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Native HIV-1 Tat Protein Targets Monocyte-Derived Dendritic Cells and Enhances Their Maturation, Function, and Antigen-Specific T Cell Responses[1]

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Vaccination of cynomolgus monkeys with the biologically active HIV-1 Tat protein induces specific Th1 responses, including CTLs. Similar responses are also induced by vaccination with *tat* DNA, but not by vaccination with inactivated Tat or Tat peptides. This suggested that the native Tat protein may act differently on APC as compared with inactivated Tat or peptide Ag. In this study, we show that biologically active Tat is very efficiently taken up by monocyte-derived dendritic cells (MDDC) in a time (within minutes)- and dose-dependent (starting from 0.1 ng/ml) fashion, whereas uptake is very poor or absent with other APC, including T cell blasts and B lymphoblastoid cell lines. Although maturation of MDDC reduces their pino/phagocytic activity, mature MDDC take up Tat much more efficiently than immature cells. In addition, Tat uptake is abolished or greatly hampered by oxidation/inactivation of the protein or by performing the experiments at 4°C, suggesting that MDDC take up native Tat by a receptor-mediated endocytosis. After uptake, active Tat protein induces up-regulation of MHC and costimulatory molecules and production of IL-12, TNF-α, and β chemokines, which drive Th1-type immune response. In contrast, these effects are lost by oxidation and inactivation of the protein. Finally, native Tat enhances Ag presentation by MDDC, increasing Ag-specific T cell responses. These data indicate that native Tat selectively targets MDDC, is taken up by these cells via specialized pathways, and promotes their maturation and Ag-presenting functions, driving Th1-type immune responses. Thus, Tat can act as both Ag and adjuvant, capable of driving T cell-mediated immune responses. ***The Journal of Immunology,*** **2002, 168: 197–206.**

Tat is a regulatory protein of HIV-1 produced very early after infection and essential for virus gene expression, replication, and infectivity (1–3). During acute infection of T cells by HIV, Tat is also released in the extracellular milieu and taken up by neighbor cells (4–8), where, according to the concentration and the cell type, it can modulate cellular functions and increase virus infectivity. Specifically, upon uptake, Tat can enhance, in infected cells, virus gene expression and replication (4, 6–8), and, in uninfected cells, the expression of the β chemokine receptors CCR5 and CXCR4, favoring transmission of both macrophage and T lymphocyte-tropic HIV-1 strains (9, 10).

Evidence suggests that the immune response to Tat has a key role in the control of HIV infection. In fact, a Tat-specific immune response is present in HIV-1-infected subjects and SIV-infected monkeys, and correlates inversely with progression to the symptomatic stage of the infection (11–18). Moreover, a recent study in macaques experimentally infected with SIV indicates that the immune response to Tat, namely anti-Tat CTL, is key to control early virus replication after primary infection and exerts a selective immune pressure on the virus, leading to the appearance of slowly replicating and apparently less pathogenic escape mutants (19). Furthermore, vaccination with the biologically active Tat protein or *tat* DNA induces protection against SHIV89.6P virus replication and disease onset, which correlates with the presence of Th1 responses, including specific CTLs (20–22 and our unpublished observations). Similar protection data have been observed with a *tat-rev* vaccine delivered with viral vectors in macaques (23). In contrast, a limited containment of the infection has been observed in monkeys vaccinated with inactivated Tat or Tat peptides, in which Ab and Th responses, but no CTL, had been induced (24, 25). In contrast, the repeated intradermal (i.d.)[3] inoculation of monkeys with native and active Tat protein alone (in the absence of any adjuvant) at low doses (5–6 μg) selectively induced a Th1 response and specific CTLs in the absence of any significant Ab production (Ref. 20 and our unpublished observations).

Soluble Tat protein or specific Tat peptides have been shown to enter different cell types (4, 6–8, 26–28), to be presented with MHC class I Ag (27, 28), and to induce CTL in vivo (20). This is a property of soluble proteins called penetratins, capable of entering APC and of delivering hydrophilic compounds (29). However, little or nothing is known on the uptake and effects of native Tat, as opposed to inactivated Tat, on APC.

Monocyte-derived dendritic cells (MDDC), T cell blasts (TCB), and B lymphoblastoid cell lines (BLCL) are among the most commonly used APC. In particular, dendritic cells (DC) are the most efficient APC and are key to the induction of immune responses against viral infections (30, 31). Their function is associated with

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[3] Abbreviations used in this paper: i.d., intradermal; DC, dendritic cell; MDDC, monocyte-derived DC; BLCL, B lymphoblastoid cell line; GLP, good laboratory practice; MFI, mean fluorescence intensity; MIP, macrophage-inflammatory protein; SI, stimulation index; TCB, T cell blast; TT, tetanus toxoid.

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a high expression of MHC and costimulatory (CD40, CD80, CD86) molecules and with the production of cytokines (IL-1β, TNF-α, IL-12, IL-15), known to activate T lymphocytes, and β chemokines (macrophage-inflammatory protein (MIP)-1α, MIP-1β, RANTES). Upon encountering the Ag, DC undergo a maturation process characterized by an increase of costimulatory molecule expression and by a reduction of their phagocytic and pinocytic capability (30, 31). Furthermore, due to the up-regulation of the homing receptor CCR7 and to the down-regulation of CCR5, mature DC migrate to lymph nodes, where they present Ag to T lymphocytes (30, 31).

In this study, we show that biologically active, but not oxidized/inactivated, Tat protein is very efficiently and selectively taken up by MDDC in a dose-, time-, maturation-, and temperature-dependent fashion. Upon uptake, Tat induces in MDDC an increase of the expression of MHC and costimulatory molecules, and production of Th1 cytokines and β chemokines. All these effects are lost when Tat is oxidized and inactivated. Furthermore, native Tat enhances both allogeneic and Ag-specific presentation by MDDC, thus increasing T cell-specific immune responses. Thus, due to its capacity to efficiently enter MDDC, to enhance their functions, and to drive Th1-specific immune responses, native Tat may favor its own presentation and the induction of specific immune responses, but may also adjuvate T cell responses to other Ag.

