High-mobility group box 1 protein induces HIV-1 expression from persistently infected cells

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Background: Necrosis is a frequent condition during AIDS, notably in organs targetted by opportunistic infections. Soluble factors released by necrotic cells are important for signalling cell damage, but little is known concerning their effect on HIV-1 replication. We focused on HMGB1, an abundant component of the chromatin that is released from necrotic cells and can act as a pro-inflammatory mediator.

Materials and methods: A native form of HMGB1 was obtained from necrotic Hela cells, whereas a purified recombinant HMGB1 was generated in *Escherichia coli*. ACH-2 and U1 cells were used as models of persistent HIV-1 infection in lymphocytes and monocytes. Reactivation from latency was also investigated *ex vivo* using peripheral blood mononuclear cells (PBMC) collected from HIV-1-infected patients controlled by HAART. HIV-1 expression was quantified by enzyme-linked immunosorbent assay, real-time reverse transcription–polymerase chain reaction and branched DNA techniques. Flow cytometry and blocking experiments were used to identify the receptor used by HMGB1. Chromatin immunoprecipitation was used to investigate long-terminal repeat activation upon stimulation by HMGB1.

Results: HMGB1 increased HIV-1 transcription in chronically infected cells, a process that did not require de-novo protein synthesis. HIV-1 induction relied on HMGB1 interaction with the receptor for advanced glycation end-products. The activation pathway involved p38 and extracellular signal-related kinase as well as nuclear factor kappa B binding to the HIV-1 promoter. Finally, HMGB1 reactivated HIV-1 from latently infected PBMC collected in aviraemic HIV-infected patients.

Conclusion: This work establishes for the first time a link between necrosis and HIV-1 replication, which involves HMGB1, a soluble mediator released by damaged cells.

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Introduction

In HIV-infected patients, HAART is effective at halting HIV-1 replication and delaying the progression of AIDS [1]. However, HAART is unable to eradicate HIV totally, because replication-competent viruses persist under therapy in an integrated latent form in several cell types and anatomical sites [2,3]. Resting memory CD4 T lymphocytes are considered to be the main long-term reservoir of HIV in patients controlled by HAART. These cells do not usually release virus unless they are exposed *ex vivo* to various stimuli, including mitogens [4–6], cytokines [7–9] or phorbol ester [10,11].

The factors that induce HIV reactivation from latently infected cells have yet to be fully identified *in vivo*. Several pro-inflammatory cytokines that can be produced notably in the context of opportunistic infections are known to promote HIV-1 expression. These include IL-1, IL-6, IL-12 or TNF-α. In addition, soluble factors released by

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necrotic cells can reach high local concentrations and are thought to be important for immune activation in response to cell damage [12]. However, little is known about their direct effects on HIV replication. This is of particular relevance in the context of AIDS because necrosis is a frequent condition in organs that are targeted by devastating opportunistic diseases. Furthermore, necrosis stimulates the production of pro-inflammatory signals [12] that may in turn increase HIV replication in infected cells [13].

Of a number of molecules released by necrotic cells, we were most interested in high-mobility group box 1 (HMGB1) protein for several reasons. Although HMGB1 was initially purified from calf thymus as an abundant component of the chromatin, it is now recognized as an important pro-inflammatory mediator. Most particularly, HMGB1 can be secreted by macrophages and monocytes stimulated by bacterial lipopolysaccharide, TNF-α or IL-1β. In this form, HMGB1 acts as a delayed pro-inflammatory molecule that plays a central role during lethal endotoxaemia and sepsis [14]. Extracellular HMGB1 induces a large panel of cellular responses, including the production of several pro-inflammatory molecules such as TNF-α, IL-1β and HMGB1 itself. A large amount of HMGB1 can also be passively released from necrotic cells, but not from apoptotic cells [15]. This form of HMGB1 also acts as a soluble mediator because it activates inflammation in response to cell injury [16]. A convergent set of data suggests that HMGB1 could directly affect HIV replication or reactivation in latently infected cells. First, the three known receptors for HMGB1, namely the receptor for advanced glycation end-products (RAGE) [17], and Toll-like receptor (TLR) 2 and 4 [18] are expressed on cells targeted by HIV, such as monocytes/macrophages [18–20], memory and activated CD4 T cells [21], and dendritic cells [22]. Second, the binding of HMGB1 to its receptors induces the activation of mitogen-activated protein kinases (MAPK) in some cells [23–26], which results in the nuclear translocation of nuclear factor kappa B (NF-κB), a major transcriptional activator of HIV-1 [27].

Persistently infected cell lines of monocytic (U1) or lymphocytic (ACH-2) lineages have been used to investigate the molecular mechanisms that control post-integration latency and viral reactivation [28]. We show here for the first time that a soluble extract derived from necrotic cells efficiently stimulates HIV-1 replication in U1 and ACH-2 cells, an effect that is mainly the result of HMGB1. Using a purified recombinant form of HMGB1, we demonstrate that HIV-1 reactivation is a direct consequence of RAGE–HMGB1 interaction and does not require de-novo production of secondary mediators. HIV-1 induction by HMGB1 requires activation of p38 and extracellular signal-related kinase (ERK) MAPK, as well as NF-κB. Finally, HMGB1 was effective at reactivating HIV *ex vivo* from primary peripheral blood mononuclear cells (PBMC) obtained from HIV-infected patients controlled by HAART for more than 4 years. Altogether, these results target HMGB1 as a potent inducer of HIV-1 reactivation, notably in the context of cell injury.

Materials and methods

Cells and reagents

ACH-2 and U1 were obtained from the AIDS Research and Reference Reagent Programme. Monocytes were isolated from healthy donors' PBMC after Ficoll–Paque gradient centrifugation, sheep red blood cells rosetting [29] and adhesion. Monocyte-derived macrophages were obtained by growing monocytes for 7 days in the presence of 10 ng/ml macrophage–colony-stimulating factor. Monocytes, monocyte-derived macrophages, U1 and ACH-2 cells were maintained in RPMI medium (Gibco, Eragny, France) supplemented with 10% fetal calf serum, glutamine and antibiotics. Lipopolysaccharide, IL-1β and cycloheximide were obtained from Sigma (Lyon, France). Specific inhibitors of JNK (JNK-i: SP600125, 100 nmol), p38 (p38-i: SB203580, 1 μmol), ERK (ERK-i: PD98059, 10 μmol) and NF-κB (aurin-tricarboxylic acid; ATA, 200 μmol) were purchased from Sigma. HMGB1 immune serum S004 was obtained after rabbit immunization against the peptide PDAAKKGVVKAEK (167–180).

Necrotic cell extracts

HeLa cells (ATCC CCL2) were lysed by three cycles of freezing and thawing in phosphate-buffered saline (10 million cells per ml) [15]. Cell debris was pelleted by centrifugation and the supernatant was passed through a 0.2 μm membrane filter. The amount of HMGB1 in necrotic extracts, assessed by immunoblot was 7 μg per ml (or per 10^7 cells). HMGB1 depletion was performed by incubating 0.5 ml of necrotic cell supernatant with 100 μl anti-HMGB1 serum for 16 h at 4°C. Pre-immune serum was used as a control. Immune complexes were removed by incubation in the presence of 75 μl protein G sepharose for 1 h at 4°C under agitation.

