collected and separated by HPLC. Fractionation was simultaneously monitored at 210 and 280 nm. Fractions were collected and tested by IFN-γ ELISPOT assays.

**Synthetic peptides**

Peptides were synthesized by solid phase method and purified by HPLC to >98% purity, as previously described (20). Structure verification was performed by elemental and amino acid analysis and mass spectrometry. Peptides were dissolved in DMSO at 10 μM, kept at −20°C, and diluted in PBS before use.

**Generation of CTL cultures**

HLA A11-restricted EBV-specific CTL cultures reacting against the EBV-encoded nuclear Ag 4 (EBNA4)-derived IVDTSFVK (IVT, aa 416–424) and AVFSSRSDAK (AVF, aa 399–408) epitopes (21) were obtained from the in vitro digestion of CLG + 5 peptide. CTLs were incubated with medium alone were used as negative control, whereas CTL stimulated with PHA (Wellcome Diagnostics, Dartford, U.K.) represented the positive control. Plates were incubated for 24 h and washed, and then a biotinylated anti-IFN-γ mAb (1 μg/ml; Pierce) was added to the wells. After 60 min, the plates were washed again and HRP-conjugated streptavidin (Pierce) was added at room temperature for 45 min. Individual IFN-γ-producing cells were detected using 3-amino-9-ethylcarbazole cromogen kit (Sigma-Aldrich) and counted by ELISPOT reader (AELVIS, Hannover, Germany). The number of specific IFN-γ-secreting T cells, expressed as spot-forming cells per 10^6 cells, was calculated by subtracting the negative control values.

**ELISPOT assay**

CTLS (4 × 10^4) were seeded in triplicate on microplate 96-well unfilter (Whatman, Maidstone, Kent, U.K.) coated with an anti IFN-γ mAb (Pierce). CTLs were stimulated with 20 μl of each HPLC fraction derived from the in vitro digestion of CLG + 5 peptide. CTLs incubated with medium alone were used as negative control, whereas CTL stimulated with PHA (Wellcome Diagnostics, Dartford, U.K.) represented the positive control. Plates were incubated for 24 h and washed, and then a biotinylated anti-IFN-γ mAb (1 μg/ml; Pierce) was added to the wells. After 60 min, the plates were washed again and HRP-conjugated streptavidin (Pierce) was added at room temperature for 45 min. Individual IFN-γ-producing cells were detected using 3-amino-9-ethylcarbazole cromogen kit (Sigma-Aldrich) and counted by ELISPOT reader (AELVIS, Hannover, Germany). The number of specific IFN-γ-secreting T cells, expressed as spot-forming cells per 10^6 cells, was calculated by subtracting the negative control values.

**Cytotoxicity assay**

Target cells were labeled with Na_2^35CrO_4 for 90 min at 37°C. Cytotoxicity tests were routinely run at different E:T ratios in triplicate.
Results

Endogenously expressed Tat or exogenous native Tat protein modulates proteasome composition and activity

To evaluate the level of proteasome expression in the presence of endogenous Tat, LCL were permanently transduced with a Tat-expressing vector (MIN-Tat and MON-Tat) or the vector alone (MIN-0 and MON-0), and total cell lysates were examined by Western blot analysis with Abs specific for the constitutive α2 subunit of proteasomes. As shown in Fig. 1, all cells expressed similar amounts of proteasomes. Because LCLs constitutively express immunoproteasomes (25), we then evaluated the expression of the IFN-γ-inducible PA28α regulator and that of the catalytic β subunits LMP2, LMP7, and MECL1. Both LCL-Tat lines showed no difference in the level of expression of PA28α as compared with control cells. In contrast, Tat-expressing cells showed a marked down-regulation of LMP2 and up-regulation of LMP7 and MECL1 subunits, respectively (Fig. 1, A and B).

We then evaluated the expression of the catalytic subunits incorporated in proteasomes purified from cell lysates. Proteasomes purified from Tat-expressing cells presented a down-regulation of LMP2 and up-regulation of LMP7 and MECL1 subunits as compared with proteasomes purified from vector-transduced cells (Fig. 1, C and D), respectively. Down-regulation of LMP2 and up-regulation of MECL1 and LMP7 were also observed at transcriptional level (data not shown).

Increase of LMP7 and MECL1 and decrease of LMP2 were detected in proteasome purified from a Jurkat cell line stably transfected with Tat (JSL3-Tat) as compared with control JSL3-0 cells (Fig. 2).

These findings indicate that the endogenous expression of Tat protein modifies the catalytic subunit composition of immunoproteasomes.