Materials and Methods

HIV-1 Tat protein

HIV-1 Tat from the human T lymphotropic virus type IIIB-BH-10 (subtype B) was expressed in *Escherichia coli* and purified to homogeneity by heparin-affinity chromatography and HPLC, as described previously (7, 20, 32, 33), as a good laboratory practice (GLP) manufactured product. The purified Tat protein was fully biologically active, as tested by the rescue assays on the HLM-1 cell line carrying a Tat-defective HIV provirus, as previously published (7, 8), or by the induction of chloramphenicol acetyl transferase activity with HL3T1 cells containing the HIV long terminal repeat chloramphenicol acetyl transferase construct (34), and by several other assays with the same protein lots on endothelial cells or mice (5, 32, 33, 35). To prevent oxidation that occurs easily because Tat contains seven cysteines, the Tat protein was stored lyophilized at −80°C and reconstituted in degassed buffer before use, as described (7, 8, 35). To prevent attachment of the protein to surfaces, plastic tips and vials were previously rinsed in 0.1% PBS-BSA or in RPMI 1640 supplemented with 20 mM HEPES (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Life Technologies, Paisley, U.K.), and 15% FBS (HyClone Laboratories, Logan, UT) (complete medium). In addition, because Tat is also photo- and thermosensitive (7, 8), the handling of the protein was always performed in the dark and on ice. Experiments were also performed with Tat oxidized by exposure to the light and air for 18 h. By this procedure, Tat loses all biological activity due to conformational changes, including multimerization and aggregation of the protein with loss of the monomeric active form (7, 8, 32, 33). Different GLP lots of Tat were used with reproducible results, and in all cases endotoxin concentration was below the detection limit (<0.05 EU/μg), as determined by the *Limulus* Amoebocyte Lysate analysis (Pyrochrome, Associates of Cape Cod, Falmouth, MA).

Cell preparation and culture

MDDC were obtained from peripheral blood monocytes of 14 different healthy human donors, according to established methods (36). Briefly, PBMC were isolated by density gradient separation (Ficoll-Paque Research Grade; Pharmacia Biotech, Uppsala, Sweden). Monocytes were further purified by incubation with anti-CD14-coated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by sorting with a magnetic device (MiniMacs Separation Unit; Miltenyi Biotec), according to the manufacturer's instructions. The purity of monocytes was always >95%, as assessed by flow cytometry (FACScan; BD Biosciences, San Jose, CA).

Monocytes were induced to differentiate to DC (MDDC) by 6 days of culture in complete medium in the presence of GM-CSF (200 ng/ml; Leucomax; Novartis, Origgio, Italy) and IL-4 (100 ng/ml; PeproTech, London, U.K.). Differentiation to DC was assessed by morphologic observation and by the detection of specific surface markers (HLA-DR, CD86, CD83, CD40, CD80) by flow cytometry.

BLCL were generated by culturing human PBMC from two healthy donors for 2 h in the presence of supernatants from the EBV producer B95-8 marmoset cell line, and further expansion for at least 4 wk, as described earlier (37). TCB were obtained by stimulation of human PBMC from four different donors with 1 μg/ml PHA (Murex Diagnostics, Chatillon, France) for 3 days and further expansion for 2 wk in complete medium supplemented with 10 IU/ml rIL-2 (BD Labware, Bedford, MA), as described earlier (37).

Cellular uptake of the Tat protein

MDDC from 14 healthy donors were cultured at the density of 2×10^5/ml in complete medium in the presence of native or oxidized HIV-1 Tat or with reconstitution buffer or medium alone (negative controls) for 5, 10, 30, or 60 min at 37°C in the dark. Cells were then washed with cold medium and treated for 10 min at 37°C with trypsin-EDTA (Life Technologies) to remove any externally bound protein. After fixation and permeabilization, MDDC were stained with affinity-purified rabbit polyclonal anti-Tat IgG Ab (7, 8, 35) or rabbit IgG control Ab (ICN Biomedicals, Opera, Italy), followed by FITC-conjugated anti-rabbit Ig (Pierce, Rockford, IL). Fluorescence was analyzed by flow cytometry, and results were expressed as the percentage of positive cells as compared with isotype-stained samples. To demonstrate the specific intracytoplasmatic localization of the protein, staining with anti-Tat Ab was always performed also with nonpermeabilized MDDC. In some experiments, uptake of Tat by MDDC was also performed at both 4 and 37°C and at both 10 and 30 min of incubation. To this goal, all the steps described above, including the treatment of the cells with trypsin-EDTA, were performed with cells and solutions at 4°C.

BLCL from two healthy donors and TCB from four healthy donors were also cultured at 5×10^5/ml in complete medium in the presence of native Tat at concentration ranging from 100 to 10,000 ng/ml, reconstitution buffer, or medium alone for 30 or 60 min at 37°C in the dark, and processed as described for MDDC.

Analysis of MDDC maturation and cytokine and β chemokine production

MDDC from 10 donors were cultured at the density of 2×10^5/ml in complete medium in the absence or presence of serial concentrations of native or oxidized Tat or with reconstitution buffer. LPS from *E. coli*, serotype 055:B5 (10 μg/ml) (Sigma-Aldrich), was used as the positive control. After 18 h, the cell supernatants (from 8 of the 10 donors) were collected and stored at −70°C in small aliquots and assayed for the presence of TNF-α and IL-12 and the β chemokines RANTES, MIP-1α, and MIP-1β with commercially available kits, according to the manufacturers' instructions (Cytoscreen TNF-α and IL-12 ELISA kits, Biosource Europe, Nivelle, Belgium; Quantikine RANTES, MIP-1α, and MIP-1β, R&D Systems, Abingdon, U.K.). The cellular fraction was washed twice and analyzed for the expression of surface molecules by flow cytometry. The following mAb were used: FITC- or PE-conjugated IgG isotypes, FITC-conjugated anti-CD14 and anti-HLA-DR (BD Biosciences), FITC-conjugated anti-CD40, anti-CD80, anti-CD83, and PE-conjugated anti-CD86 (BD PharMingen, San Diego, CA).

Mixed allogeneic cultures

MDDC (2×10^5 cells/ml) were incubated for 18 h with LPS (positive control), native Tat protein (10 μg/ml), or reconstitution buffer. Then they were washed and cultured in complete medium (containing 5% FBS) in 96-well plates together with monocyte-depleted allogeneic PBL (2×10^5/well) at ratios ranging from 1:10 to 1:640. After 6 days, [^{3}H]thymidine was added for additional 16 h, and samples were harvested onto glass fiber filters (Printed Filtermat A; Wallac, Turku, Finland) and counted with a Betaplate (Wallac), and the values were expressed in cpm.

Ag-specific presentation assays

MDDC (2×10^5 cells/ml) from three healthy donors were incubated for 18 h with active Tat protein (10 μg/ml) or reconstitution buffer and then cultured in complete medium in the presence of 5% FBS, together with autologous PBL (2×10^5/well), at a ratio of 1:20 in the presence of 5 μg/ml tetanus toxoid (TT; Pasteur Merieux Connaught, Willowdale, Canada). After 6 days, [^{3}H]thymidine was added for an additional 16 h, samples were harvested, and cpm were counted, as reported above. The stimulation indexes (SI) were the ratios between counts from DC-PBL cocultures and those from PBL alone.

