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Human immunodeficiency virus 1 favors the persistence of infection by activating macrophages through TNF

Eric Guillemard, Catherine Jacquemot, Fabienne Aillet, Nathalie Schmitt, Françoise Barré-Sinoussi, Nicole Israël*

Unité de Biologie des Rétrovirus, Institut Pasteur, 75724 Paris Cedex 15, France

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Abstract

Macrophages play a major role in HIV-1 persistence. In the present paper, we demonstrate that the absence of apoptosis in HIV-1-infected primary human monocyte-differentiated macrophages (MDM) correlates with an increase in anti-apoptotic (Bcl-2 and Bcl-x_L) and a decrease in pro-apoptotic (Bax and Bad) proteins. This is associated with macrophage activation as shown by tumor necrosis factor (TNF) production and NF-κB activation upon infection. TNF production was shown to be involved in the upregulation of Bcl-2 and Bcl-x_L because this increase was abolished by an anti-TNF anti-serum or an inhibitor of TNF synthesis. In parallel, inhibition of TNF production induced an increase in the number of apoptotic cells. Furthermore, using an inhibitor of NF-κB activation, we demonstrated that TNF-induced upregulation of Bcl-x_L and Bcl-2 occurs, respectively, through a NF-κB-dependent and an NF-κB-independent pathway.

Keywords: Macrophages; HIV-1; Persistence; TNF; NF-κB; Apoptosis; Bcl-2; Bcl-x_L; Bax; Bad

Introduction

Macrophages, a main non-T-cell reservoir for HIV (Gartner et al., 1986), are sites of infection in several organs and particularly in the brain, and play a critical role in HIV pathogenesis (Collman et al., 2003). Studies performed both in human patients and in an animal model show that at a late stage of infection, macrophages constitute a major source of virus in lymphoid tissues of HIV-infected patients (Orenstein et al., 1997) or in simian immunodeficiency virus/HIV-1 chimera (SHIV)-infected macaques (Igarashi et al., 2001). In particular, several studies demonstrated the capacity of infected macrophages to induce apoptosis in both CD4 and CD8 T lymphocytes (Mahlknecht and Herbein, 2001). Therefore, HIV persistence in macrophages plays a key role in virus spreading and disease progression.

Furthermore, monocytes or macrophages behave as a reservoir refractory to antiretroviral treatment. In patients, under prolonged HAART, exhibiting undetectable peripheral viral load, HIV-DNA was found in monocytes and viral production can be activated in these cells by diverse bacterial products (Lambotte et al., 2000). This is consistent with the fact that antiretrovirals including protease inhibitors have been shown in vitro to be less active in chronically HIV-infected macrophages than in lymphocytes (Crowe et al., 1989; Perno et al., 1998). The molecular mechanisms that regulate the effects of HIV infection are different in T cells and macrophages. Upon HIV infection, whereas apoptosis has been proposed as a crucial mechanism of T cell destruction (Selliah and Finkel, 2001), resistance to virus-induced cell death was shown to be a key feature of macrophages. However, the mechanisms involved in the survival capacity of HIV-infected macrophages resulting in sustained viral persistence are not currently understood. Key arguments are provided by observations made on T lymphocytes. For instance, in HIV-infected patients, Bcl-2 expression was

* Corresponding author. Unité de Biologie des Rétrovirus, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cedex 15, France. Fax: +33 1 45 68 89 57.
E-mail address: nisrael@pasteur.fr (N. Israël).

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shown to decrease in both blood CD4 (Regamey et al., 1999) and CD8 T cells (Boudet et al., 1996; Regamey et al., 1999) correlating with a higher propensity of these cells to undergo spontaneous apoptosis in culture (Akari et al., 2001). Several in vitro studies carried out in primary cells or lymphocytic cell lines also described an increase of production of the pro-apoptotic protein Bax (Castedo et al., 2001; Genini et al., 2001). We therefore hypothesized that HIV infection induces an increase of anti-apoptotic and a decrease of pro-apoptotic proteins of the Bcl-2 family to strengthen the survival capacity of macrophages. Indeed, in a previous report, we showed that HIV infection induces an upregulation of Bcl-2 at the transcriptional level in monocytic U 937 cells and that this increase in Bcl-2 synthesis correlates with cell survival (Aillet et al., 1998). We also showed that downregulation of Bcl-2 in blood monocytes from HIV-infected patients was observed during the asymptomatic phase of infection followed by a gradual increase of Bcl-2 levels in the late stage of the disease (Elbim et al., 1999).

