Implications of the release of high-mobility group box 1 protein from dying cells during human immunodeficiency virus type 1 infection in vitro

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Plasma levels of high-mobility group box 1 protein (HMGB1) are elevated during the course of human immunodeficiency virus type 1 (HIV-1) infection and the molecule has an impact on virus replication. This study investigated the mode of cell death and release of HMGB1 during HIV-1 infection in vitro. MT4 cells and primary CD4 T cells were infected with HIV-1 isolates, and HMGB1 release was monitored in relation to cytopathic effects (CPE) and apoptosis. HMGB1 release from cells was analysed by Western blotting. For MT4 cells, an enzyme-linked immunosorbent spot (ELISPOT) assay was adapted to measure the release during necrosis. Lactate dehydrogenase (LDH) activity was quantified using a commercial assay. Flow cytometry was used to determine the level of infection and apoptosis. MT4 cells were ≥90 % infected at 48 h post-infection (p.i.). CPE was first observed at 60 h and correlated with release of HMGB1, LDH activity and caspase-3 (C3) activation. HMGB1 spots were clearly detected by ELISPOT assay at 72 h p.i. Annexin V and C3 staining showed that apoptosis was substantially involved in HIV-1-related cell death. Addition of Z-VAD (a caspase inhibitor) in a single dose at 24 or 40 h p.i. decreased both the number of caspase-positive cells and the release of HMGB1. Infection of primary CD4 T cells showed a 22 % (median) infection rate at 96 h. Related CPE corresponded to LDH and HMGB1 release. Both necrosis and apoptosis contributed to HMGB1 liberation during HIV-1-induced cell death and the protein could induce tumour necrosis factor-α release from peripheral mononuclear blood cells. These data imply that passive HMGB1 release contributes to the excessive immune activation characteristic of HIV-1 pathogenesis.

INTRODUCTION

High mobility group box 1 protein (HMGB1) is present ubiquitously in cell nuclei and plays a role in nucleosome stabilization and transcription processes (Dumitriu et al., 2005). The protein can be released into the extracellular matrix in either an active or a passive manner (Lotze & Tracey, 2005) to signal tissue injury and initiate inflammatory responses (Scaffidi et al., 2002). Thus, HMGB1 is liberated from necrotic cells, but it can also be secreted from activated macrophages (Bonaldi et al., 2003; Wang et al., 1999), endothelial cells and natural killer cells (Semino et al., 2005). Extracellular HMGB1 acts as a pro-inflammatory cytokine and is one of the main prototypes of the damage-associated molecular pattern molecules (Harris & Raucci, 2006). Suggested receptors for HMGB1 are RAGE (receptor for advanced glycation end products) and also Toll-like receptor 2 (TLR2) and TLR4 (Park et al., 2004). Elevated levels of HMGB1 have been described in a wide range of clinical conditions associated with acute and chronic inflammation (Alleva et al., 2005; Nowak et al., 2007; Yasuda et al., 2006). HMGB1 has also been proposed as a molecule that could have an impact on the pathogenesis of virus infections related to viral cytopathic effect (CPE) and to activation of the immune cells (Alleva et al., 2008; Chen et al., 2008; Chu & Ng, 2003; Kosai et al., 2008; Wang et al., 2006).

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We have shown previously that HMGB1 is involved in the immune activation that is a hallmark of human immunodeficiency virus type 1 (HIV-1) infection. We and others have shown that HMGB1, acting as a cytokine, can modulate HIV-1 replication in cells of monocytic origin (Cassetta et al., 2009; Nowak et al., 2006; Thierry et al., 2007). In addition, plasma levels of HMGB1 were found to be elevated during chronic HIV-1 infection, with the highest concentration in patients with clinical complications (Nowak et al., 2007).

The purpose of this study was to investigate the release of HMGB1 from HIV-1-infected cells in vitro and to elucidate this process in the context of apoptosis and necrosis in HIV-1 infection.