Statistical analysis

Statistical analysis was performed by the two-tailed Student *t* test.

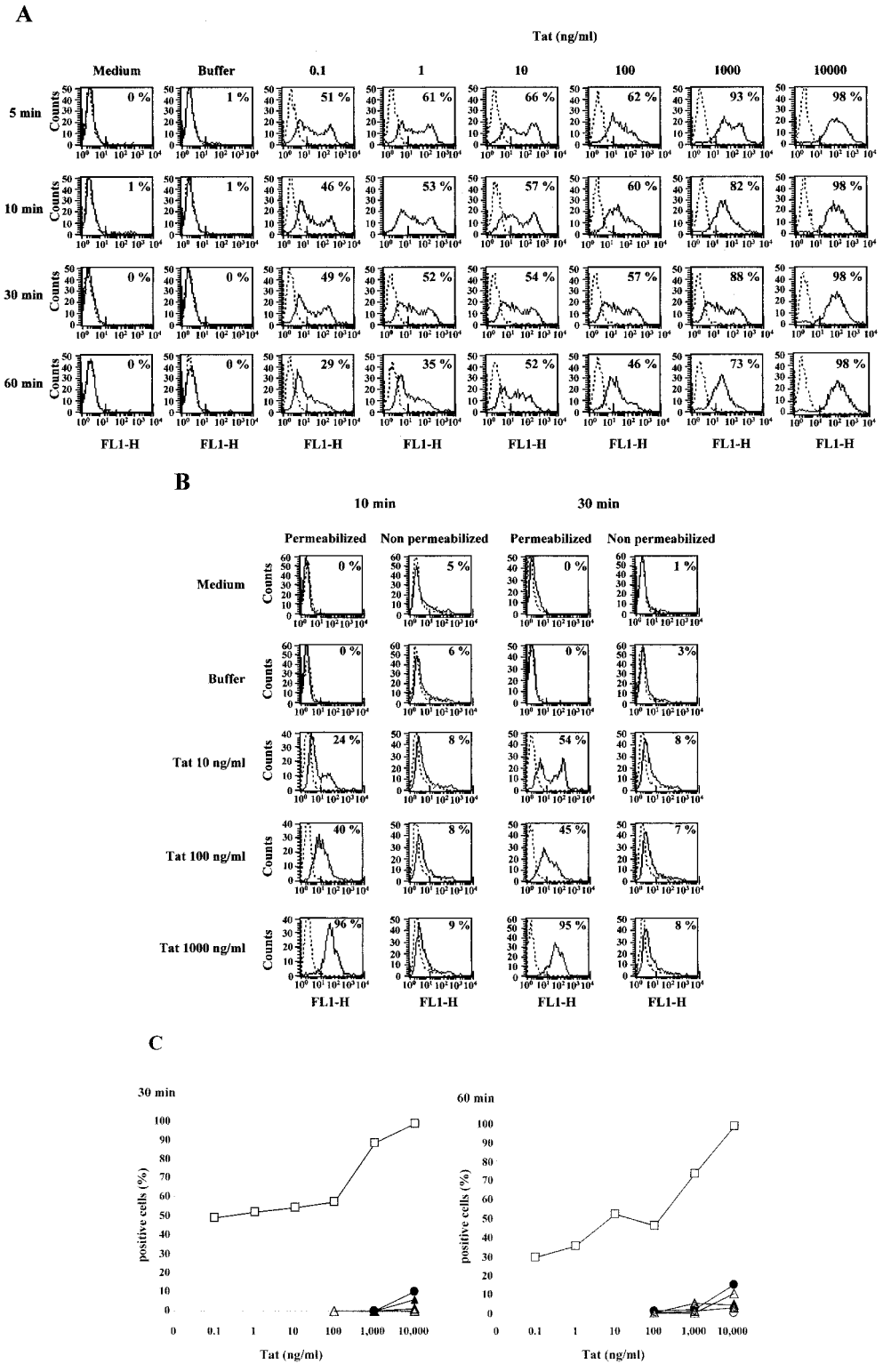

FIGURE 1. Native Tat is efficiently and selectively taken up by MDDC, but not by BLCL or TCB. *A*, MDDC were incubated with serial concentrations (0.1–10,000 ng/ml) of the native Tat protein, medium, or reconstitution buffer for 5, 10, 30, and 60 min, respectively. Cells were then processed, washed, fixed, and permeabilized, as described in *Materials and Methods*. Intracytoplasmatic Tat content was evaluated by flow cytometry after staining with specific affinity-purified rabbit anti-Tat polyclonal Ab (or isotype control), followed by secondary FITC-conjugated anti-rabbit Ab. The percentage of positive cells (as compared with isotype-stained samples) is reported in the boxes. Data are from one representative donor of 14 tested, whose levels of Tat uptake were closest to the median of the values observed with all donors tested at both 10 min (49, 52, 49, 70, 94, and 98% positive cells for 0.1, 1, 10, 100, 1,000, and 10,000 ng/ml) and 30 min (49, 45, 54, 65, 95, and 98% positive cells for 0.1, 1, 10, 100, 1,000, and 10,000 ng/ml), respectively. *B*, MDDC were incubated with the native Tat protein (10–1,000 ng/ml) for 10 or 30 min, as reported above, and both permeabilized and not permeabilized cells were analyzed by FACS. *C*, BLCL from two donors (circles) and TCB for four donors (triangles) were incubated for 30 or 60 min in the presence of native Tat protein (100–10,000 ng/ml) and stained for intracellular Tat detection, as reported above. Data are compared with those of MDDC (squares) cultured at the same times and with the same doses of the protein and chosen as a representative example, because Tat uptake with this donor resulted at levels closest to the median from 11 different donors tested (54%, range 17–91%; 65%, range 27–91%; and 95%, range 83–99%, of positive cells, at 10, 100 and 1,000 ng/ml Tat, after 30 min of culture, respectively).