HIV infection is characterized by a general state of immune activation as shown by the increase of expression of multiple factors or markers of activation in T cells and monocytes or macrophages (Gougeon et al., 2000; Herbein and O'Brien, 2000). In particular, tumor necrosis factor (TNF) as well as soluble receptors TNF-R2 was found to be produced at high levels in the serum of HIV-infected patients (Herbein and O'Brien, 2000). Peripheral blood monocytes were reported as a possible source of TNF production because these cells isolated from AIDS patients were shown to spontaneously release TNF (Wright et al., 1988). In vitro, infection by HIV-1 induces TNF production in monocyte-derived macrophages (MDMs) (Godard and Chermann, 1998; Le Naour et al., 1994a; Weissman et al., 1994). In addition, the transcription factor NF-κB is activated upon HIV infection in both monocytic cells and primary macrophages (Choe et al., 2002; Mc Elhinny et al., 1995). Furthermore, several reports clearly demonstrate that NF-κB activity prevents apoptosis in various cellular models, including lymphocytic and macrophagic cell types (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996, 1998; You et al., 2001). Therefore, TNF-induced NF-κB activity during HIV infection might be involved in inhibition of apoptosis and survival of macrophages. One of the mechanisms involved in this protective activity might be the induction of expression of the anti-apoptotic proteins of the Bcl-2 family. Consistent with this, an NF-κB-dependent TNF-induced production of anti-apoptotic factors such as Bcl-x (Chen et al., 2000; Cheng et al., 2000; Tamatani et al., 1999) or Bfl-1/A1 (Cheng et al., 2000) was reported in various animal or human cell types. In addition, Bcl-2 was also described as a TNF-inducible protein (Burgmaier et al., 2000; Genestier et al., 1995; Tamatani et al., 1999), possibly involving a NF-κB-dependent mechanism (Tamatani et al., 1999).

In the present paper we investigated the modulation of pro- and anti-apoptotic molecules of the Bcl-2 family in blood monocyte-derived macrophages (MDMs) upon HIV infection. We demonstrate that infection induces macrophage activation through TNF and NF-κB and leads to a particular status of Bcl-2-related molecules, a key determinant of HIV persistence in these cells.

Results

HIV-1 infection does not induce apoptosis or cytotoxicity in macrophages

A small number of apoptotic MDMs (less than 3%) was detected among control cells, and this rate was not modified among infected cells. Indeed, no variation of DNA condensation or production of nuclear apoptotic bodies was seen after staining with Hoechst dye in cells infected with HIV-1_{BaL} or HIV-1_{BXO8} (see the curves of p24 observed with HIV-1_{BaL} in Fig. 1, panel c). In addition, HIV infection did not induce any DNA fragmentation as measured by TUNEL analysis (data not shown). Furthermore, Western blot analysis of Caspase-3, a terminal protease of the caspase cascade, revealed no variation of the amount of the procaspase form p32 upon infection in MDMs, and we did not detect any production of the p11 and p20 peptides normally generated by the cleavage necessary for the induction of Caspase-3 activity (data not shown). Likewise, no cytotoxicity was detected upon infection as assessed by titration of the release of lactate dehydrogenase in the supernatants of MDMs (data not shown). These results confirmed survival of infected MDMs in our experimental conditions.

Bcl-2 and Bcl-x_L expressions are increased in macrophages upon HIV-1 infection

In parallel experiments, we measured the expression level of the anti-apoptotic factors Bcl-2 and Bcl-x_L in MDMs. Data derived from control and infected cells, although coming from the same Western blot, were presented separately in panels A and B of Fig. 1 to facilitate comparison of the kinetics of expression of these molecules under control or infection conditions. We first observed that adherence of monocytes to plastic transiently induced an increase in Bcl-2 and Bcl-x_L expression (data not shown). This induction was still present for Bcl-2 when the cells had differentiated into macrophages at J0 (corresponding to 7 days post adherence of monocytes) as shown in Fig. 1Aa. In control cells, Bcl-2 and Bcl-x_L returned to basal level whereas infection transiently induced higher expression levels (Figs. 1Aa and Ba). In the five assays presented in Fig. 1Ab, densitometric analysis showed an increase of Bcl-2 expression that reached a maximum level between 10 and 18 days of infection with a 2- to 22-fold increase (median = 5 for five