RESULTS

High level of infection in MT4 cells

MT4 cells were infected with the HIV-1 HXB2 isolate (HIV-1HXB2), and the level of infection was assessed by p24 staining. Flow cytometry revealed that, at 48 h post-infection (p.i.), more than 80 % of the cells resided within the viable population gate (Fig. 1a). At 72 h, two distinct cell populations were observed in the infected cultures, indicating an increased level of death of the infected cells (Fig. 1b). At 48 h after inoculation, more than 90 % of the gated cells were positive for HIV p24 antigen (Fig. 1c). CPE was visualized as the formation of syncytia and cell degradation, with the earliest CPE present at 60 h p.i. (Fig. 1d). Additionally, HIV-1 replication was confirmed by an increased level of reverse transcriptase (RT) activity in the culture supernatant from 48 to 72 h p.i. (data not shown).

Apoptotic cell death confirmed by staining of viable MT4 cells

To characterize the cell death further, infected and uninfected cells were stained with annexin V and 7-amino-actinomycin D (7-AAD). Cells positive for both annexin V and 7-AAD were characterized as non-viable due to primary or secondary necrosis. In two separate experiments, higher levels of doubly positive infected cells were found at 72 h (54 and 57 %, respectively; Fig. 2b, data from the second experiment not shown) compared with 48 h (9 and 13 %, respectively; Fig. 2a). This increase implied that the majority of the cells had lost their outer
membrane integrity at 72 h. A substantial apoptosis component in the death of the HIV-1-infected MT4 cells was also suggested by an increased number of annexin V-positive, 7-AAD-negative cells, corresponding to early apoptotic cells. Apoptosis was further confirmed by caspase 3 (C3) single staining with levels reaching 66 and 48 %, respectively, in duplicate experiments at 72 h compared with 48 h (19 and 15 %; Fig. 2a, b).

Fig. 2. Cell death and HMGB1 release in HIV-1-infected MT4 cells. (a, b) Flow cytometric analysis of annexin V/7-AAD (dot plots) and single staining of active C3 (histograms; uninfected controls shaded) expression in MT4 cells during HIV-1 infection. The figures demonstrate the differences between infected and uninfected cells at 48 (a) and 72 (b) h p.i. Two independent experiments were performed with similar results. Representative data from one experiment are shown. All analyses were performed on 20 000 live events. (c) Measurement of LDH activity in HIV-1HXB2-infected MT4 cell supernatants at 24, 48 and 72 h p.i. The LDH ratio of infected : uninfected supernatants increased with time and the means ± s of three independent experiments are shown. The increase was 0.2-fold from 24 to 48 h and 2-fold from 24 to 72 h. (d) Western blot analysis of supernatants and cell lysates from uninfected (−) and HIV-1 HXB2-infected (+) MT4 cells at 24–72 h p.i. Cell supernatants and lysates of MT4 cells were subjected to Tris/glycine 10–20 % gel electrophoresis and the blotted proteins were detected with anti-HMGB1 polyclonal antibody (1 μg ml⁻¹). In cell supernatants, HMGB1 was barely detectable at 48 h, but was clearly detected in infected cells at 72 h compared with uninfected cells. In infected and uninfected cell lysates at 48 h, the HMGB1 band was similarly weak, whilst at 72 h, infected cell remnants had less HMGB1 than uninfected cells. 3F/T, necrotic control (three freeze/thaw cycles); L, pre-stained SDS-PAGE standard. (e) ELISPOT assay visualizing passive release of HMGB1 from HIV-1-infected MT4 cells at 72 h p.i. Images in the upper row show HMGB1 spots in an infected well (+) and the lack of spots in a corresponding uninfected control (−). The lower left panel shows the results for an uninfected control where necrosis was elicited by osmotic damage (H₂O). The active release of HMGB1 from RAW 264.7 cells was captured 24 h after stimulation with LPS (10 μg ml⁻¹) (lower right panel). Each well contained 2500 cells. One representative experiment out of three is presented.
Increased lactate dehydrogenase (LDH) activity during the course of infection

The loss of plasma membrane integrity was determined by measuring the level of LDH activity in the supernatants. In three independent experiments, increased activity of cytosolic LDH over time was found in the supernatants from infected cells (24 h: 0.74, 0.89, 1.14; 48 h: 1.14, 0.96, 1.33; 72 h: 3.53, 2.13, 2.64), compared with uninfected cells, indicating LDH leakage following cell lysis (Fig. 2c). The increase in LDH was 0.2-fold from 24 to 48 h, and 2-fold from 24 to 72 h.