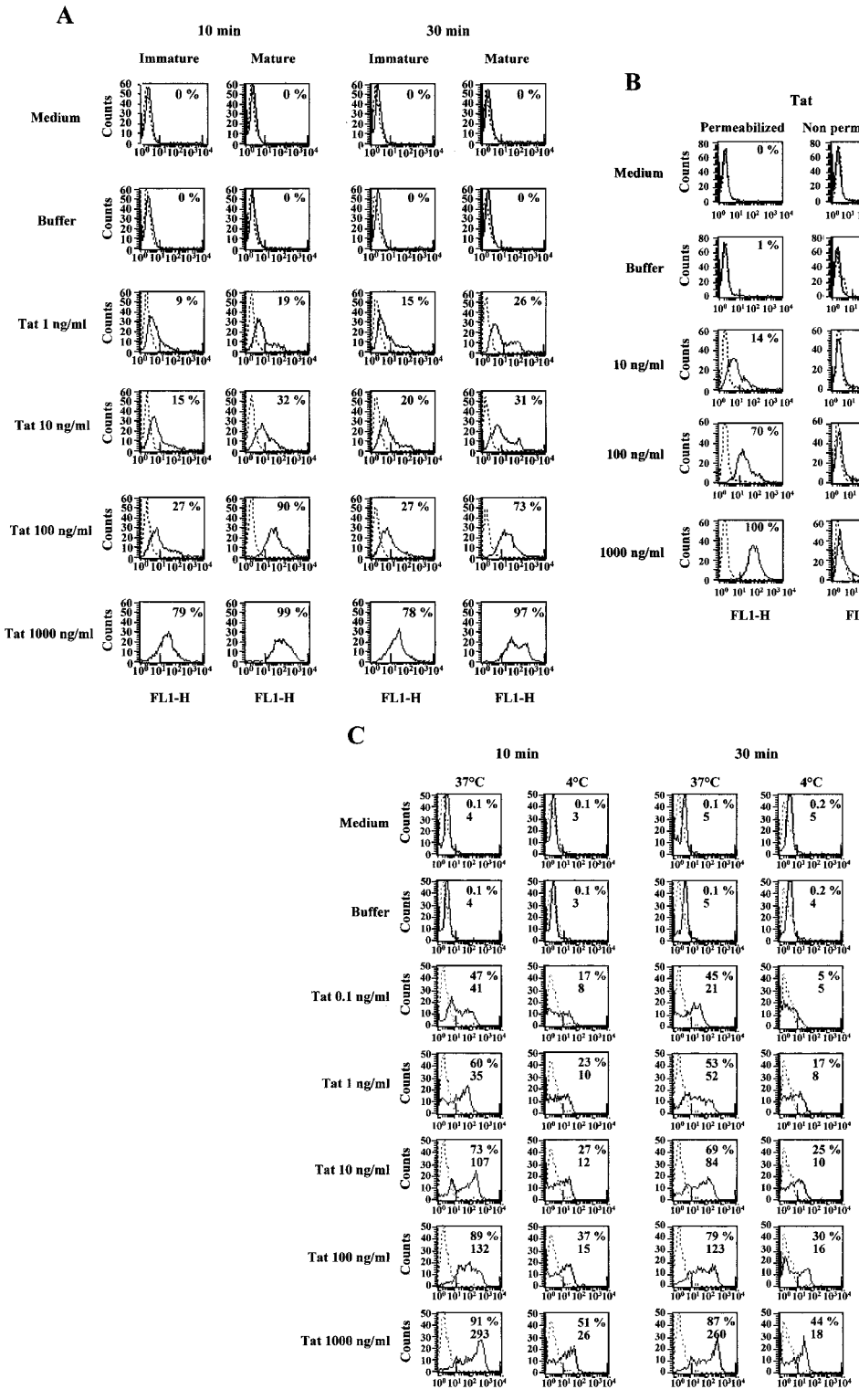

FIGURE 2. Uptake of Tat by MDDC increases with cell maturation, but it is lost by oxidation/inactivation of the protein or by performing the uptake at low temperatures. *A*, MDDC were induced or not to maturation with LPS for 18 h, and then incubated for 10 and 30 min in the presence of the native Tat protein (1–1000 ng/ml), as reported above. The data shown are from a donor who had lower uptake levels than the median values of all donors tested and that was chosen because well highlights the increase of Tat uptake induced by cell maturation. *B*, MDDC were incubated for 10 min in the presence of the native or the oxidized (by exposure to light and air for 18 h) Tat protein (10–1000 ng/ml) and processed, as reported above. The biological activity of native vs oxidized Tat used in the experiments is reported in Table I. *C*, MDDC were incubated for 10 or 30 min in the presence of native Tat protein (0.1–1000 ng/ml) at 37 or 4°C and processed, as reported above. The data from a representative experiment of three performed (with different donors) are shown. The percentage of positive cells and the MFI are reported in the boxes.

Results

Native Tat is efficiently taken up by MDDC, but not by TCB and BLCL

The uptake of active Tat by MDDC, TCB, and BLCL was evaluated by intracellular immunofluorescence in flow cytometry, using a specific affinity-purified polyclonal Ab on permeabilized cells. Fig. 1 shows the results of a representative donor whose levels of Tat uptake represented the median of the values obtained from 14 donors tested. Tat uptake by MDDC was very efficient and occurred in a dose- and time-dependent fashion (Fig. 1*A*). Uptake was already evident with the lowest dose of Tat utilized (0.1 ng/ml). Regardless of the Tat concentration tested, the level of staining always peaked after 5 min of incubation and was reduced after 60 min, most likely due to the processing of the protein. However, uptake of Tat remained high (98%) up to 60 min of incubation at the highest dose of Tat (10 μg/ml) used. No staining was observed with cells incubated in medium alone or reconstitution buffer (Fig. 1*A*). In addition, the Tat detected was almost entirely intracellular because no staining was observed after 10 or 30 min of incubation of Tat with nonpermeabilized cells (Fig. 1*B*). Similar dose and time kinetic of Tat uptake by MDDC was reproducibly observed with different protein lots.

In contrast to MDDC, uptake of active Tat by TCB and BLCL was much less efficient. In fact, little or no specific intracellular staining was observed with both cell types at concentrations of Tat up to 10 μg/ml after 30 or 60 min of incubation with values, at the highest dose of Tat, much lower than those obtained with MDDC (0–15 and 3–10% for BLCL and TCB, respectively, vs 98%) (Fig. 1*C*). Thus, efficient uptake of active Tat is a selective feature of MDDC.

Tat uptake by MDDC enhances with cell maturation and is lost by oxidation and inactivation of the protein or by low temperatures

Immature MDDC take up Ag by phagocytosis and pinocytosis (30, 31). Mature DC lose these activities while acquiring strong Ag presentation capability. To verify whether cell maturation affects the uptake of native Tat, MDDC were induced to maturate with LPS. Mature MDDC expressed higher levels of HLA-DR, CD83, and CD86 surface markers (data not shown). Both immature or mature cells were then used for the uptake experiments. Tat uptake was highly increased by MDDC maturation at all protein concentrations tested (Fig. 2*A*). In fact, incubation of mature MDDC with low Tat concentrations gave levels of intracellular staining similar to those observed in immature cells with the highest doses of Tat (Figs. 1*A* and 2*A*).