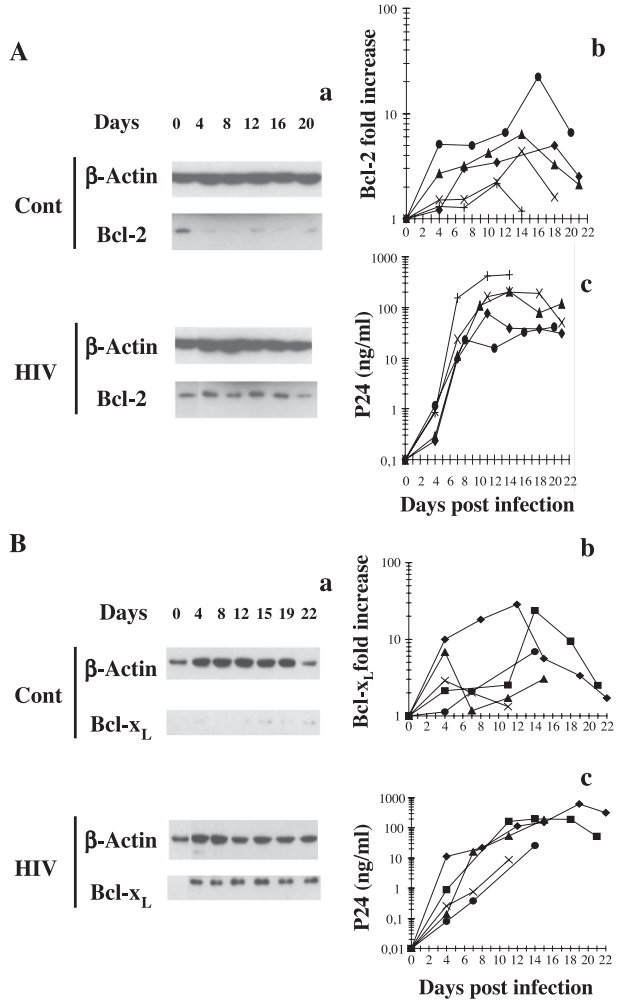

Fig. 1. Effect of HIV-1 infection on Bcl-2 (panel A) and Bcl-x_L (panel B) expression in MDMs. MDMs were left uninfected (control) or were infected by HIV-1_{BaL} at day 0 and analyzed for Bcl-2 or Bcl-x_L expression and viral production during 20 or 22 days. Western blot analysis of Bcl-2 (panel Aa) and Bcl-x_L (panel Ba) in control and infected MDMs was performed in comparison with β-Actin as a protein of constant expression. Fold increase of the expression of Bcl-2 (panel Ab) and Bcl-x_L (panel Bb) upon infection was measured by densitometric analysis. Viral production was determined by titration of p24 in cell supernatants (panel c). For panels b and c, curves correspond to kinetics of infection performed with cells from five different donors. Symbols used in both panels refer to the same donors. The data used in panel a correspond to the circle symbol in panels b and c for Bcl-2, and to the diamond symbol for Bcl-x_L.

assays). Thereafter, levels decreased but remained above those of control cells at least up to 3 weeks postinfection. These results were obtained with the HIV-1_{BaL} strain, but the same conclusion was made when infection was performed using the primary isolate HIV-1_{BXO8} (data not shown). In this case, fold increase of Bcl-2 upon infection albeit significant (2–3.2) was lower than that observed with HIV-1_{BaL} infection. For this reason and to emphasize the response, we used the HIV-1_{BaL} strain for the rest of the experiments.

Bcl-x_L expression increased upon infection (Fig. 1Bb) with a 2.9- to 28.3-fold increase ratio at the peak of expression (median = 6.9 for five assays) (Fig. 1Bb). Bcl-x_L expression also tended to decrease after prolonged kinetics of infection. In parallel, virus production was evaluated by p24 titration and this production reached a plateau especially at longer times of infection (Figs. 1Ac and Bc). It is interesting to stress that for short times of infection (1 or 2 days), for which no p24 production was detectable, we did not observe any increase of production of the proteins Bcl-2 and Bcl-x_L in infected macrophages (data not shown). The evolution of the slope of p24 (between day 0 and 6) correlates with the evolution of the levels of Bcl-2 or Bcl-x_L, emphasizing the role of infection in the modulation of Bcl-2 proteins. Therefore, this increase only starts with the onset of HIV production, thus precluding possible effects of proteins contained in the viral stock solutions. Increase of expression of anti-