HIV-1 infection of MT4 cells induces HMGB1 release

HMGB1 was extracted from HIV-1HXB2-infected supernatants using heparin–Sepharose beads. The HMGB1 band was clearly detected by Western blotting at 72 h p.i. (Fig. 2d). The increasing amount of HMGB1 in the supernatants corresponded to the loss of the protein from the cell lysates (Fig. 2d). The liberation of HMGB1 during infection of MT4 cells was not due to the high level of infection found in our cell system, as similar results were reproduced with lower infectious doses of HIV-1HXB2. Furthermore, this process was independent of the cell type or virus strain, as similar results were obtained upon infection of Jurkat cells with the HIV-1NL4-3 isolate (data not shown). Enzyme-linked immunosorbent spot (ELISPOT) analysis demonstrated HMGB1 spots in the HIV-1-infected MT4 cells at 72 h p.i. (Fig. 2e). The analysis at the earlier time point (48 h p.i.) resulted in 5–10 spots per 1000 HIV-1-infected cells in the wells. At 72 h p.i. we could detect 40 ± 14 spots per 1000 infected cells compared with 6 ± 3 spots per 1000 mock-infected cells (mean ± SD, n = 3). Necrosis due to osmotic cell damage resulted in 234 ± 14 spots per 1000 cells. We also noticed that spots detected during passive HMGB1 release were larger and more diffuse compared with spots due to lipopolysaccharide (LPS) stimulation of RAW cells, consistent with earlier reports (Wahamaa et al., 2007).

Kinetics of HMGB1 release from MT4 cells

In order to further characterize the kinetics of HMGB1 release from MT4 cells, a more detailed investigation with expanded time points was carried out by Western blotting. The supernatants were collected pre-incubation and at 24, 48, 54, 60, 72 and 96 h p.i. HMGB1 release started at 54 h, was substantially higher at 60 h and increased thereafter in a time-dependent manner (Fig. 3a). The uninfected controls had only a weak signal compared with the corresponding infected supernatants. Densitometry analysis of the HMGB1 bands showed an increased ratio of infected : uninfected cells over time (48 h: ratio 1.3; 72 h: ratio 3.6; Fig. 3a, graph).

Elevated levels of C3 activity in MT4 cells

In five separate experiments, we followed the morphological changes of the MT4 cell population as defined by FCS/SSC as well as by C3 kinetics (p24/C3) in HIV-1-infected cells using flow cytometry. The infected cells slowly started to disintegrate at 48 h with a decreasing number of viable cells within the given gate (mean 82 %), and this decrease continued throughout infection at 54 h (mean 77 %), 60 h (mean 57 %) and 72 h (mean 48 %) (Fig. 3b). The mock-infected cells remained >86 % viable throughout the experiment. The vast majority of cells were HIV-1 infected (p24 positive) at 48 h p.i. and we could follow the increasing number of cells that were C3 positive at 54 h p.i. (48 h: mean 21 %; 72 h: mean 49 %, Fig. 3c), indicating a continuous apoptosis process corresponding to the cell morphological changes. Thereafter, the C3 activity remained stable (60 h: mean 47 %; 72 h: mean 51 %, Fig. 3c).

Z-Val-Ala-Asp(OMe)-CH$_2$F (Z-VAD) inhibits C3 activity and the release of HMGB1

In order to evaluate whether apoptosis and cell death in our model could be modulated, we treated MT4 cells with the broad-spectrum caspase inhibitor Z-VAD. Z-VAD inhibited the cell population changes in infected cells, as determined by flow cytometry. A single dose of Z-VAD prevented the disintegration of cells into two populations; instead, the cells remained within the viable cell gate at all time points: 54 h (mean 83 %), 60 h (mean 86 %) and 72 h (mean 78 %) (Fig. 4a). Moreover, C3 activity was inhibited in infected MT4 cells compared with non-Z-VAD-treated infected cells at 54 h (mean 12 %), 60 h (mean 15 %) and 72 h (mean 10 %) (Fig. 4b). Not surprisingly, Z-VAD treatment resulted in a reduction in LDH and RT activity in the corresponding supernatants (data not shown). Western blot analysis of supernatants collected at 48, 54, 60, 72 and 96 h showed that the release of HMGB1 was also substantially inhibited (40 ± 5 %; estimated by densitometry) after addition of Z-VAD at 24 or 40 h p.i. (Fig. 4c) compared with controls.