HIV-1 monitoring

p24 antigen and viral genomic RNA were respectively measured by enzyme-linked immunosorbent assay (ELISA; HIV-1 p24 Innogenetics ELISA Kit; Ingen, Rungis, France) and the branched DNA technique (Versant HIV-1 RNA 3.0 assay; Bayer, Puteaux, France), according to the manufacturer's procedure. A real-time reverse transcription–polymerase chain reaction (PCR) assay was used to quantify multiply spliced and unspliced viral RNA, as previously described [30].

Purification of the recombinant human HMGB1 protein

Recombinant human HMGB1 was purified from *Escherichia coli* BL21(DE3) pLysS transformed with

pET15b-6His-HMGB1. The expression vector encoded for the full-length human HMGB1 fused to a poly-histidine tag at its N-terminus. Protein expression and purification were performed as previously described [31]. The removal of contaminanting bacterial lipopolysaccharide was performed using Triton X-114, as described [31]. Residual lipopolysaccharide, quantified using the E-toxate assay (Sigma) was less than 100 fg per microgram rhHMGB1.

Immunoblotting assays

Total proteins were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond nitrocellulose; Amersham Biosciences, Saclay, France). Membranes were incubated for 1 h with blocking buffer (Tris-buffered saline, 0.2% Tween, 5% milk), washed and incubated for 1 h with a 1 : 2000 dilution of anti-HMGB1 serum or a 1 : 400 dilution of anti-RAGE antibodies (Santa Cruz, Heidelberg, Germany). Detection of the primary antibodies was performed using a 1 : 2000 dilution of a donkey anti-rabbit immunoglobulin-horseradish peroxidase conjugate (Amersham). Immune complexes were detected using ECL detection reagents (Amersham).

Measurement of TNF-α production

TNF-α release was measured using the human TNF-α ELISA kit (Peprotech, London,UK) according to the manufacturer's procedure.

Fluorescence-activated cell sorter analysis of HMGB1 membrane receptors

Direct immunofluorescence was performed using phycoerythrin-conjugated anti-TLR-2 and FITC-conjugated anti-TLR-4. RAGE detection was performed using a rabbit-anti-RAGE and an FITC-conjugated goat-anti-rabbit antibody. All antibodies were purchased from Santa Cruz. The expression of cell surface molecules was analysed by flow cytometry (FACSCalibur; Becton Dickinson, Le Pont de Claix, France).

Chromatin immunoprecipitation assay and nuc-1 analysis

The chromatin immunoprecipitation assay was described previously [30], using an antibody against NF-κB p50 (Santa Cruz SC114X). A non-immune rabbit IgG was used as a negative control. The immunoprecipitated as well as the input DNA were subjected to quantification by real-time PCR in the nuc-1 region of the HIV-1 long-terminal repeat (LTR) [30]. Results are expressed as the fraction of immunoprecipitated DNA for each condition.

Patients and ex-vivo experiments

Six HIV-1-infected volunteers under antiretroviral therapy were selected for good treatment compliance and a viral load of less than 50 copies/ml for more than 4 years. These patients had received HAART for at least 5 years, a treatment that was unmodified for more than one year. The antiretroviral therapy associates two nucleoside analogue reverse-transcriptase inhibitors and either a non-nucleoside analogue reverse-transcriptase inhibitor or a protease inhibitor. The median CD4 T-cell count of the volunteers was 723 cells/μl (minimum 419; maximum 961). After informed consent, 50 ml peripheral blood were drawn and PBMC were isolated by Ficoll gradient centrifugation. HIV-1 cultures were performed from 2.10^6 CD8 depleted (Miltenyi Biotec, Paris, France) PBMC, in the presence or absence of recombinant HMGB1. Cultures were maintained for 35 days in RPMI medium supplemented with 10% fetal calf serum, glutamine and antibiotics, with or without IL-2 (50 ng/ml; Roche, Meylan, France). Half medium was renewed every week. Cells activated by phytohaemagglutinin A (PHA) (2.5 μg/ml; Sigma) and IL-2 were used as a positive control for HIV-1 outgrowth. HIV-1 reactivation was monitored every week by quantification of HIV RNA in cell culture supernatants, using the bDNA technique.

Statistical evaluation

Unless specified, values are expressed as the mean of at least three independent experiments. Confidence intervals are presented in the figures, with a P value of less than 0.05 or less than 0.01 as indicated.

Results

HMGB1 released from necrotic cells activates HIV-1 expression

To determine the impact of an endogenous signal associated with cell injury on HIV-1 expression, we generated soluble necrotic extracts from HeLa cells. Western blot analysis confirmed the presence of large amounts of HMGB1 in these extracts (Fig. 1a). HMGB1-depleted extracts were obtained by two rounds of immunodepletion using specific anti-HMGB1 antibodies.

HMGB1 has previously been shown to activate TNF-α secretion from macrophages and monocytes [32]. To evaluate the cytokine activity of HMGB1 derived from necrotic extracts, HMGB1-depleted and mock-depleted necrotic extracts were assayed for their ability to induce TNF-α release from human primary monocytes. As shown in Figure 1b, necrotic extracts exhibited a strong activity that was dramatically reduced, but not totally abrogated, after immunodepletion with anti-HMGB1 antibodies. These experiments confirmed that necrotic extracts contained an active, pro-inflammatory form of HMGB1, as well as other pro-inflammatory molecules.

ACH-2 and U1 cells are widely used to investigate HIV-1 postintegration latency in T and monocytic cells,