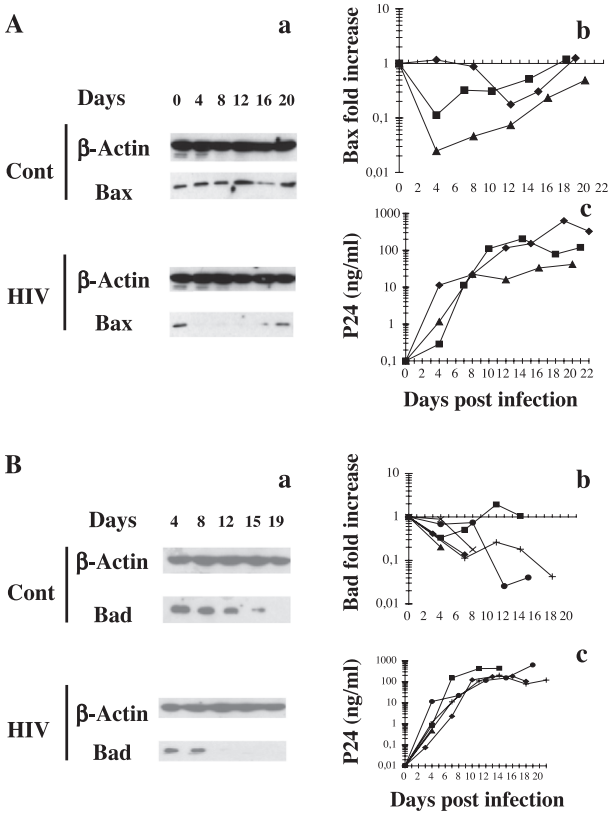

Fig. 2. Effect of HIV-1 infection on Bax (panel A) and Bad (panel B) expression in MDMs. MDMs were left uninfected (control) or were infected by HIV-1_{BaL} at day 0 and analyzed for Bax or Bad expression and viral production during 19 or 20 days. Western blot analysis of Bax (panel Aa) and Bad (panel Ba) in control and infected MDMs was performed in comparison with β-Actin as a protein of constant expression. Fold decrease of the expression of Bax (panel Ab) and Bad (panel Bb) upon infection was measured by densitometric analysis. Viral production was determined by titration of p24 in cell supernatants (panel c). For panels b and c, curves correspond to kinetics of infection performed with cells from three different donors for Bax and five different donors for Bad. Symbols used in both panels refer to the same donors. The data used in panel a correspond to the triangle symbols in panels b and c for Bax, and to the circle symbols for Bad.

apoptotic factors occurred early during infection, being detected at the first time point (day 4), when viral production was still limited (maximum of 1 ng/ml of p24). Similar results were obtained with MDMs differentiated in the presence of M-CSF and 15% FCS instead of human SAB (data not shown).

In conclusion, the absence of apoptosis under HIV infection correlated with an upregulation of the anti-apoptotic factors Bcl-2 and Bcl-x_L.

Bax and Bad expressions are decreased in MDMs upon HIV-1 infection

In parallel, we also investigated the modulation of pro-apoptotic members of the Bcl-2 family by HIV-1 infection in MDMs. Our data clearly show that Bax expression decreased during infection (Figs. 2Aa and b) and then gradually increased toward levels observed in control cells. Bax expression reached a minimum that was 5.7–50 times lower in infected MDMs than in control cells (median = 10 for three assays). Similarly, Bad expression was reduced upon HIV infection in MDMs (Figs. 2Ba and b) with a fold decrease ratio from 3 to 38.8 (median = 6.5 for six assays). Similar to the modulation of anti-apoptotic factors, the decrease of both Bax and Bad expression generally started early during infection when viral production was still at 1 ng of p24 per milliliters or below (Figs. 2Ac and Bc). Analyses of Bcl-x_s expression in five independent experiments showed that this truncated pro-apoptotic form of Bcl-x_L was produced neither in control nor in infected MDMs (data not shown).

Increased expression of anti-apoptotic factors upon HIV-1 infection correlates with the induction of NF-κB activity and TNF production

In a previous study, we demonstrated that differentiation of thymocytes into mature single-positive CD4 cells conferred to these cells a high capacity to survive HIV-1 infection in the thymus (Guillemard et al., 2001). This capacity correlates with both a high basal level of Bcl-2 production, which is further increased by infection, and a high level of NF-κB activity, which is under the control of TNF released into the thymic microenvironment (Chêne et al., 1999a, 1999b; Guillemard et al., 2001). Thus, a similar correlation could be envisioned in macrophages. Therefore, we investigated the activation level of MDMs under control and HIV-1 infection conditions by measuring kinetics of both NF-κB activity and TNF production. We first observed that the adherence of monocytes to plastic induced a transient cellular activation with an induction of NF-κB activity (data not shown) and of TNF secretion (between 30 and 45 pg/ml). As shown in Fig. 3A by EMSA analysis, control-differentiated macrophages still exhibited a strong NF-κB activity (p50–p65 heterodimer), which gradually decreased during culture. By comparison, in infected cells, we observed a higher level of NF-κB activity at each time point of the kinetics (Fig. 3A), indicating a reinduction of NF-κB activity. As shown by densitometric analysis, this increase was followed by a decrease toward control values after 2–3 weeks post-infection (Fig. 3B). At the highest levels of induction, NF-κB activity was 2–7.6 higher upon infection than in control conditions (median = 3.7 for five assays). In parallel, titration by ELISA in culture supernatants showed constitutive production of TNF in MDMs that gradually decreased with time (Fig. 4). Here also HIV-infection triggered a TNF increase mostly in the first days of infection. Therefore, NF-κB activity could be directly related to TNF secretion. Thus activation of MDMs was further increased upon HIV-infection. Because the kinetics of cellular activation correlated with the modulations of Bcl-2 and Bcl-x_L in infected MDMs, we investigated the involvement of NF-κB and TNF in the modulation of these anti-apoptotic proteins.