HIV-1-infected primary CD4$^+$ T cells release HMGB1

Acute infection of primary CD4$^+$ T cells was established with two of the three tested viral isolates. We were unable to infect primary cells using the HXB2 strain, which is a CXCR4 co-receptor-utilizing isolate adapted to inoculation of T-cell cultures. Both HIV-1$_{BAL}$ and HIV-1$_{HIV}$ viruses yielded infection and C3 activity in the four donors at 96 h p.i. (% p24 and C3; Fig. 5a); donor A (HIV-1$_{BAL}$): 22 and 9; donor B (HIV-1$_{BAL}$): 22 and 6; donor C (HIV-1$_{BAL}$): 45 and 15, (HIV-1$_{HIV}$) 43 and 13; donor D (HIV-1$_{BAL}$): 7 and 9, (HIV-1$_{HIV}$): 7 and 3. CPE could be seen by 48 h (HIV-1$_{BAL}$) and 72 h (HIV-1$_{HIV}$). We could detect HMGB1 in the supernatants of infected cells at 48 h p.i. compared with uninfected cells (HMGB1 band density ratio of infected : uninfected cells: 1.2–1.3). The latter started to leak HMGB1 at 72 h, although to a substantially lower extent than the infected cells, as shown by Western blots at
96 h p.i (ratio: 1.7–2.1) (Fig. 5b). The pattern of LDH release was similar to that observed with the MT4 cells (data not shown). In contrast to the results from the MT4 cell experiments, we did not notice a substantial increase in C3 activation in HIV-1-infected primary CD4+ T cells (mean 9%; Fig. 5a). Treatment with Z-VAD had an effect on C3 activation (Fig. 5a) but not on HMGB1 release from the primary cells (data not shown).
HMGB1 released from HIV-1-infected cells induces tumour necrosis factor (TNF-α) release from peripheral mononuclear blood cells (PBMCs)

The MT4 cell culture supernatants collected at 72 h after HIV-1 infection were tested for their effect on TNF-α secretion using human PBMCs. The HMGB1 concentration in the supernatants was estimated to exceed 10 mg ml⁻¹ by Western blotting. To address the issue of HMGB1 contribution to the pro-inflammatory effect, we depleted HMGB1 by immune precipitation using anti-HMGB1 monoclonal antibody 2G7 or heparin–Sepharose beads (Fig. 6a).

MT4 cell (HIV⁺) culture supernatants were able to induce TNF-α release from PBMCs (mean 1959 pg ml⁻¹) after overnight (16 h) stimulation compared with controls (164 pg ml⁻¹). This stimulatory effect was substantially decreased (mAb 2G7 680 pg ml⁻¹; heparin beads 241 pg ml⁻¹) but not totally reduced after immune depletion of HMGB1 (Fig. 6b). Moreover, pre-incubation of PBMCs with anti-RAGE antibodies for 1.5 h eliminated the stimulatory activity of added cell supernatant (555 pg ml⁻¹) in the same way as HMGB1 depletion. These experiments indicated that the extracellular milieu after HIV-1 infection contains an active pro-inflammatory form of HMGB1.

DISCUSSION

Immune activation has been accepted as the driving force of disease progression during HIV-1 infection. The cause of this phenomenon has been discussed, but is far from unicomponental (Douek et al., 2009; Hunt, 2007). The inflammatory milieu, created by the excessive immune activation, not only facilitates virus replication and spreading but is also responsible for the increased appearance of inflammatory conditions, such as athero-
sclerosis and osteoporosis in HIV-1-infected patients. By controlling virus replication with highly active antiretroviral therapy, immune activation can be decreased (Moanna et al., 2005).