Tat contains seven cysteines, and is very sensitive to oxidation, which causes conformational changes and loss of biological activity. To verify the role of conformation and biological activity of Tat in the uptake process by MDCC, the protein was exposed to air and light, the loss of biological activity was tested (Table I), and active or inactive Tat protein was then compared in the uptake experiments. As shown in Fig. 2*B*, no staining was observed with oxidized Tat up to 1 μg/ml of the protein, and, at this concentration, only a very low Tat-specific staining was detected as compared with native Tat. Thus, Tat must have native conformation and biological activity for efficient uptake by MDDC.

Low temperature reduces all energy-dependent processes, including receptor-mediated endocytosis. To verify the effect of low temperature on Tat uptake by MDDC, experiments were performed in parallel at both 4 and 37°C (Fig. 2*C*). At 4°C, Tat uptake by MDDC, both at 10 and 30 min, resulted greatly reduced at all doses of the protein used, as compared with 37°C and as both the percentage of stained cells and the mean fluorescence intensity (MFI). This was particularly evident at the lower doses of Tat (0.1–100 ng/ml) at which MDDC staining was extremely reduced or abolished at both 10 and 30 min, as compared with 37°C (Fig. 2*C*). Because MDDC maturation that is associated with a reduced pino/phagocytic activity increases Tat uptake, whereas oxidation/inactivation of the protein or low temperatures greatly reduces it to levels comparable with those observed with BLCL and TCB, it is conceivable that uptake of native Tat by MDDC is mediated by specific receptors and entry mechanisms that are not related to the pino/phagocytic activity of the cells.

Native, but not oxidized, Tat induces MDDC maturation

To evaluate the effect of the Tat protein on MDDC maturation, the surface expression of MHC, HLA-ABC, and HLA-DR, and of the costimulatory molecules CD40, CD80, CD86, and CD83 was analyzed by flow cytometry on cells cultured for 18 h in the presence of the protein, complete medium, reconstitution buffer, or LPS (positive control). Experimental data obtained with 10 different donors indicated that Tat induces a dose-dependent enhancement of the expression of MHC and costimulatory molecules in the absence of any cell toxicity (Table II). A marked increase of the MFI was observed for HLA-ABC (3 of 6 donors, average 37%), for HLA-DR (10 of 10, average 53%), for CD40 (6 of 10, average 35%), for CD80 (8 of 8, average 54%), for CD83 (9 of 10, average 177%), and for CD86 (10 of 10, average 146%). Reconstitution buffer or medium alone did not change the expression levels of the molecules analyzed. Of note, oxidation of Tat markedly reduced the capacity of the protein to up-regulate MHC and costimulatory molecules on MDDC (Table III). Thus, only native Tat promotes the maturation of MDDC.

Table I. *Oxidation of Tat removes its biological activity*[a]

Cell Line	Buffer	Tat (ng/ml) 500	1,000	2,500	5,000	10,000	Native or Oxidized
HLM-1	14	434	491	971	1507	5028	Native
	19	21	22	40	34	117	Oxidized
HL3T1	0	29	34	79	81	187	Native
	0	3	2	2	4	6	Oxidized

[a] Tat was oxidized by exposure to air and light. Biological activity of the native and oxidized protein was then tested (at doses from 500 to 10,000 ng/ml) by measuring the capacity of rescuing the replication of Tat-defective HIV provirus in the HLM-1 cell line (7, 8) or of inducing CAT activity in the HL3T1 cell line containing the LTR-CAT construct (34). Values of the p24 (measured in picograms per milliliter) from culture supernatants of HLM-1 cells and CAT activity from HL3T1 cell extracts (percentage of acetylation/100 μg of protein) are reported.

Table II. *Native Tat enhances the expression of HLA and costimulatory molecules on MDDC*[a]

	Donor Code	MFI		% Increase vs Control	
		Medium	Buffer	Tat	LPS
HLA-ABC	B19	392	332	78.3	74.0
	B24	291	309	49.2	140.9
	B26	304	297	15.8	32.6
	B38	130	127	63.8	168.5
	B53	276	272	2.2	48.9
	B55	251	259	13.9	9.2
HLA-DR	B19	145	192	27.1	84.8
	B24	164	143	60.8	48.8
	B26	175	177	60.5	60.6
	B38	464	366	98.1	85.6
	B40	1040	1127	38.9	58.9
	B43	415	408	27.7	ND
	B44	687	692	31.4	ND
	B45	847	797	40.4	ND
	B53	471	493	94.7	94.5
	B55	313	303	47.2	30.7
CD40	B19	53	51	23.5	77.4
	B24	60	58	41.4	103.3
	B26	42	43	16.3	45.2
	B38	70	62	95.2	64.3
	B40	85	76	60.5	76.5
	B43	48	48	−6.3	ND
	B44	69	68	14.7	ND
	B45	87	76	18.4	ND
	B53	82	80	52.5	75.6
	B55	34	36	38.9	14.7
CD80	B19	8	8	62.5	350.0
	B24	16	16	43.8	143.8
	B26	10	9	44.4	90.0
	B38	21	19	100.0	109.5
	B40	27	23	73.9	63.0
	B43	9	8	25.0	ND
	B44	18	18	27.8	ND
	B45	21	21	52.4	ND
CD83	B19	7	6	166.7	442.9
	B24	9	7	257.1	200.0
	B26	8	9	55.6	62.5
	B38	5	6	233.3	520.0
	B40	12	10	310.0	233.3
	B43	8	9	22.2	ND
	B44	9	13	7.7	ND
	B45	7	10	90.0	ND
	B53	3	6	150.0	433.3
	B55	6	5	480.0	ND
CD86	B19	81	76	82.9	133.3
	B24	35	41	48.8	77.1
	B26	128	129	48.1	55.5
	B38	95	103	266.7	288.0
	B40	25	24	95.1	144.0
	B43	75	74	41.9	ND
	B44	70	75	80.0	ND
	B45	51	50	166.0	ND
	B53	15	14	535.7	693.3
	B55	37	40	97.5	32.4

[a] Cells were exposed for 18 h to native Tat (0.02–20 μg/ml), reconstitution buffer, complete medium, or LPS (10 μg/ml), stained with fluorochrome-conjugated mAb, and then analyzed by flow cytometry. The expression of the surface molecules on MDDC from 10 different donors is reported as the percentage increase of the MFI of cells exposed to Tat (at the dose which gave the maximum increase) or LPS as compared to the MFI of those exposed to Tat buffer or medium, respectively.