Because the HIV Tat protein is efficiently taken up by cells (2–6), we tested the effect of exogenous Tat protein on proteasomes in MIN and MON LCLs cultured in the absence or presence of increasing concentrations (0.01–1 μg/ml) of the native Tat protein. After treatment, proteasomes purified from Tat-treated cells showed a down-regulation of LMP2 and an up-regulation of LMP7 and MECL1, as compared with proteasomes purified from untreated cells (Fig. 3). These results demonstrate that both endogenously expressed Tat and exogenous native Tat protein modify the subunit composition of immunoproteasomes.

To investigate whether the differences in subunit composition correlated with differences in enzymatic activity, we analyzed the cleavage specificity of equal amounts of proteasomes isolated from MIN-Tat, MON-Tat cells, or control cells. Chymotryptic, tryptic-like, and postacidic activities were all augmented in proteasomes purified from cells expressing Tat, as compared with control cells (Fig. 4). This is in agreement with the pattern of expression of the three catalytic subunits in Tat-expressing cells, because activation of LMP7 and MECL1 expression is associated with increased chymotryptic and tryptic activities, whereas a reduction of LMP2 expression is associated with an increased postacidic activity, respectively (10, 26, 27).

Tat modifies the generation and recognition of CTL peptide epitopes derived from EBV latent Ags

Because proteasomes play a key role in the generation of CTL epitopes, we investigated the effect of Tat on the presentation of dominant or subdominant CTL epitopes using as models LCLs endogenously expressing Tat or treated with the Tat protein.

LCLs were chosen because they express all EBV latent Ags, including EBNA1, 2, 3, 4, 5, and 6, and LMP1 and 2. These Ags, except for the nuclear Ag 1, are all targets of CTL and contain a large number of well-characterized CTL epitopes (28). Therefore, we evaluated the killing of LCLs expressing the immunodominant
IVT and AVF HLA-A11-presented epitopes (21), and the subdominant YLQ and CLG HLA-A2-presented epitopes, respectively (22, 23). To this end, the HLA-2- and HLA-A11-positive MIN LCL and the HLA-A2-positive MON LCL, transduced or not with Tat, were used as targets in cytotoxic assays using CTL cultures specific for IVT, AVF, CLG, and YLQ epitopes (Fig. 5). As expected, IVT- and AVF-specific CTLs efficiently lysed A11-matched LCL (21). In contrast, YLQ- and CLG-specific CTLs recognized the target cells less efficiently (22–24). This is due to the poor expression of these two epitopes on the surface of EBV-infected B cells (28). However, a decrease in IVT- and AVF-specific CTL killing and an increase in YLQ- and CLG-specific killing were observed in Tat-expressing cells as compared with control cells. No killing of the HLA-A11-negative MON LCL, either expressing Tat or the empty vector, was observed by using IVT- and AVF-specific CTL cultures (Fig. 5).

In a second set of experiments, we evaluated the CTL killing of MIN LCL untreated or treated with 0.1 μg/ml Tat protein. As expected from the results of the previous experiments, LCLs treated with Tat were less sensitive to IVT- and AVF-specific CTL killing, but were lysed more efficiently by YLQ- and CLG-specific CTLs (Fig. 6).

These findings suggested that the effect of Tat on proteasome composition and enzymatic activity results in changes in epitope presentation, leading to increased CTL recognition of subdominant epitopes.

**Efficient in vitro generation of the subdominant CLG epitope by proteasomes purified from Tat-expressing cells**

To confirm whether proteasomes from Tat-expressing cells are able to generate subdominant epitopes more efficiently, we analyzed the in vitro degradation of a CLG peptide precursor containing 5 aa at the C terminus (CLG + 5), corresponding to the wild-type sequence of the LMP2 Ag. Proteasomes were purified from MIN-Tat or MIN-0, and the in vitro CLG precursor degradation was evaluated by HPLC analysis. We observed that after 2 h, proteasomes isolated from control cells degraded 20% of the CLG + 5 peptide precursor, while proteasomes purified from Tat-expressing cells degraded 80% of the CLG + 5 peptide (Fig. 7A). This level of degradation was only reached by proteasomes from control cells after 24 h (data not shown).

We then determined the presence of CLG peptides/precursors by testing the HPLC-fractionated digestion products for their capacity to activate CLG-specific CTLs by IFN-γ ELISpot assays. Fractions purified from digests obtained after 30, 60, and 90 min of incubation, respectively, did not activate CTL responses (data not shown), suggesting that they did not contain any active peptide. In contrast, fractions 4 and 8, which were obtained after 120 min of degradation by proteasomes purified from MIN-Tat, stimulated CLG-specific CTL responses (Fig. 7B). This indicated that such fractions contained the CLG epitope or a longer antigenic epitope. In contrast, only a weak CLG-specific CTL response was observed with HPLC fraction 8, obtained after 120 min of degradation by proteasomes purified from MIN-Tat, stimulated CLG-specific CTL responses after 24 h of incubation, respectively, did not activate CTL responses (data not shown).