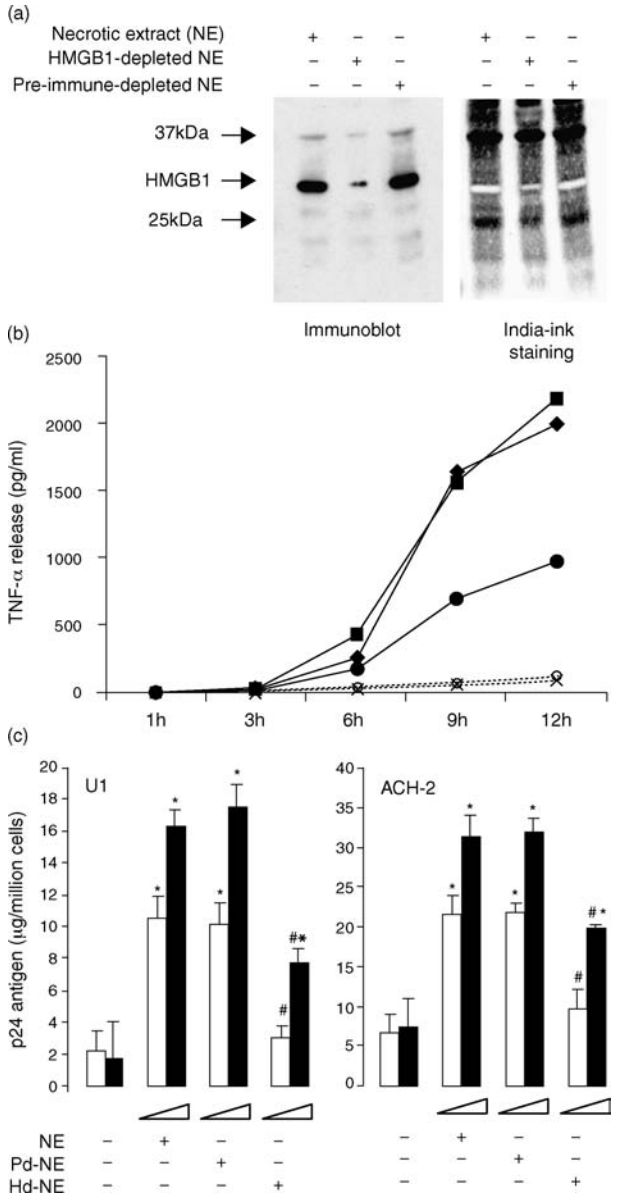

Fig. 1. HMGB1 released from necrotic cells induces HIV-1 expression in chronically infected cells. (a) Soluble necrotic extracts (NE) were subjected to immunodepletion using specific anti-HMGB1 antibodies or the corresponding pre-immune serum. The amount of HMGB1 in NE, assessed before immunodepletion by immunoblot was 7 μg per ml (not shown). The presence of HMGB1 in untreated, HMGB1-depleted or pre-immune-treated necrotic extracts (10 μl or 10^5 eq cells per lane) was assessed by immunoblot and India-ink staining. (b) Primary human monocytes were exposed to necrotic extracts (150 μl) depleted or not with anti-HMGB1 or pre-immune serum. TNF-α secretion was evaluated by ELISA at different timepoints. –×– Untreated; –○– phosphate-buffered saline; —■— NE; —◆— pre-immune-depleted NE; —●— HMGB1-depleted NE. (c) U1 and ACH-2 cells were exposed to increasing amounts of necrotic extracts (NE), depleted with anti-HMGB1 (Hd-NE) or with pre-immune serum (Pd-NE). Before immunodepletion, the concentration of HMGB1 in culture medium was 200 ng/ml (□) and 1 μg/ml (■). The release of p24 antigen was measured by enzyme-linked immunosorbent assay in cell culture supernatant after 72 h. Data are expressed as the mean of p24 antigen by million cells, obtained from three independent experiments. Confidence interval analysis is shown. $^*P < 0.05$ compared with non-stimulated cells; $^\#P < 0.05$ compared with cells stimulated with non-depleted NE.

respectively. They both exhibit a low level of basal HIV-1 expression that can be increased by various chemical or biological agents. ACH-2 and U1 cells were cultivated in the presence of HMGB1-depleted or undepleted necrotic extracts. As indicated in Figure 1c a strong increase in p24 antigen production was observed when these cells were exposed to undepleted necrotic extracts. Again, this effect was dramatically reduced, but not totally abrogated, when the extracts were first immunodepleted with specific anti-HMGB1 antibodies. Therefore our results demonstrated that HMGB1 derived from necrotic extracts activated HIV-1 expression in ACH-2 and U1 cells, although other cellular components may also contribute to viral reactivation.

Activation of HIV-1 by HMGB1 is mediated by RAGE

To confirm that HMGB1 alone was able to induce HIV-1 in ACH-2 and U1 cells, a recombinant form of human HMGB1 (rhHMGB1) was produced and purified from *E. coli*. rhHMGB1 was biologically active as it could stimulate TNF-α release from primary human monocytes as previously described (data not shown) [31,32].

To test whether purified HMGB1 could activate HIV-1 expression in U1 and ACH-2, these cells were incubated for 24 or 72 h with increasing amounts of rhHMGB1, and viral expression was quantified by measuring intra and extracellular p24 antigen. A dose-dependent increase in the production of p24 antigen was observed in both U1 and ACH2 cells with HMGB1 concentrations ranging from 100 ng/ml to 10 μg/ml (Fig. 2a), demonstrating that a recombinant purified form of HMGB1 was able to activate HIV-1 replication in these cells. Importantly, similar concentrations are known to be active on monocytes/macrophages [31,32], dendritic cells [33,34], smooth muscle cells [35,36], endothelial cells [26] or enterocytic cells [24].

Three receptors for HMGB1 have been described, namely RAGE, TLR-2 and TLR-4 [17,18]. Their presence at the surface of ACH-2 and U1 cells was first investigated by flow cytometry analysis (Fig. 2b). RAGE was the only receptor to be significantly expressed at the surface of U1 and ACH-2 cells (72.5 and 45.2 % of the cells, respectively). Western blot analysis confirmed