We have proposed that HMGB1 contributes to immune activation in HIV-1-infected patients (Nowak et al., 2007).

This pro-inflammatory protein is present in plasma during HIV-1 infection and is capable of inducing virus replication and cytokine secretion (Nowak et al., 2006, 2007). The question of whether HMGB1 acts alone or in complexes with other molecules (such as bacterial products and cytokines) is a matter for investigation (Bianchi, 2009).

Although uncomplexed HMGB1 may have an inhibitory effect on HIV-1 replication (Cassetta et al., 2009), this molecule bound to LPS or CpG DNA shows potent pro-inflammatory and HIV-inductive effects. The fact that both bacterial products and HMGB1 are detected in the plasma of HIV-positive individuals correlating with disease progression provides a theoretical background for complex formation in vivo settings (Brenchley et al., 2006; Cassetta et al., 2009).

The present study clearly showed that infected T cells passively release HMGB1 during membrane disintegration and cell death in connection with HIV-1 replication. Both apoptosis and necrosis led to the liberation of HMGB1 during virus infection of MT4 cells. Hence, we could observe CPE, as well as detecting HMGB1 in an ELISPOT assay that is claimed to capture released HMGB1 only during the necrotic process (Wahamaa et al., 2007). As Z-VAD, a broad-spectrum caspase inhibitor, had a substantial inhibitory effect on cell death and HMGB1 release, the relationship to HIV-1-induced apoptosis was established.

The differences observed between HMGB1 release and the Z-VAD effect in the MT4 cell system and in primary CD4+ T cells can be explained by the differences in cell infectivity and cytopathogenicity of the HIV-1 strains used in these experiments. Additionally, we showed that HMGB1 present in cell supernatants had a biological activity, as extracts containing HMGB1 were able to induce TNF-α secretion from PBMCs.

The leakage of HMGB1 from virus-infected cells has been reported previously for West Nile virus and dengue virus as well as for herpes simplex virus type 2 in vitro infections.
and has been connected with necrosis (Chen et al., 2008; Chu & Ng, 2003; Gaillard et al., 2008). These findings are in line with the initial dogma that only necrosis leads to passive release of HMGB1 (Scaffidi et al., 2002). However, apoptosis can also lead to this leakage, as described recently (Bell et al., 2006; Qin et al., 2006). Under conditions of apoptotic clearance deficiency such as systemic lupus erythematosus, non-ingested apoptotic cells may undergo secondary necrosis leading to the release of HMGB1 in complexes with nucleosomes (Urbonaviciute et al., 2008). We presume that this can be the case not only under in vitro conditions but also for the in vivo situation of HIV-1 infection.

The process of cell death during HIV-1 infection involves both necrosis and apoptosis (Bolton et al., 2002; Gougeon, 2003; Plymale et al., 1999). The CD4+ T-cell loss can be due to direct destruction by the virus itself. However, HIV-1-induced bystander CD4+ T-cell killing is now recognized as central to immunodeficiency (Varbanov et al., 2006). This process is due to apoptosis that is at least partially driven by excessive inflammation. Moreover, necrosis that is a frequent condition in organs targeted by opportunistic diseases during AIDS could be an additional source of HMGB1 release during the late stages of HIV-1 disease.

In conclusion, we have shown that HMGB1 is passively released during cell death caused by HIV-1 infection and has a biological activity. The mechanism of release in our in vitro model may be at least partially due to apoptosis/secondary necrosis as implicated by the effect of caspase inhibitor. We postulate that passive release of HMGB1 and other damage-associated molecular pattern molecules during HIV-1 infection should be considered an interesting mechanism that connects cell death with immune activation not only at the systemic level but also in local compartments where virus replication occurs.

**METHODS**

**Cells.** The MT4 cell line (transformed with human T-cell lymphotropic virus type 1; MRC ARP017) was cultured in complete medium (RPMI 1640, 20 mM HEPES buffer without L-glutamine; Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS), 20 μg gentamicin ml-1, 2 mM L-glutamine and 0.1% sodium bicarbonate (Gibco).