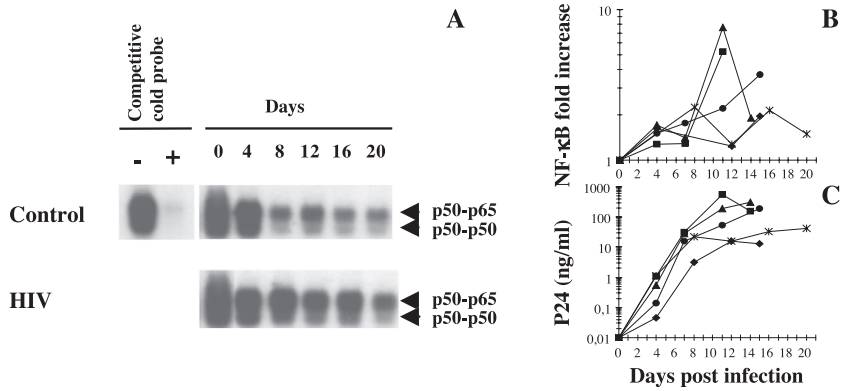

Fig. 3. Effect of HIV-1 infection on NF-κB activity in MDMs. MDMs were left uninfected (control) or were infected by HIV-1_{BaL} at day 0 and analyzed for NF-κB activity and viral production during 20 days. (A) Analysis of NF-κB by EMSA was carried out in control and infected MDMs. Specific binding was controlled by competition with a cold DNA probe as described in Materials and methods. (B) Fold increase of the activity of NF-κB upon infection was measured by densitometric analysis of p50–p65 band level. (C) Viral production was determined by titration of p24 in cell supernatants. For B and C, curves correspond to kinetics of infection performed with cells from five different donors. Symbols used in both panels refer to the same donors. The data used in panel A correspond to the cross symbol in panels B and C.

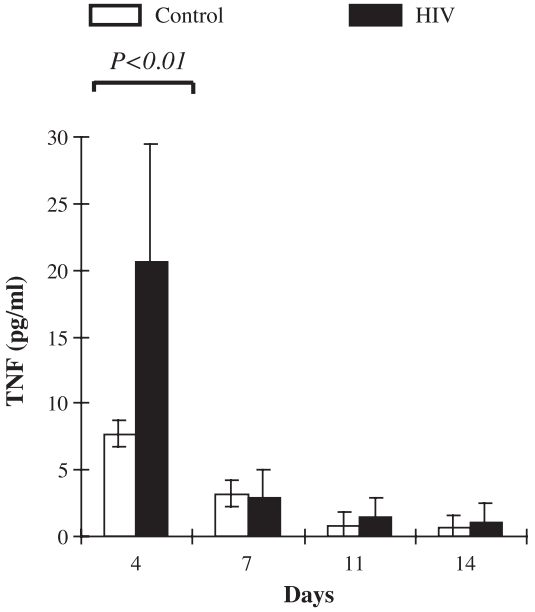

Fig. 4. Effect of HIV-1 infection on TNF production in MDMs. MDMs were left uninfected (control) or were infected by HIV-1_{BaL} at day 0 and cell supernatants were collected for analysis of TNF production by ELISA during 14 days. Data are given with standard deviations for each time point of the kinetics as a mean of independent assays performed with cells from five different donors. *P* value was determined using the nonparametric test of Mann–Whitney.

TNF produced by MDMs upregulates Bcl-2 and Bcl-x_L expression and prevents apoptosis

We first analyzed the role of TNF in the expression of Bcl-2 and Bcl-x_L by measuring the level of both proteins by Western blot analysis in MDMs treated with recombinant human TNF. As shown in Fig. 5, TNF induces an increase in expression of both Bcl-2 and Bcl-x_L in MDMs after 2 or 72 h of treatment ($P < 0.04$ in three independent experiments). We then investigated the role of endogenous TNF produced during infection by analyzing expression of Bcl-2 and Bcl-x_L in HIV-1-infected MDMs cultivated in the presence of an anti-TNF anti-serum or an inhibitor of TNF synthesis RP55778 (Le Naour et al., 1994a; Weissman et al., 1993). Our results demonstrate that both the anti-TNF serum or the inhibitor RP55778 decreased the expression of both Bcl-2 and Bcl-x_L in infected MDMs (Fig. 6A), with however a higher efficiency for RP55778. This was confirmed by densitometric analysis in three independent experiments ($P < 0.04$, data not shown). Indeed, RP55778 treatment was shown to markedly decrease HIV replication (Fig. 6B, left panel). Treatment with anti-TNF serum induced no significant decrease in HIV replication (Fig. 6B, right panel), whereas down-regulation of Bcl-2 and Bcl-x_L was observed. Of note, we did not observed any modulation of the pro-apoptotic protein Bax either under TNF treatment or in condition of inhibition of TNF production (data not shown).