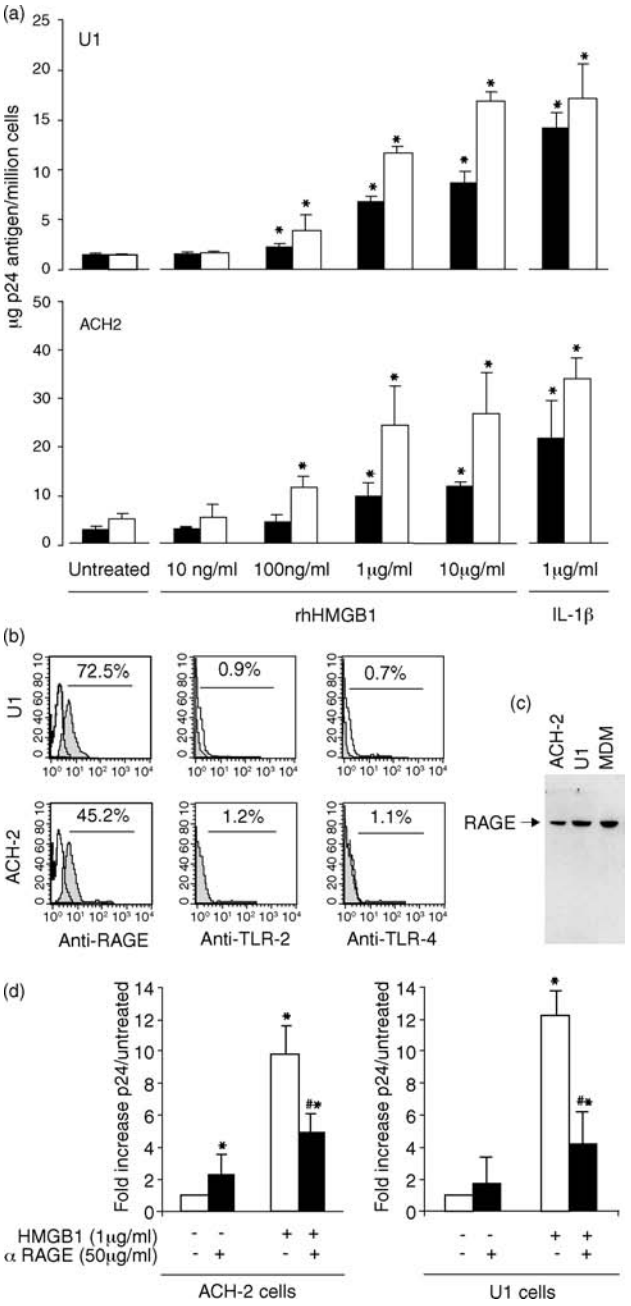

Fig. 2. HMGB1–RAGE interaction is required to induce HIV-1 expression. (a) U1 and ACH-2 cells were exposed to increasing amounts of recombinant human HMGB1. Intracellular and secreted HIV-1 p24 antigen was measured at 24 and 72 h, respectively. HIV induction by IL-1β was used as a positive control. Data are expressed as the average amount of p24 antigen per million cells, obtained from three independent experiments. Confidence interval analysis is shown ($^{*}P < 0.01$). □ Secreted p24 antigen; ■ intracellular p24 antigen. (b) Flow cytometry analysis was performed to detect Toll-like receptor (TLR) 2, TLR-4 and the receptor for advanced glycation end-products (RAGE) at the surface of U1 and ACH-2 cells. The shaded histograms represent the binding of anti-TLR-2, anti-TLR-4 and anti-RAGE, and open histograms show binding of the respective isotype controls. (c) RAGE expression was detected by immunoblot (40 µg total protein extract per lane) in U1, ACH-2, and human monocyte-derived macrophages (MDM). (d) U1 or ACH-2 cells were incubated with 50 µg/ml anti-RAGE antibodies and then exposed to 1 µg/ml rhHMGB1. Intracellular p24 antigen was measured by enzyme-linked immunosorbent assay after 24 h. Data are expressed as the fold increase in p24 antigen compared with control cells, treated with an unrelated isotype control antibody. Data were obtained from three independent experiments and confidence interval analysis is shown. $^{*}P < 0.01$ compared with non-stimulated cells; $^{\#}P < 0.01$ compared with HMGB1-stimulated cells pre-incubated with control antibody.

that anti-RAGE antibodies recognized a single polypeptide of the expected mobility (Fig. 2c).

To confirm that the HMGB1-dependent activation of HIV-1 was mediated through RAGE, ACH-2 and U1 cells were incubated with anti-RAGE or irrelevant antibodies before being exposed to rhHMGB1. As shown in Figure 2d, the activation of HIV-1 by HMGB1 was inhibited by anti-RAGE antibodies, which demonstrated that the HMGB1-dependent activation of HIV-1 was mainly, if not exclusively, caused by its interaction with RAGE in U1 and ACH-2 cells.

HMGB1 activates HIV-1 transcription without de-novo protein synthesis

In macrophages and monocytes, HMGB1 stimulates the release of pro-inflammatory cytokines, such as TNF-α or IL-1β, that are known to induce HIV replication [32]. Therefore, we wondered whether the activation of HIV-1 expression by HMGB1 was direct or if it was part of an autocrine/paracrine loop mediated by cytokines produced in response to HMGB1.

We first quantified TNF-α release from ACH-2 and U1 cells exposed to HMGB1. Whereas ACH-2 cells did not secrete detectable amount of TNF-α, U1 cells exhibited a time-dependent release of TNF-α in response to HMGB1. Importantly, the addition of the protein synthesis inhibitor cycloheximide completely abrogated the production of TNF-α, whether or not the cells were treated with HMGB1 (Fig. 3a). Then, we quantified the transcription of multiply spliced and unspliced viral RNA in cells incubated with HMGB1 in the presence or in the absence of cycloheximide. A dramatic increase of both RNA species was observed from 2 to 8 h after the addition of rhHMGB1 (Fig. 3b). Once again, this transcription pattern was not modified by cycloheximide, which demonstrated that the transcriptional activation of HIV-1 by HMGB1 did not require the synthesis of cellular cytokines and therefore occurred through a direct activation pathway.

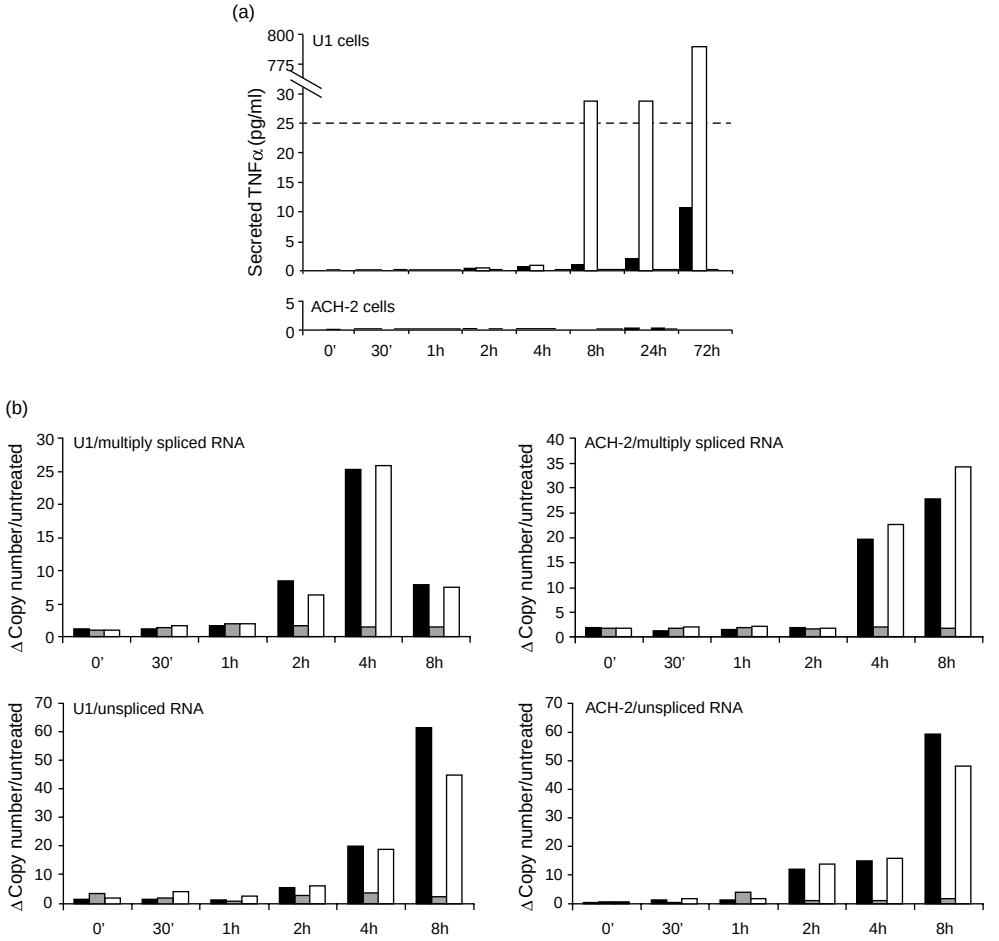

Fig. 3. Induction of HIV-1 by HMGB1 does not require de-novo protein synthesis. (a) TNF-α secretion was quantified at several timepoints after the incubation of U1 and ACH-2 cells with 1 μg/ml recombinant human HMGB1, in the absence or presence of cycloheximide (10 μmol). ■ Untreated; □ rhHMGB1; ▨ cycloheximide; ▩ rhHMGB1 plus cycloheximide. (b) Kinetics of HIV-1 multiply-spliced and unspliced transcripts after rhHMGB1 (1 μg/ml) induction were analysed in the absence or presence of cycloheximide. Real-time reverse transcription–polymerase chain reaction was performed at various times after induction, and results are expressed as the ratio of copy numbers, compared with untreated negative controls. Results are representative of two independent experiments. ■ rhHMGB1; ▨ cycloheximide; □ rhHMGB1 plus cycloheximide.