CD4+ T cells were enriched by mixing 10 ml buffy coat from healthy blood donors with 1 ml RosetteSep Human CD4+ T Cell Enrichment Cocktail (Stemcell Technologies). The purity of the cells was determined by flow cytometry (CD4, CD45, CD3 and CD20; BD Biosciences). Cells were frozen in FCS and 10% DMSO or directly cultured. Briefly, T25 cell-culture flasks (Nunclon; Thermo Fisher Scientific) were pre-coated with a monoclonal anti-human CD3 antibody (10 μg ml-1, clone OKT3; Ortho Biotech) in PBS and incubated overnight at +4 °C. The following day, 10–15×10^6 cells were cultured in basic medium (RPMI 1640 supplemented with 10% FCS, 0.1% penicillin and streptomycin, 100 U interleukin-2 ml-1 (Novartis) and soluble monoclonal anti-human CD28 (2 μg ml-1; BD Biosciences)) at 37 °C. The level of cell activation was measured by flow cytometry (CD25 and CD69; BD Biosciences) before infection of the cells.

**HIV-1 infection of MT4 cells.** The MT4 cells were split 24 h before infection to obtain actively dividing cells and transferred to T75 Nunc A/S flasks (Thermo Fisher Scientific). On the day of infection, cells were washed and cell viability was assessed with trypan blue prior to stimulation. Cells (3×10^6 or 1.5×10^6) and 500 or 250 μl HIV-1 strain HXB2 [1.6×10^4 50% tissue culture infectious doses (TCID50) ml-1; MRC] were added to an infection tube (Nolato Cervo Group) to a total volume of 800 or 400 μl, respectively, and pre-incubated for 3 h. After pre-incubation, the cell pellet was resuspended and transferred to the wells of a 12-well plate (Corning) in a total volume of 2000 or 1000 μl. Culture supernatant was collected at the indicated time points.

The caspase inhibitor Z-VAD (Calbiochem) was added to the wells as a single dose at a final concentration of 50 μM. The optimal working concentration was determined empirically in a series of control experiments (data not shown).

**HIV-1 infection of CD4+ T cells.** Activated CD4+ T cells were washed and resuspended in basic medium. Cells (10–15×10^6) were counted and transferred to separate vials to be infected with HIV-1BAL (2.6×10^6 50% tissue culture infectious doses (TCID50) ml-1), HIV-1INN (1.75×10^5 50% tissue culture infectious doses (TCID50) ml-1), both from NIH AIDS Research and Reference Reagent Program) or the HXB2 isolate. Uninfected controls were prepared in parallel. One hundred microlitres of the HIV-1BAL and HIV-1INN strains and 1 ml HXB2 were pre-incubated with CD4+ T cells in basic medium for 3 h. Following pre-incubation, the vial contents were transferred to a T25 cell-culture flask and incubated for 24 h. Cells were then washed twice, dissolved in basic medium at a final concentration of 1×10^6 cells ml-1 and transferred to 12-well plates (Corning).

**HIV-1 p24 staining of MT4 cells and CD4+ T cells.** The level of HIV-1 infection in MT4 cells and CD4+ T cells was determined by intracellular staining for the HIV-1 p24 antigen. Cells were fixed using a Cytofix/Cytoperm kit (BD Biosciences Pharmingen) and stained with a phycoerythrin (PE)-conjugated anti-p24 antibody (clone KC57-RD1; Beckman Coulter). Analyses were performed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo (Treestar) or Cell Quest (BD) software.

**Microscopy of cell cultures.** HIV-1-induced CPE was monitored carefully throughout the course of infection by studying cell morphology under an optical microscope. Images were captured with an inverted Nikon Eclipse TS100 microscope (Nikon Coolpix 4500; Nikon Instruments). Necrotic cells were obtained by three cycles of freezing/thawing in complete medium.

**Detection of HMGB1 by Western blotting**

**Sample preparation.** Heparin–Sepharose CL-6B beads (GE Healthcare) were prepared as recommended by the manufacturer. The bead solution (100 μl) was transferred to a vial containing 800 μl clarified culture supernatant (20,000 g, 2 min). The mixture was incubated for 2 h at 4 °C and then washed twice at 5000 g for 1 min. Finally, 60 μl Laemmli sample buffer and β-mercaptoethanol (Bio-Rad) was added and the pellet was boiled at 95 °C for 5 min. Samples were either processed directly by separating on a 10–20% Tris/glycine gel (Invitrogen) or stored at -20 °C.