Table III. *Oxidized-inactivated Tat does not enhance the expression of HLA and costimulatory molecules on MDDC*[a]

	MFI		Tat (% increase vs control)	Native or Oxidized
	Medium	Buffer		
HLA-DR	313	303	46.9	Native
			10.6	Oxidized
CD40	34	36	38.9	Native
			8.3	Oxidized
CD83	6	5	480.0	Native
			40.0	Oxidized
CD86	37	40	97.5	Native
			15.0	Oxidized

[a] MDDC were exposed for 18 h to native or oxidized-inactivated Tat (0.02–20 μg/ml) and stained and analyzed as described in the legend to Table II. Data are from a representative donor. MDDC cultured in the presence of native or oxidized Tat were always viable, not differing from those treated with medium or reconstitution buffer (data not shown).

Native, but not oxidized Tat enhances the production of Th1-type cytokines and β chemokines by MDDC

To evaluate the effects of Tat on DC activation, the production of the cytokines IL-12 and TNF-α, known to activate immune cells and to induce Th1-type responses (38), and of the β chemokines RANTES, MIP-1α, and MIP-1β that are known mediators of immune responses (39), was assessed by ELISA in the supernatants of cells cultured for 18 h with the protein, reconstitution buffer (negative control), or LPS (positive control) (Fig. 3). Incubation with native Tat induced a dose-dependent increase of the levels of IL-12 and TNF-α, reaching, at the highest dose of Tat, an increase of 23-fold ($p < 0.02$) for IL-12 and 20-fold ($p < 0.03$) for TNF-α as compared with cells treated with buffer alone. Similarly, Tat markedly enhanced, in a dose-dependent fashion, the production of RANTES (10-fold, $p < 0.02$), MIP-1α (97-fold, $p < 0.005$), and MIP-1β (15-fold, $p < 0.01$). The reconstitution buffer had no effects, whereas LPS markedly enhanced the production of both cytokines and β chemokines.

In contrast to the effects of native Tat protein, oxidized Tat did not increase the production of IL-12 or TNF-α (data not shown). Thus, only native Tat increases the production and secretion of Th1 cytokines and β chemokines by MDDC.

Native Tat increases allogeneic presentation by MDDC

To evaluate the effect of Tat on the Ag-presenting capacity of MDDC, cells were exposed to the Tat protein, reconstitution buffer, complete medium or LPS (positive control), and cultured with allogeneic PBL at serial cell:cell ratios (Fig. 4). This assay was chosen because, although not specific for a given Ag, it provides adequate information on the overall Ag-presenting function of DC. Untreated MDDC induced some levels of proliferation of allogeneic lymphocytes, depending on the number of APC used. However, the proliferative response of allogeneic PBL was significantly enhanced by MDDC pulsed with Tat (3.3-fold, $p < 0.01$, at the highest DC/PBL ratio), reaching levels similar to those induced by LPS (3.8-fold, $p < 0.005$) at the same cell to cell ratio. In contrast, no enhancement of allogeneic lymphocyte proliferation was observed by treatment of MDDC with reconstitution buffer (Fig. 4). Thus, native Tat increases DC presentation function.

Native Tat enhances the presentation of recall Ag by MDDC and specific T cell responses

The effect of Tat on the Ag-specific presenting capacity of MDDC was evaluated by transiently treating the cells with the protein,

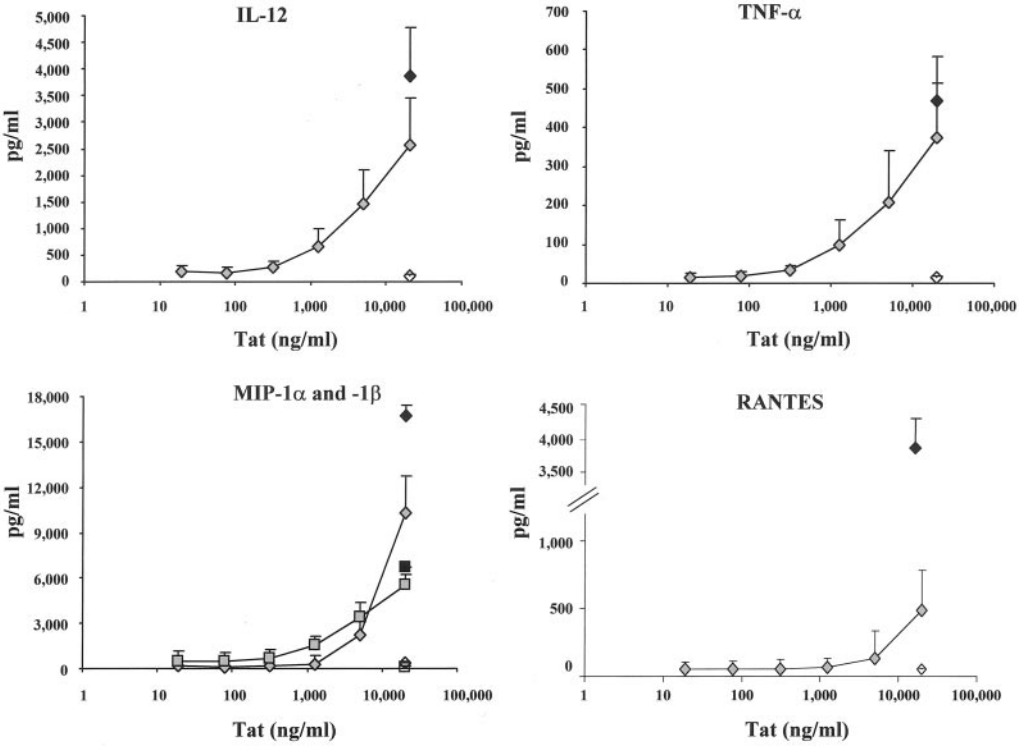

FIGURE 3. Tat enhances the production of the cytokines IL-12 and TNF-α and of the β chemokines MIP-1α, MIP-1β, and RANTES by MDDC. Supernatants of cells exposed for 18 h to serial concentrations of Tat (20–20,000 ng/ml), reconstitution buffer, complete medium, or LPS (positive control) were assayed for the levels of IL-12, TNF-α, MIP-1α (diamonds), MIP-1β (squares), and RANTES. Shaded, open, and filled symbols represent values from cells treated with Tat, buffer, or LPS, respectively. Data are reported as the mean values ($\pm$ SEM) from eight different donors and are expressed in picograms per milliliter. A very poor cytokine or β chemokine production was induced by oxidized/inactivated Tat protein (data not shown).

reconstitution buffer, or complete medium, and culturing them together with autologous lymphocytes in the presence of the recall Ag TT. As shown in Fig. 5, untreated MDDC induced TT-specific proliferation of autologous lymphocytes in two of the three donors analyzed (SI 15.4 and 7.3, respectively), and this effect was enhanced by their treatment with Tat (SI 27.9 and 12.5, respectively), but not with reconstitution buffer (SI 13 and 5.9, respectively). Tat-treated MDDC did not induce lymphocyte proliferation to TT in the subject who did not respond to the recall Ag. Thus, Tat can boost specific T cell responses to other Ag.