These results indicate that proteasomes purified from Tat-expressing cells exhibit different proteolytic activity, capable of efficiently generating subdominant peptide epitopes.

**Discussion**

We have shown in this study that the HIV-1 Tat protein, an early product of HIV-infected cells, modifies the subunit composition...
and activity of proteasomes. In particular, we demonstrated that proteasomes in cells of B and T cell origin, expressing an endogenous Tat or treated with a biologically active Tat protein, present an up-regulation of the LMP7 and MECL1 subunits and a down-modulation of the LMP2 subunit. Changes in proteasome subunit composition induced by Tat result in the increase of all three major proteolytic activities of the proteasomes.

Selective down-regulation of LMP2 by viral gene products has already been observed (29, 40). Of note, the LMP2 gene is under the control of the STAT1-IFN regulatory factor-1 (IRF-1) complex that, after binding with LMP2 promoter, induces transcription. It is therefore of interest that the Tat protein associates with IRF-1 (30), and it is tempting to speculate that this may result in IRF-1 sequestration and down-regulation of LMP2 transcription and expression.

Of note, Tat-dependent LMP2 down-regulation, both at protein and RNA levels (data not shown), did not affect the incorporation of MECL1 into proteasomes. LMP2 incorporation into immunoproteasomes is an early event during assembly, and it has been shown that subsequent incorporation of MECL1 normally requires LMP2 (31, 32). This is due to a cooperative assembly process (33) mediated by not yet defined MECL1 and LMP2 propeptide-protein interactions. We speculate that Tat may also play a role in the assembly of proteasomal subunits by interacting with MECL1 propeptide, resulting in MECL1 incorporation without the need of LMP2. Indeed, it has recently been shown that Tat interacts with both α subunits and LMP7 and MECL1 subunits of the proteasome (14). Tat has also been shown to inhibit the proteolytic activity of 20S proteasome by competing with PA28 regulator for binding to 20S (15, 16). In contrast with these data, no effect on the expression of PA28 in Tat-expressing cells nor on the activity of immunoproteasomes treated in vitro with Tat protein was detected (data not shown). However, we used a monomeric fully active Tat derived from the human T cell leukemia virus-IIIB isolate (BH10 clone, subtype B), which lacks Asp67, shown by Huang et al. (16) to be required for the interaction with the 20S core particle.

We also showed that changes of the proteolytic activities of proteasomes in Tat-expressing LCLs or in LCLs treated with the Tat protein correlate with a different presentation of EBV-derived epitopes. In particular, Tat decreases the presentation of two immunodominant CTL epitopes (IVT and AVF) presented by HLA-A11 molecules, and increases the presentation of two subdominant epitopes (YLQ and CLG) presented by HLA-A2. In fact, proteasomes from Tat-expressing cells are more efficient both in the degradation of a CLG peptide precursor and in the generation of immunogenic CLG peptide fragments. A similar phenomenon has been observed for an HLA-A2-presented epitope expressed in melanoma cells (34), suggesting that the presence of LMP2 may specifically affect the range of peptides presented by some HLA class I alleles (i.e., HLA-A2). Further confirming the critical role of the LMP2 subunit in the generation of CTL epitopes, it has been demonstrated that influenza-specific CTL responses to the two most dominant determinants decrease in LMP2 knockout mice, whereas responses to two subdominant epitopes are greatly enhanced (35).

Changes in immunodominance may be particularly relevant in vaccination strategies aimed at the control of viral infections and tumors. In fact, a decrease in the presentation of immunodominant epitopes concomitant with an increase of subdominant and cryptic epitopes may be beneficial for the elimination of virally infected or tumor cells. Indeed, it is well established that immunodominant epitopes are very prone to mutations and to CTL escape (36, 37), while subdominant epitopes are more conserved and may induce protective immune responses (38, 39).

In conclusion, our results demonstrate that the HIV-1 Tat protein modulates proteasome composition and activity, and that this affects the generation of peptide Ags recognized by CTLs. These
data may have important implication in the immune recognition of HIV-infected cells, the CTL control of HIV-associated viral infections and malignancies, and the immunogenicity of Tat itself. We have shown recently that vaccination with Tat protein or tat DNA protected monkeys against challenge with pathogenic simian HIV, and that protection correlated with Th1 responses and CTL activity (7, 8). Furthermore, we have shown that the Tat protein induces maturation of DC and increases both allogeneic and recall Ag presentation by DC (6). These observations, together with the findings presented in this work, suggest that native Tat is not only an Ag, but also a novel adjuvant capable of modulating CTL epitope hierarchy and responses against heterologous Ags favoring the generation of subdominant CTL epitopes. Therefore, the Tat protein may represent an important tool for broadening the spectrum of the epitopes recognized by CTLs, and for increasing the chances to prevent the appearance of CTL escape in vaccine strategies against HIV, and more in general against intracellular pathogens and tumors.

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