We then measured the level of apoptosis of MDMs treated with RP55778 by TUNEL analysis. As shown in

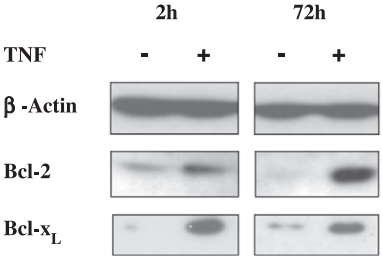

Fig. 5. Effect of recombinant TNF on Bcl-2 and Bcl-x_L expression in MDMs. Control MDMs were first cultivated for 11 days. Cells were then treated for 2 h with recombinant TNF at 40 ng/ml and were analyzed by Western Blot for Bcl-2 and Bcl-x_L expression in comparison with β-Actin as a protein of constant expression. The blots presented here are representative of experiments carried out with cells from three different donors.

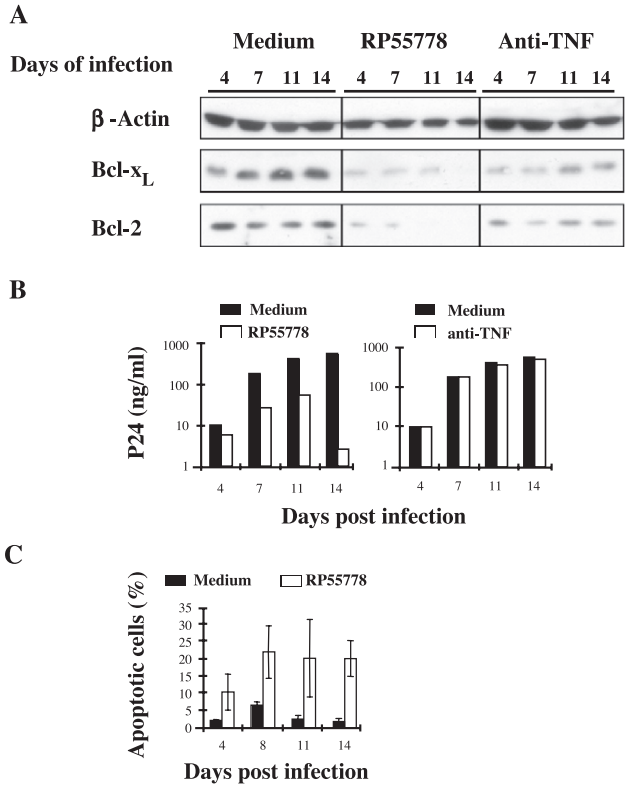

Fig. 6. Role of endogenous TNF in the regulation of Bcl-2 and Bcl-x_L expression and apoptosis in HIV-1-infected MDMs. (A) MDMs were infected by HIV-1_{BaL} and analyzed by Western Blot for Bcl-2 and Bcl-x_L expression in comparison with β-Actin as a protein of constant expression. Infected cells were cultivated in regular medium or in the presence of the TNF-synthesis inhibitor RP55778 or an anti-TNF serum. (B) Viral production was determined by titration of p24 in cell supernatants. (C) Infected MDMs seeded in Lab-Tek chamber slides and treated or not with the TNF synthesis inhibitor RP55778 were used for detection of apoptotic cells by TUNEL analysis. Results are given as apoptotic cell counts per well, as a mean of duplicate with standard deviation. The data presented here are representative of two (C) to three (A and B) experiments carried out with cells from different donors.

Fig. 6C, treatment increased the percentage of apoptotic cells in infected MDMs. This was shown both by TUNEL labeling and by the presence of nuclear apoptotic bodies in Hoechst-counterstained cells (data not shown). Therefore, downregulation of the anti-apoptotic molecules Bcl-2 and Bcl-x_L in the presence of RP55778 was associated with an increase of apoptosis. Thus, TNF produced upon infection is involved in the positive control of expression of both Bcl-2 and Bcl-x_L, which prevents apoptosis in infected MDM. This increase in expression of the anti-apoptotic proteins was diminished when the anti-TNF serum was used (see Discussion).

TNF-induced NF-κB activity is involved in positive regulation of Bcl-x_L but not of Bcl-2

We then determined whether TNF-induced NF-κB activity was involved in the regulation of Bcl-2 or Bcl-x_L expression. For this purpose, we first measured the activation level of NF-κB activity in the presence of anti-TNF serum or RP55778, as described above. Our results show that NF-κB activity in infected macrophages was decreased in both conditions (Fig. 7A), confirming the role of TNF in the induction of this activity.