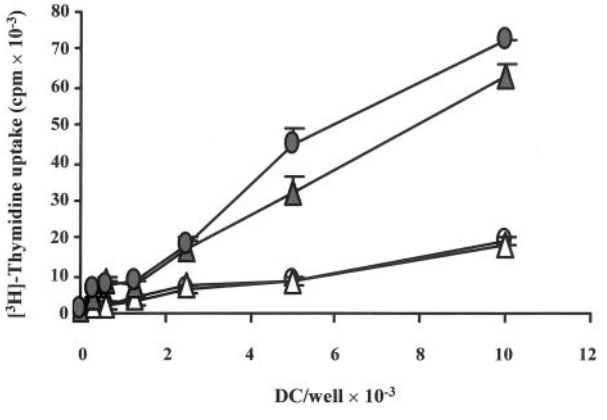

FIGURE 4. Tat enhances allogeneic Ag presentation by MDDC. MDDC were exposed for 18 h to complete medium (containing 5% FBS) (○), LPS (●), reconstitution buffer (△), or native Tat (10 μg/ml) (▲), and cultured together with allogeneic PBL. [^{3}H]Thymidine uptake was measured after 6 days of culture to evaluate lymphocyte proliferation. Data are from a representative experiment and have been reproduced with other three different donors. Means and SEM are reported.

Discussion

We and others have recently shown that vaccination with native Tat protein, *tat* DNA, or *tat* expression vectors protected monkeys against challenge with pathogenic viruses in animal models (20–23). In contrast, vaccination of monkeys with inactivated Tat protein or with Tat peptides induced only partial protection against challenge (25) or protection only against nonpathogenic viruses (24), which correlated with the presence of Ab responses and Th2 responses in the absence of CTL. In contrast, protection by vaccination with native Tat protein or *tat* DNA correlated with specific Th1 responses, including CTLs. In this regard, we observed that repeated inoculations of low doses (5–6 μg) of biologically active Tat given i.d. in the absence of adjuvant induced protection against virus challenge and Th1 responses with specific CTLs (20, 21) in the absence of a significant Ab production. These immunological results were recently confirmed in a new vaccination protocol in which native Tat alone was repeatedly inoculated i.d. in four monkeys (B. Ensoli, unpublished observations), and are comparable with those induced by i.m. vaccination with *tat* DNA in a published (22) and in an ongoing study (B. Ensoli, unpublished observations).

This suggested that the active Tat protein may target APC and drive Th1-type cellular responses, as occurs for an intracellularly expressed protein delivered by a DNA plasmid. This was also suggested by the known properties of active Tat protein or peptides to 1) enter cells of various types (4, 6–8, 26–28), 2) localize in the nuclei and *trans* activate virus or cellular gene expression upon cellular uptake (4, 6–8, 40), 3) bind via its RGD region to the $\alpha_v\beta_3$

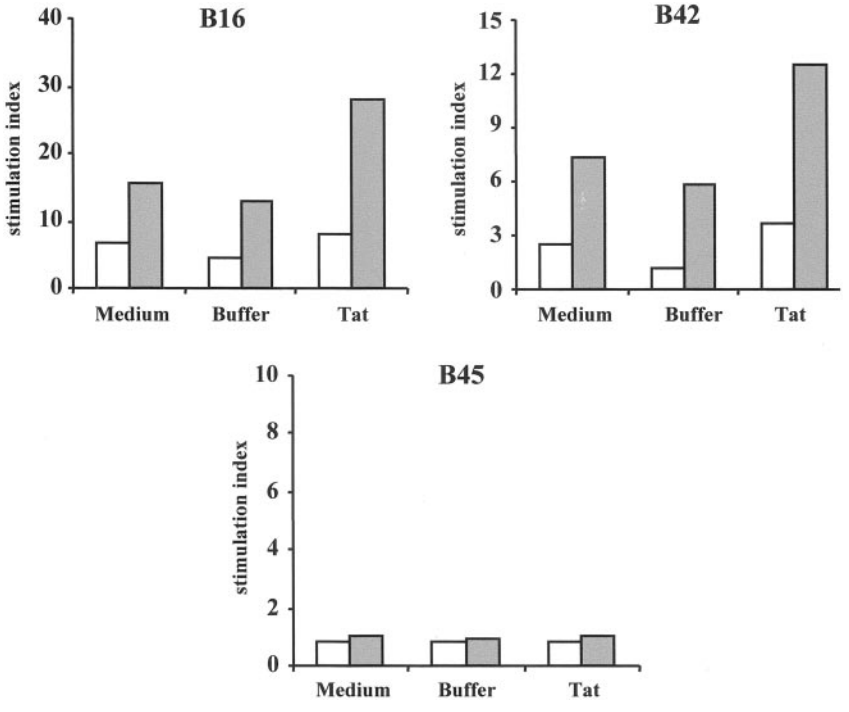

FIGURE 5. Tat increases TT-specific presentation by MDDC to primed PBL-enhancing specific T cell responses. MDDC from three healthy donors (B16, B42, and B45; two of them (B16, B42) responsive to TT in proliferation assays) were exposed for 18 h to native Tat (10 μg/ml), reconstitution buffer, or complete medium (containing 5% FBS), and cultured together with autologous lymphocytes (ratio 1:20) in the absence (open bars) or in the presence (filled bars) of TT (5 μg/ml). [^{3}H]Thymidine uptake was measured after 6 days of culture to evaluate lymphocyte proliferation. Values are expressed as SI (see *Materials and Methods*).

and $\alpha_5\beta_1$ integrins and via its basic region to heparan sulfate proteoglycans of activated endothelial cells that also function as APC (6, 32, 33, 41, 42), 4) be very efficiently taken up by activated endothelial cells expressing integrin receptors and much less efficiently by nonactivated cells (6, 7), 5) interact with DC and inhibit apoptotic bodies engulfment via the binding to the $\alpha_v\beta_3$ integrin (43, 44), 6) interact with monocytes, inducing an increase of proinflammatory cytokines and promoting their chemotactic function (45–47), and 7) be processed and presented by APC with MHC class I molecules (27, 28) and, therefore, induce a CTL response as other penetratins can do (29). Notably, a biologically active Tat was required to observe these effects.