HMGB1-mediated induction of HIV-1 involves p38 and extracellular signal-related kinase, and nuclear factor kappa B activation

The HMGB1–RAGE interaction has previously been shown to activate a transduction pathway involving several MAPK and NF-κB [23,25]. To evaluate the relative contribution of three important MAPK, respectively JNK, p38 and ERK, U1 and ACH-2 cells were exposed to rhHMGB1 in the presence of specific inhibitors [37,38], respectively, JNK-i, p38-i and ERK-i. As shown in Fig. 4a, p38-i and ERK-i completely abrogated the induction of HIV by rhHMGB1, whereas JNK-i only induced a partial reduction in HIV-1 activation. A similar pattern was observed when the viral activation was induced by IL-1β, a known activator of MAPK (51–52). In contrast, the direct activation of HIV-1 LTR by the histone deacetylase inhibitor trichostatin A [39] was unaffected by the MAPK inhibitors.

We subsequently analysed NF-κB binding to the HIV-1 promoter LTR upon cell stimulation by HMGB1. Two NF-κB binding sites have been mapped in the LTR close to nuc-1, a nucleosome that plays a central role in the control of LTR-dependent transcription [30,40,41]. We previously designed a chromatin immunoprecipitation assay to investigate the binding of NF-κB p50 to this region [30]. As shown in Figure 4b, a four to eightfold increase in NF-κB binding to nuc-1 was observed in cells treated with HMGB1 compared with controls. The recruitment of NF-κB was directly associated with HMGB1 activity because it was also observed in the presence of cycloheximide.

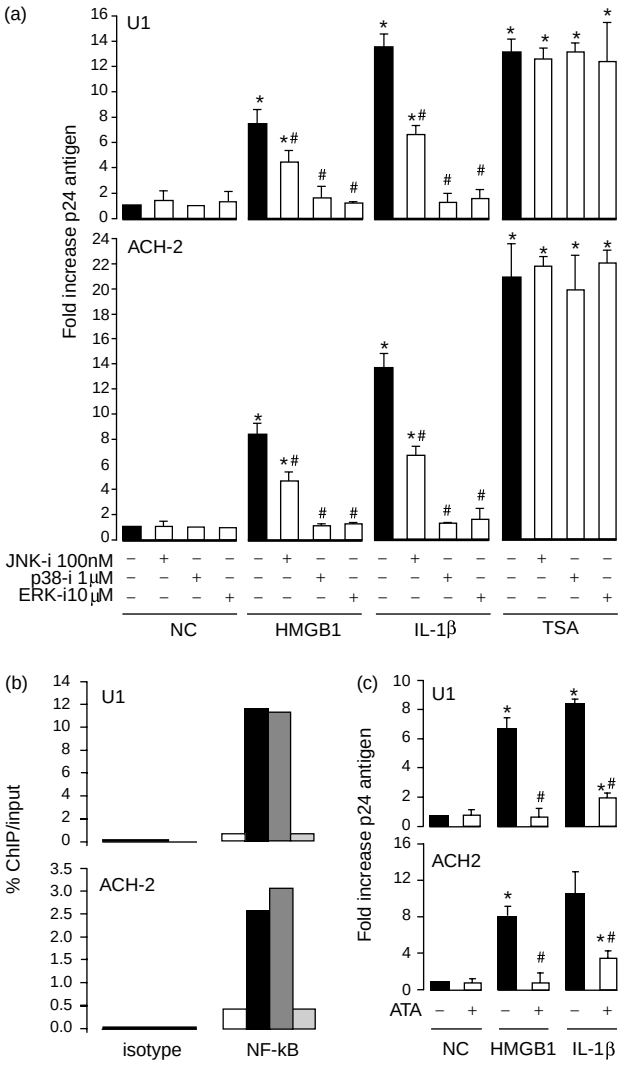

Fig. 4. p38, extracellular signal-related kinase and nuclear factor kappa B are required for HIV-1 induction by HMGB1. (a) Specific inhibitors of the mitogen-activated protein kinases (MAPK) JNK, p38 and extracellular signal-related kinase (ERK) were added to U1 and ACH-2 cells, 1 h before HIV-1 induction by recombinant human HMGB1 (1 μg/ml), IL-1β (1 ng/ml) or trichostatin A (TSA; 1 ng/ml). Intracellular p24 antigen was measured by enzyme-linked immunosorbent assay (ELISA) after 24 h. Data are expressed as the fold increase of p24 antigen compared with untreated cells (NC). Results are the mean of three independent experiments. Confidence interval analysis is shown. $^{*}P < 0.05$ compared with NC; $^{\#}P < 0.05$ compared with HMGB1-induced cells without inhibitors. (b) U1 and ACH-2 cells were incubated for 4 h with rhHMGB1 (1 μg/ml) in the presence or absence of cycloheximide (10 μM). Formaldehyde crosslinked chromatin was subjected to chromatin immunoprecipitation analysis, using a specific antibody directed against anti-p50 nuclear factor kappa B (NF-κB) or an irrelevant control antibody. One-tenth of the input and the immunoprecipitated chromatin were analysed by quantitative real-time polymerase chain reaction using primers located in the nuc-1 region of the 5′long-terminal repeat HIV-1 promoter. Results are expressed for each condition as the fraction of immunoprecipitated chromatin. □ Negative control; ■ HMG; ▩ HMG plus cycloheximide; ▨ cycloheximide. (c) U1 and ACH-2 cells were exposed to rhHMGB1 or IL-1β in the presence or absence of 200 μM aurin-tricarboxylic acid (ATA). Intracellular p24 antigen was measured by ELISA after 24 h. Data are expressed as the fold increase of p24 antigen compared with untreated cells (NC). Results are the mean of three independent experiments. Confidence interval analysis is shown. $^{*}P < 0.05$ compared with NC; $^{\#}P < 0.05$ compared with HMGB1-induced cells without ATA.

To confirm the involvement of NF-κB in the proviral activation induced by HMGB1, ACH-2 and U1 cells were exposed to rhHMGB1 in the presence of ATA, a chemical inhibitor of NF-κB [42]. IL-1β was used as a positive control because it is known to activate HIV-1 expression through a NF-κB-dependent pathway [40]. As illustrated in Figure 4c, ATA greatly reduced the activation of HIV by IL-1β and totally abrogated HIV induction in rhHMGB1-treated cells.