Bcl-2 and Bcl-x_L proteins were then detected in MDMs treated with sesquiterpene lactone, which prevents the degradation of IκB inhibitory molecules through inhibition of the IKK activity (Hehner et al., 1998, 1999). This inhibitor was used at concentrations that did not induce cytotoxicity (data not shown), as shown by analysis of Lactate Deshydrogenase release in cell supernatants. As shown in Fig. 7B, sesquiterpene lactone strongly inhibited NF-κB activity in HIV-infected MDMs in a dose-dependent manner. This inhibition was accompanied by a decrease in Bcl-x_L expression that was not observed for Bcl-2. Rather, sesquiterpene lactone tended to increase Bcl-2 expression. Therefore, NF-κB is involved in the positive regulation of Bcl-x_L but not of Bcl-2 in HIV-infected MDMs. Positive regulation of Bcl-2 by TNF is thus likely mediated by an NF-κB-independent pathway whereas TNF-induced Bcl-x_L expression occurs through an NF-κB-dependent pathway.

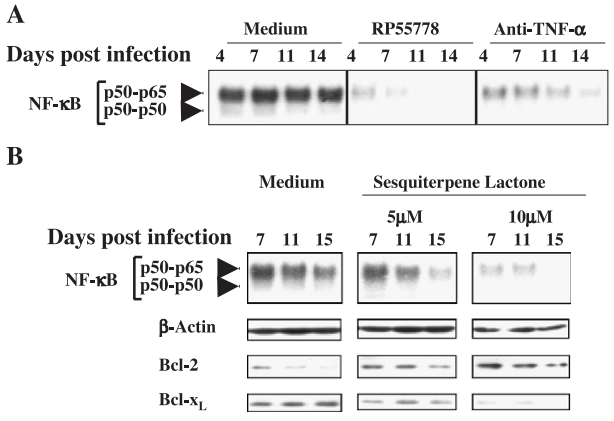

Fig. 7. Role of NF-κB in the expression of Bcl-2 and Bcl-x_L in HIV-1-infected MDMs. (A) NF-κB activity was measured in infected MDMs incubated in the presence of the TNF-synthesis inhibitor RP55778 or an anti-TNF serum. (B) NF-κB activity and Bcl-2 and Bcl-x_L expression were measured in infected MDMs incubated in the presence of various concentrations of the NF-κB inhibitor, sesquiterpene lactone. Analysis of NF-κB activity was carried out by EMSA and analysis for Bcl-2 and Bcl-x_L expression was done by Western blot in comparison with β-Actin as a protein of constant expression. The data presented here are representative of three experiments carried out with cells from different donors.

Discussion

In the present paper, we investigated the mechanisms responsible for the high survival capacity of HIV-infected macrophages that could contribute to the persistent infection observed in patients. Our in vitro studies demonstrate that the absence of apoptosis in HIV-1-infected monocyte-derived macrophages (MDMs) correlates with an increase in the expression of the anti-apoptotic proteins Bcl-2 and Bcl-x_L and a decrease in expression of the apoptotic proteins Bax and Bad upon infection. Our data show that this anti-apoptotic pattern is associated with an increase in the activation state of MDMs. This was shown by an increased TNF production upon infection, which in turn induced an increase in NF-κB activity. By the use of an anti-TNF antiserum and an inhibitor of TNF synthesis RP55778, we demonstrate that this increase in TNF production is involved in the upregulation of both Bcl-2 and Bcl-x_L, correlating with a protection against apoptosis in infected MDMs. In addition, sesquiterpene lactone, a direct inhibitor of NF-κB activation, decreases Bcl-x_L but not Bcl-2 expression in HIV-1-infected MDMs. Therefore, TNF-mediated upregulation of Bcl-x_L and Bcl-2 expression during infection occurs, respectively, through an NF-κB-dependent and an NF-κB-independent pathway. It is of note that RP55778 is always more efficient than the anti-TNF antiserum in all the assays we used and particularly in its effect on apoptosis. We interpret this result as a consequence of the known dual activity of TNF, which elicits both an anti-apoptotic activity through NF-κB activation, and a pro-apoptotic activity through induction of the caspase cascade. The respective importance of these two pathways regarding the survival or apoptosis decision varies from one cell type to another and has not been specifically studied in macrophages. For example, we showed in mature thymocytes that the thymic microenvironment (IL-7) induces the expression of TNFR-2, therefore inhibiting apoptosis, whereas the constitutive TNF-R1 leads to apoptosis (Guillemard et al., 2001). Under these conditions, inhibiting TNF activity will interfere with both pathways, but the respective effect of the antiserum on activation of the TNF-R1 and the TNF-R2 receptors is difficult to predict. In addition, direct cell to cell transmission of TNF might be

one of the reasons of the limited activity of the antiserum. On the other hand, RP5578 has been shown to inhibit TNF synthesis, probably at the level of secretion (Le Naour et al., 1994a), therefore acting before TNF is produced, and leading to inhibition of all downstream pathways. As expected, RP55778 has been previously shown in HIV-infected macrophages to decrease both viral and cytokine production (Le Naour et al., 1994b).