Altogether these in vitro and in vivo data suggested that the effect of Tat on APC might vary according to its conformation and biological activity. Therefore, we analyzed the uptake of native or oxidized/inactivated Tat by different types of APC, including MDDC, BLCL, and TCB. Furthermore, we evaluated the effects of both native and oxidized/inactivated Tat on the function of MDDC, which are the most efficient among the APC, and key for the induction of antiviral immune responses (30, 31).

We first studied the capability of MDDC of specifically taking up soluble active Tat and found that this is very efficient, since most of the Tat is taken up by the cells very rapidly with a peak after 5–10 min, depending on the concentration of Tat given to the cells. In contrast, the uptake of active Tat by TCB or BLCL is very poor, requiring very high protein concentrations (10 μg/ml) and much longer time of incubation and, even under these conditions, most Tat remains bound to the cell surface and does not enter cells. Furthermore, active Tat is also rapidly processed by MDDC, as indicated by the reduction of the intracellular staining after 30 min of incubation.

Mature MDDC are able to take up Tat 10- to 100-fold more efficiently than immature cells, as indicated by the values of intracellular staining observed with low concentrations of Tat (100 ng/ml) as compared with those observed with 1 or 10 μg/ml Tat with immature cells. In addition, Tat uptake requires a native conformation and full biological activity of the protein. Oxidation of Tat by exposure to light and air abolishes or markedly reduces (by ~100-fold) the uptake observed with Tat by MDDC. Interestingly, the type of uptake by MDDC observed with oxidized Tat is similar or identical to that of native Tat with TCB and BLCL. Furthermore, at a temperature of 4°C, uptake of Tat by MDDC is markedly reduced at all the doses of the protein, and this is particularly evident at low Tat concentrations (0.1–100 ng/ml), suggesting that Tat uptake is an energy-dependent process.

Taken together, these data indicate that Tat targets MDDC, and they suggest that the selective and efficient uptake of Tat by MDDC is not mediated by the high pino/phagocytic activity of these cells, but it requires specialized uptake pathways that are selectively expressed by immature MDDC and at higher levels by mature MDDC. Furthermore, the different uptake observed at low vs high concentrations of Tat with MDDC indicates the presence of at least two different uptake pathways, the first one occurring at low Tat concentrations (up to 100 ng/ml) and the other one observed at higher Tat concentrations (>100 ng/ml). In this regard, the low temperature (4°C) reduced or abolished particularly the uptake of low doses of Tat (0.1–100 ng/ml), suggesting the involvement of a receptor-mediated endocytosis.

Of note, endothelial cells activated by IFN-γ, IL-1β, and TNF-α, but not nonactivated cells, bind and take up native Tat very efficiently and in fashion similar to MDDC (Refs. 7, 32, and 33, and data not shown). Tat uptake by endothelial cells occurs via the RGD domain of Tat that binds the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins (the classical receptors for vitronectin and fibronectin) and via the basic region of Tat that binds heparan sulfate proteoglycans of the cell surface and extracellular matrix (8, 32, 33, 35, 41, 42). In this respect, integrin antagonists block endothelial cell uptake of low (up to 100 ng/ml), but not high Tat concentrations (>1 μg/ml). Of interest, binding to the same integrins also mediates the effects of Tat on activated endothelial cells, and these effects are abolished by oxidation/inactivation of the protein, or by specific Ab, or by competitor peptides or ligands (6, 7, 32, 33, 35, 41). Preliminary data indicate that also for MDDC, the uptake of low concentrations

of Tat is blocked by specific anti-integrin mAb or competitor ligands, while that of high Tat concentrations is only partially reduced. This suggests that integrins mediate Tat entry into MDDC through a receptor-mediated endocytic pathway.

To investigate the effects of Tat on MDDC phenotype and function, cell viability, surface molecule expression, cytokine and β chemokine production, and Ag-presenting function were evaluated after 18 h of incubation of MDDC with Tat. A dose-dependent enhancement of the surface expression of HLA-ABC, HLA-DR, CD40, CD80, CD86, and CD83 on MDDC was found in most of the donors examined. However, this effect was observed with native, but not with oxidized, Tat. In addition, Tat induced a dose-dependent increase of the production of both IL-12 and TNF-α, cytokines essential for driving a Th1-type response (38), and of the β chemokines RANTES, MIP-1α, and MIP-1β, which are key players in the effector phase of the lymphocyte response (39). Importantly, in both cases, the levels were comparable with those induced by the known activator LPS. Again, oxidized Tat did not induce these effects.

Active Tat also enhanced the Ag-presenting function of MDDC, increasing the proliferative response of T cells to allogeneic and recall Ag.

Thus, active Tat is selectively and efficiently taken up and processed by MDDC, induces their maturation, and promotes their capacity of presenting Ag, eliciting immune responses toward a Th1 pattern, and increasing T cell responses to other Ag. Taken together, these properties indicate that active Tat is not only an Ag but also a potent T cell adjuvant.

Our data are in apparent contrast with reports showing that Tat exerts toxic and immunosuppressive effects (48–51). However, in most of these studies, high amounts of Tat were used, and quality controls of Tat composition, endotoxin content, biological activity, and specificity were not shown, hampering both interpretation and comparison of the data. In our hands, different lots of a GLP product of a monomeric form, fully active at picomolar concentrations and endotoxin free, have never exerted toxic and/or immunosuppressive effects in vitro (with primary endothelial cells, PBMC, MDDC, CD4 and CD8 T cells, T and B cell lines, fibroblasts) (5–8, 20, 22, 32, 33, 35, 41), nor in vivo after inoculation in nude mice or guinea pigs (33, 35), or after immunization in mice or monkeys (Refs. 20, 21, and 52, and B. Ensoli, unpublished observations). Similarly, no toxic effects have ever been observed in animals with AIDS (our unpublished observations) or after vaccination with *tat* DNA in monkeys or in HIV-1-infected humans (22, 23, 53), indicating that vaccination with Tat is safe. Moreover, no toxicity or immunosuppressive effects were observed by mucosal or systemic immunization of mice with Tat and heterologous Ag (B. Ensoli, unpublished observations).

The promoting effects of Tat on DC functions and its capability of being presented in association with MHC class I molecules may explain the type of immune response and the protective role exerted by vaccination with the soluble active Tat protein. Because the induction of Th1 responses and CTL correlates with reduced viral load also with Env-based vaccines, the data presented suggest that native Tat protein or *tat* DNA should be exploited to drive or to increase Th1 immune responses and CTL activity also against other HIV Ag to support an effective and long-lasting antiviral immunity capable of controlling virus replication and blocking disease onset.

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