Altogether these experiments demonstrated that ERK, p38 and NF-κB play a crucial role in the molecular events that promote provirus expression in U1 and ACH-2 cells after exposure to HMGB1.

HMGB1 induces HIV-1 outgrowth from peripheral blood mononuclear cells collected in aviraemic HIV-infected patients controlled by HAART

The ability of HMGB1 to induce the replication of quiescent HIV was finally analysed *ex vivo* in PBMC from HIV-infected individuals efficiently controlled by HAART. Six patients were selected because they had been aviraemic for more than 4 years under unmodified HAART. Two million CD8-depleted PBMC were cultured in various conditions for a 5-week period (Fig. 5). In the absence of PHA or HMGB1, no viral outgrowth was detected, except in one case (patient 4) in which a moderate HIV production was observed (less than 200 copies/ml) after 4 weeks of culture in the presence of IL-2. Stimulation with PHA induced the recovery of replication-competent virus for five out of six patients' PBMC. Importantly, HMGB1 was at least as efficient as PHA at reactivating HIV because it induced virus outgrowth for five out of six PBMC cultures, with a viral production similar (patients 4, 5 and 6) or even higher (patients 2 and 3) than that observed with PHA-treated cells. Interestingly, HMGB1 could also reactivate HIV-1 in the absence of IL-2 (patients 2, 3, 5 and 6).

Discussion

Cell necrosis delivers endogenous danger signals that are critical for initiating the pro-inflammatory process as well

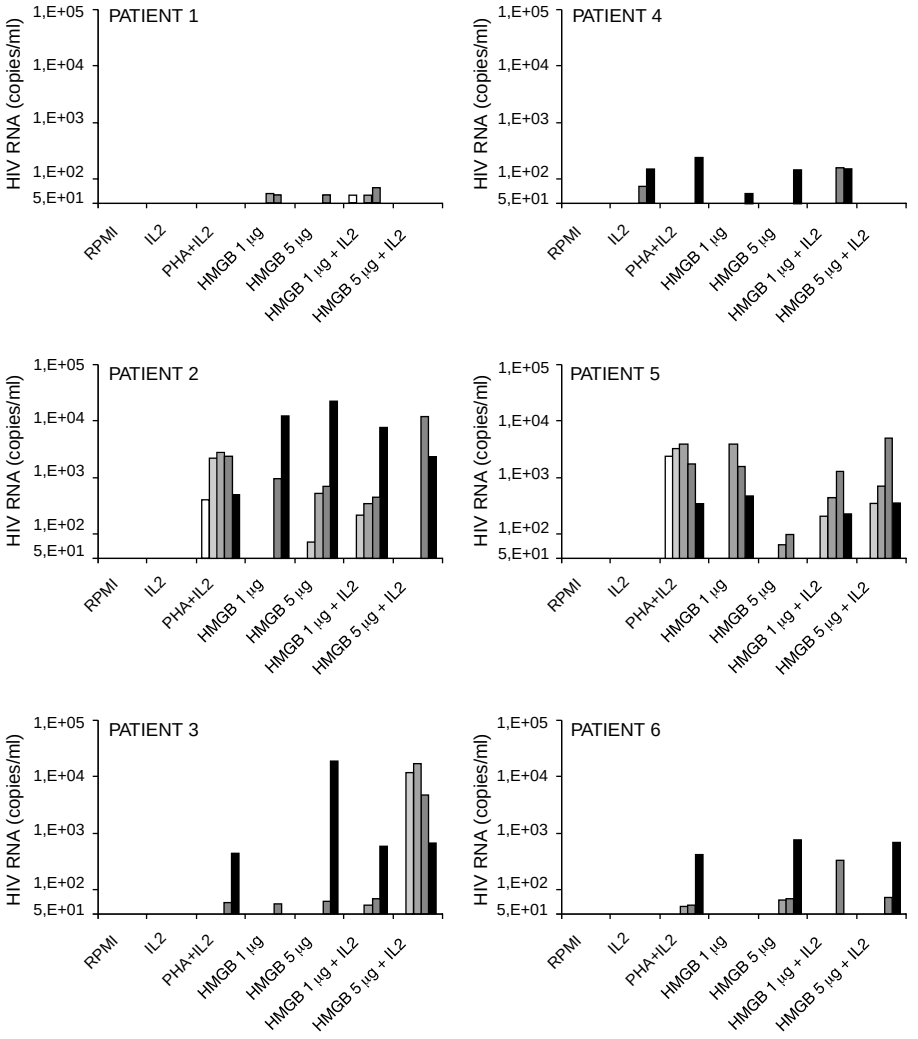

Fig. 5. HIV recovery by HMGB1 in peripheral blood mononuclear cells from aviraemic patients controlled by HAART. Two million CD8-depleted peripheral blood mononuclear cells were cultured for 35 days in various conditions, as indicated in the figure. HIV-1 outgrowth was evaluated every week by quantification of viral RNA in the cell supernatant, using a branched DNA technique. PHA, Phytohaemagglutinin A. □ day 8; ■ day 14; □ day 21; □ day 28; ■ day 35.

as stimulating immune response. We show here that signals associated with cell injury also efficiently activate HIV-1 expression in persistently infected cells. This effect was largely, although not exclusively, a result of the release of HMGB1 protein by necrotic cells. HMGB1 is secreted by various cells in response to lipopolysaccharide or pro-inflammatory cytokines, but its passive release by necrotic cells could provide a far larger source for its activity *in vivo*. The minimal concentration (100 ng/ml) required to activate HIV-1 expression in U1 and ACH-2 cells is in the upper range of the concentrations of HMGB1 that have been measured in the serum during sepsis [43]. However, much higher concentrations can be reached in the context of local inflammation or in damaged tissues, as recently reported in the lung (20 µg/ml in epithelial lining fluid) [44].

Nowak and colleagues [45] recently reported that HMGB1 reactivated HIV-1 in U1 but not in ACH-2 cells. This apparent discrepancy could partly be explained by the high basal level of HIV-1 expression in the ACH-2 clone used in their study compared with U1 cells.

Using fluorescence-activated cell sorter analysis and blocking experiments, we demonstrated that HIV-1 activation by HMGB1 was mediated by RAGE in U1 and ACH-2 cells. However, the activation of TLR-2-dependent pathways by HMGB1 may also be important *in vivo*, because many target cells for HIV, including monocytes [46] and memory CD4 T lymphocytes [21], express this receptor. The activation of TLR-2 by HMGB1 is likely to induce HIV-1 because stimulation of this receptor with bacterial antigens activates HIV-1 replication [47].