Cell suspensions containing 3×10^5 cells were thawed and washed twice. The cell pellets were dissolved in a mixture of 1× RIPA buffer with PBS (1:1) (Abdurahman et al., 2007) and passed through a
syringe (Microlane; BD Biosciences). The cell lysate was denatured in Laemmli sample buffer and boiled at 95 °C for 5 min. Samples were either used directly or stored at −20 °C.

**Western blotting.** Equal volumes of the above prepared samples and pre-stained standards (Bio-Rad) were resolved on 10–20 % Tris/glycine gels and transferred onto nitrocellulose or PVDF membranes (Invitrogen). The membranes were then incubated with rabbit anti-HMGB1 antibody (1 μg ml⁻¹; BD Biosciences Pharmingen) at 4 °C overnight. As a secondary antibody, horseradish peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare) was used at 1:20,000 dilution and incubated for 45 min at room temperature. The proteins were visualized using ECL reagents (GE Healthcare). The band density of HMGB1 in culture supernatants was evaluated using ImageJ software (http://rsb.info.nih.gov/ij/).

**HMGB1 ELISPOT assay.** An HMGB1 ELISPOT assay was performed as described by Wahamaa et al. (2007) with minor modifications. Briefly, the ELISPOT plate was pre-coated with mouse anti-HMGB1 monoclonal antibody 2G7 (20 μg ml⁻¹; a generous gift from Critical Therapeutics Inc., USA). HIV-1-infected MT4 cells as well as controls were added to the wells at 42 h p.i. Three different cell concentrations were used in duplicate for MT4 cells (mock and infected); 2500, 5000 and 10,000 cells/well. Each assay included a control of RAW cells seeded at a concentration of 2500 cells per well and stimulated with 10 μg LPS (Sigma) ml⁻¹ for 6 or 24 h. Necrosis controls were obtained by adding 150 μl sterile water to mock wells 4 h prior to detection. The cells were collected at time points corresponding to 48 and 72 h p.i. (separate plates) and the ELISPOT was continued as described previously (Wahamaa et al., 2007). The high- and low-density spots were analysed using an AID ELISPOT Reader System (ELISPOT 3.2.3; AID).

**Immune precipitation of HMGB1 from supernatants and stimulation of PBMCs.** Cell supernatants obtained after 72 h culture of HIV-1-infected MT4 cells (four independent experiments) were pooled. The fractions (400 μl) diluted with PBS (1:1) were incubated overnight at 4 °C with 4 μg 2G7 mAb or 100 μl heparin-Sepharose beads. A corresponding amount of IgG2b mAb (Sigma) was used as an isotype control. The complexes were removed from the supernatants by 2 h incubation with Protein A/G Plus-Agarose beads (Santa Cruz Biotechnology), followed by centrifugation as suggested by the manufacturer. PBMCs were purified by a standard Ficoll-Paque (GE Healthcare) centrifugation procedure and seeded in 96-well plates at a concentration of 4 × 10⁵ cells per well. Supernatant from infected MT4 cells (75 μl) or corresponding volumes of controls were added to the wells in duplicates. Where indicated, cells were pre-incubated with polyclonal anti-RAGE antibodies (2 μg ml⁻¹; Santa Cruz Biotechnology) for 1.5 h. LPS from Escherichia coli O55:B5 (Sigma) was added at 10 ng ml⁻¹. Cell culture supernatants were harvested after overnight (16 h) incubation and stored at −20 °C.

TNF-α concentration was evaluated using a human TNF-α ELISA kit (R&D Systems) according to the manufacturer’s recommendations.

**LDH assay.** The release of LDH into the supernatants was estimated using a CytoTox 96 assay system (Promega).

**HIV-1 RT activity assay.** HIV-1 replication was confirmed by analysing RT activity in supernatants using a Lentri RT Activity Assay kit (Cavidi).

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