Previous studies have provided evidence that HIV-1 expression induced by phorbol myristate acetate in ACH-2 and U1 cells resulted at least partly from the stimulation of TNF-α release by phorbol myristate acetate [48,49]. Similarly, it was important to determine whether HMGB1 activity resulted from the direct activation of RAGE-dependent pathways or whether it relied on the production of secondary cytokines, including TNF-α. Although TNF-α was secreted in response to HMGB1, at least in U1 cells, HIV-1 transcriptional activation was unaffected by cycloheximide in HMGB1-stimulated cells. These experiments indicate that the de-novo synthesis of TNF-α or other cytokines is not required, at least in these cells. Nonetheless, the ability of HMGB1 to induce various pro-inflammatory cytokines may also contribute to activate HIV-1 replication *in vivo*. Notably, nanogram concentrations of HMGB1 can act synergistically with IL-1β and IL-2 to drive IFN-γ production from human PBMC and natural killer cells [16].

We analysed the molecular pathways that lead to HIV activation and proved that the MAPK p38 and ERK play a crucial role in HIV-1 activation by HMGB1. We also demonstrated that HMGB1 activation was associated with the recruitment of NF-κB to the LTR. The nuclear translocation of NF-κB could follow the activation of MAPK p38 or ERK through the HMGB1–RAGE interaction, which is strongly reminiscent of activation pathways that have been described in macrophages and endothelial cells exposed to HMGB1 [26,32]. The complete reversion of the HMGB1-induced activation of HIV-1 by ATA, a known inhibitor of NF-κB, confirms the key role of NF-κB in the process that links HMGB1 exposure and HIV-1 reactivation.

We finally performed preliminary experiments to evaluate whether HMGB1 could reactivate HIV from latently infected PBMC. Our results indicate that HMGB1 is at least as efficient as PHA in activating provirus expression, alone or in combination with IL-2. Whether this activity is directly linked to HMGB1, as suggested by the constitutive expression of TLR-2 on memory (CD45RO+) CD4 T lymphocytes [21], or results from its ability to induce various cytokines is currently under investigation.

This work demonstrates that HMGB1 is a potent activator of HIV expression that may play an important role by increasing viral replication when necrosis occurs. This may be especially relevant during AIDS, in which cell injury is frequent in organs targetted by opportunistic diseases. Furthermore, HIV itself could participate in cell injury because both necrosis and apoptosis were observed during HIV-mediated cell killing [50,51]. The effective contribution of HMGB1 in HIV replication *in vivo* is currently under investigation in our laboratory. In that respect, a recent report correlated increased levels of circulating HMGB1 in HIV patients [52] with high viral loads and T-cell depletion, which strengthens the hypothesis that HMGB1 might contribute significantly to AIDS pathogenesis.

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References

1. Gulick RM, Mellors JW, Havlir D, Eron JJ, Gonzalez C, McMahon D, *et al*. **Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy.** *N Engl J Med* 1997; **337**:734–739.
2. Schrager LK, D'Souza MP. **Cellular and anatomical reservoirs of HIV-1 in patients receiving potent antiretroviral combination therapy.** *JAMA* 1998; **280**:67–71.
3. Pierson T, McArthur J, Siliciano RF. **Reservoirs for HIV-1: mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy.** *Annu Rev Immunol* 2000; **18**:665–708.
4. Chun TW, Stuyver L, Mizell SB, Ehler LA, Mican JA, Baseler M, *et al*. **Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy.** *Proc Natl Acad Sci U S A* 1997; **94**:13193–13197.
5. Finzi D, Hermankova M, Pierson T, Carruth LM, Buck C, Chaisson RE, *et al*. **Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy.** *Science* 1997; **278**:1295–1300.
6. Wong JK, Hezareh M, Gunthard HF, Havlir DV, Ignacio CC, Spina CA, Richman DD. **Recovery of replication-competent HIV despite prolonged suppression of plasma viremia.** *Science* 1997; **278**:1291–1295.
7. Chun TW, Engel D, Mizell SB, Ehler LA, Fauci AS. **Induction of HIV-1 replication in latently infected CD4+ T cells using a combination of cytokines.** *J Exp Med* 1998; **188**:83–91.
8. Scripture-Adams DD, Brooks DG, Korin YD, Zack JA. **Interleukin-7 induces expression of latent human immunodeficiency virus type 1 with minimal effects on T-cell phenotype.** *J Virol* 2002; **76**:13077–13082.
9. Wang FX, Xu Y, Sullivan J, Souder E, Argyris EG, Acheampong EA, *et al*. **IL-7 is a potent and proviral strain-specific inducer of latent HIV-1 cellular reservoirs of infected individuals on virally suppressive HAART.** *J Clin Invest* 2005; **115**:128–137.
10. Korin YD, Brooks DG, Brown S, Korotzer A, Zack JA. **Effects of prostratin on T-cell activation and human immunodeficiency virus latency.** *J Virol* 2002; **76**:8118–8123.
11. Kulkosky J, Culnan DM, Roman J, Dornadula G, Schnell M, Boyd MR, Pomerantz RJ. **Prostratin: activation of latent HIV-1 expression suggests a potential inductive adjuvant therapy for HAART.** *Blood* 2001; **98**:3006–3015.
12. Savill J, Dransfield I, Gregory C, Haslett C. **A blast from the past: clearance of apoptotic cells regulates immune responses.** *Nat Rev Immunol* 2002; **2**:965–975.
13. Decrion AZ, Dichamp I, Varin A, Herbein G. **HIV and inflammation.** *Curr HIV Res* 2005; **3**:243–259.

14. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, *et al*. **HMG-1 as a late mediator of endotoxin lethality in mice.** *Science* 1999; **285**:248–251.
15. Scaffidi P, Misteli T, Bianchi ME. **Release of chromatin protein HMGB1 by necrotic cells triggers inflammation.** *Nature* 2002; **418**:191–195.
16. DeMarco RA, Fink MP, Lotze MT. **Monocytes promote natural killer cell interferon gamma production in response to the endogenous danger signal HMGB1.** *Mol Immunol* 2005; **42**:433–444.
17. Hori O, Brett J, Slattery T, Cao R, Zhang J, Chen JX, *et al*. **The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system.** *J Biol Chem* 1995; **270**:25752–25761.
18. Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, Abraham E. **Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein.** *J Biol Chem* 2004; **279**:7370–7377.
19. Kuniyasu H, Yano S, Sasaki T, Sasahira T, Sone S, Ohmori H. **Colon cancer cell-derived high mobility group 1/amphoterin induces growth inhibition and apoptosis in macrophages.** *Am J Pathol* 2005; **166**:751–760.
20. Kokkola R, Andersson A, Mullins G, Ostberg T, Treutiger CJ, Arnold B, *et al*. **RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages.** *Scand J Immunol* 2005; **61**:1–9.
21. Xu D, Komai-Koma M, Liew FY. **Expression and function of Toll-like receptor on T cells.** *Cell Immunol* 2005; **233**:85–89.
22. Hemmi H, Akira S. **TLR signalling and the function of dendritic cells.** *Chem Immunol Allergy* 2005; **86**:120–135.
23. Huttunen HJ, Fages C, Kuja-Panula J, Ridley AJ, Rauvala H. **Receptor for advanced glycation end products-binding COOH-terminal motif of amphoterin inhibits invasive migration and metastasis.** *Cancer Res* 2002; **62**:4805–4811.
24. Sappington PL, Yang R, Yang H, Tracey KJ, Delude RL, Fink MP. **HMGB1 B box increases the permeability of Caco-2 enterocytic monolayers and impairs intestinal barrier function in mice.** *Gastroenterology* 2002; **123**:790–802.
25. Park JS, Arcaroli J, Yum HK, Yang H, Wang H, Yang KY, *et al*. **Activation of gene expression in human neutrophils by high mobility group box 1 protein.** *Am J Physiol Cell Physiol* 2003; **284**:C870–C879.
26. Fiuza C, Bustin M, Talwar S, Tropea M, Gerstenberger E, Shelhamer JH, Suffredini AF. **Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells.** *Blood* 2003; **101**:2652–2660.
27. Hiscott J, Kwon H, Genin P. **Hostile takeovers: viral appropriation of the NF-kappaB pathway.** *J Clin Invest* 2001; **107**:143–151.
28. Folks TM, Justement J, Kinter A, Dinarello CA, Fauci AS. **Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line.** *Science* 1987; **238**:800–802.
29. Jondal M, Holm G, Wigzell H. **Surface markers on human T and B lymphocytes I A large population of lymphocytes forming nonimmune rosettes with sheep red blood cells.** *J Exp Med* 1972; **136**:207–215.
30. Thierry S, Marechal V, Rosenzwajg M, Sabbah M, Redeuilh G, Nicolas JC, Gozlan J. **Cell cycle arrest in G2 induces human immunodeficiency virus type 1 transcriptional activation through histone acetylation and recruitment of CBP, NF-kappaB, and c-Jun to the long terminal repeat promoter.** *J Virol* 2004; **78**:12198–12206.
31. Li J, Wang H, Mason JM, Levine J, Yu M, Ulloa L, *et al*. **Recombinant HMGB1 with cytokine-stimulating activity.** *J Immunol Methods* 2004; **289**:211–223.
32. Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H, *et al*. **High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes.** *J Exp Med* 2000; **192**:565–570.
33. Semino C, Angelini G, Poggi A, Rubartelli A. **NK/iDC interaction results in IL-18 secretion by DCs at the synaptic cleft followed by NK cell activation and release of the DC maturation factor HMGB1.** *Blood* 2005; **106**:609–616.
34. Dumitriu IE, Baruah P, Bianchi ME, Manfredi AA, Rovere-Querini P. **Requirement of HMGB1 and RAGE for the maturation of human plasmacytoid dendritic cells.** *Eur J Immunol* 2005; **35**:2184–2190.
35. Jaulmes A, Thierry S, Janvier B, Raymondjean M, Marechal V. **Activation of sPLA2-IIA and PGE2 production by high mobility group protein B1 in vascular smooth muscle cells sensitized by IL-1{beta}.** *Faseb J* 2006; **20**:1727–1729.
36. Degryse B, Bonaldi T, Scaffidi P, Muller S, Resnati M, Sanvito F, *et al*. **The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells.** *J Cell Biol* 2001; **152**:1197–1206.
37. Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, *et al*. **SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase.** *Proc Natl Acad Sci U S A* 2001; **98**:13681–13686.
38. Davies SP, Reddy H, Caivano M, Cohen P. **Specificity and mechanism of action of some commonly used protein kinase inhibitors.** *Biochem J* 2000; **351**:95–105.
39. Van Lint C, Emiliani S, Ott M, Verdin E. **Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation.** *Embo J* 1996; **15**:1112–1120.
40. Quivy V, Van Lint C. **Diversity of acetylation targets and roles in transcriptional regulation: the human immunodeficiency virus type 1 promoter as a model system.** *Biochem Pharmacol* 2002; **64**:925–934.
41. Verdin E, Paras P Jr, Van Lint C. **Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation.** *Embo J* 1993; **12**:3249–3259.
42. Sharma RK, Garg BS, Kurosaki H, Goto M, Otsuka M, Yamamoto T, Inoue J. **Aurine tricarboxylic acid, a potent metal-chelating inhibitor of NFkappaB-DNA binding.** *Bioorg Med Chem* 2000; **8**:1819–1823.
43. Sunden-Cullberg J, Norrby-Teglund A, Rouhiainen A, Rauvala H, Herman G, Tracey KJ, *et al*. **Persistent elevation of high mobility group box-1 protein (HMGB1) in patients with severe sepsis and septic shock.** *Crit Care Med* 2005; **33**:564–573.
44. Ueno H, Matsuda T, Hashimoto S, Amaya F, Kitamura Y, Tanaka M, *et al*. **Contributions of high mobility group box protein in experimental and clinical acute lung injury.** *Am J Respir Crit Care Med* 2004; **170**:1310–1316.
45. Nowak P, Barqasho B, Treutiger CJ, Harris HE, Tracey KJ, Andersson J, Sonnerborg A. **HMGB1 activates replication of latent HIV-1 in a monocytic cell-line, but inhibits HIV-1 replication in primary macrophages.** *Cytokine* 2006; **34**:17–23.
46. Heggelund L, Muller F, Lien E, Yndestad A, Ueland T, Kristiansen KI, *et al*. **Increased expression of toll-like receptor 2 on monocytes in HIV infection: possible roles in inflammation and viral replication.** *Clin Infect Dis* 2004; **39**:264–269.
47. Equils O, Schito ML, Karahashi H, Madak Z, Yarali A, Michelsen KS, *et al*. **Toll-like receptor 2 (TLR2) and TLR9 signaling results in HIV-long terminal repeat trans-activation and HIV replication in HIV-1 transgenic mouse spleen cells: implications of simultaneous activation of TLRs on HIV replication.** *J Immunol* 2003; **170**:5159–5164.
48. Biswas P, Poli G, Kinter AL, Justement JS, Stanley SK, Maury WJ, *et al*. **Interferon gamma induces the expression of human immunodeficiency virus in persistently infected promonocytic cells (U1) and redirects the production of virions to intracytoplasmic vacuoles in phorbol myristate acetate-differentiated U1 cells.** *J Exp Med* 1992; **176**:739–750.
49. Poli G, Kinter A, Justement JS, Kehrl JH, Bressler P, Stanley S, Fauci AS. **Tumor necrosis factor alpha functions in an autocrine manner in the induction of human immunodeficiency virus expression.** *Proc Natl Acad Sci U S A* 1990; **87**:782–785.
50. Plymale DR, Tang DS, Comardelle AM, Fermin CD, Lewis DE, Garry RF. **Both necrosis and apoptosis contribute to HIV-1-induced killing of CD4 cells.** *AIDS* 1999; **13**:1827–1839.
51. Lenardo MJ, Angleman SB, Bounkeua V, Dimas J, Duvall MG, Graubard MB, *et al*. **Cytopathic killing of peripheral blood CD4(+) T lymphocytes by human immunodeficiency virus type 1 appears necrotic rather than apoptotic and does not require env.** *J Virol* 2002; **76**:5082–5093.
52. Nowak P, Treutiger CJ, Andersson A, Sonneborg A. *Contribution of HMGB1 protein to innoactivation in HIV-1 infection*. Rio de Janeiro: International AIDS Society; 2005.