Regarding sesquiterpene lactone, it has been described as an inhibitor of the IKK complex, and more recent data (Zhang et al., in press) suggest that it also acts by inhibiting TNF-alpha-mediated NF-κB activation via disrupting the recruitment of the IKK complex to the TNF receptor. The observed effects are consistent with an inhibitory effect of RP55778 on TNF production and of sesquiterpene lactone on NF-κB activation. In addition, a recent paper (Liu et al., 2004) indicates that TNF is responsible for survival of noninfected macrophages through induction of NF-κB, which is consistent with the results presented here.

In a previous report we showed that monocytic U937 cells infected with HIV-1 exhibit transient downregulation of Bcl-2 expression, which correlates with an increased apoptosis. Later, during infection, apoptosis decreased due to HIV-induced upregulation of Bcl-2 at the transcriptional level, allowing cell survival of HIV-productive cells (Aillet et al., 1998). A similar pattern of Bcl-2 modulation was observed in vivo because monocytes from HIV-infected patients showed transient downregulation of Bcl-2 in the asymptomatic phase of infection (Elbim et al., 1999). We report here that, in contrast with the monocytic cell type, fully differentiated macrophages exhibit a substantial increase in the expression of the anti-apoptotic proteins Bcl-2 and Bcl-x_L upon HIV infection, with no transient downmodulation. One of the possible explanations for the kinetic differences observed between monocytes and macrophages could be their level of activation, that is, the induction of NF-κB and TNF, which we demonstrated here to be responsible for the regulation of anti-apoptotic factors in macrophages. Indeed, differentiation of monocytes into macrophages was clearly reported to be accompanied by induction of the p50–p65 and p50–RelB active forms of NF-κB (Conti et al., 1997; Lewin et al., 1997). In addition, this higher activation level in macrophages correlates with a higher capacity, compared to monocytes, to produce inflammatory cytokines such as TNF, as observed here or under inducing conditions such as LPS treatment (Gessani et al., 1993). In fully activated macrophages, our data show that the upregulation of anti-apoptotic factors or the downmodulation of apoptotic proteins occurs very early during infection.

The particularly high propensity of macrophages to respond to HIV infection by rapid induction of protective mechanisms makes these cells a potent persistent viral reservoir. The fact that during infection, the level of anti-apoptotic proteins decreased in parallel with the level of cell activation suggests two hypotheses. The first one is that the level of Bcl-2–Bcl-x_L remained sufficiently high to be compatible with cell survival and a stabilized virus production, as we previously showed in U937 cells (Aillet et al., 1998). The other possibility is that modulation of expression of these anti-apoptotic factors might be an early event, crucial for the onset of HIV production in macrophages, and that other mechanisms would later be involved in the long-term maintenance of cell survival.

In conclusion, we identified crucial mechanisms involved in the survival of HIV-infected primary macrophages. The high level of cell activation and the inflammatory context in AIDS patients could therefore participate in the establishment of this viral reservoir.

Materials and methods

Isolation and culture of blood monocyte-derived macrophages

Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors by centrifugation on a Ficoll-Hypaque gradient (Pharmacia). Blood monocytes were obtained by centrifuging PBMCs on a Percoll gradient (Amersham Pharmacia Biotech). Purified monocytes were allowed to adhere to plates and differentiate into macrophages for 7 days in complete RPMI containing 10% of human serum AB (SAB) (Valbiotech). These monocyte-derived macrophages (MDMs) constitute a 95% pure population as revealed by immunostaining with anti-CD14 (Becton-Dickinson), anti-CD11b, and anti-CD16 antibodies (Immunotech-Coulter) (Clarkson and Ory, 1988; Passlick et al., 1989).

Infection of macrophages

Infections were performed either with the HIV-1_{BaL} strain or the monocytotropic primary isolate HIV-1_{BXO8} (provided by H. Fleury, Bordeaux 2 University, France). HIV-1_{BaL} was maintained and HIV-1_{BXO8} amplified once in cord blood PBMC cultures. The obtained stock solutions of viruses were used at a final dilution of 1/100 to limit the effects of the proteins eventually produced by PBMCs. Infection of MDMs was performed at J0 (corresponding to 7 days post adherence of monocytes) at a multiplicity of infection of 10^{-3} for 1 h at 37 °C for both viruses. Both infected and uninfected cells were then washed three times with RPMI 1640 and put in culture in complete RPMI supplemented with 15% FCS. For kinetics of infection, the medium was replaced every 3–4 days. The HIV-1 $p24^{gag}$ antigen concentration was determined in culture supernatants using a $p24^{gag}$ antigen detection kit (Coulter HIV-1 p24 antigen assay) according to the instructions of the manufacturer.

Antibodies used for Western blot analysis

The following antibodies were used for the detection of specific human proteins by Western blot: mouse anti ß-Actin (AC-74) (Sigma), mouse anti-Bcl-2 (Bcl-2/100) (BD Pharmingen), rabbit anti-Bax, anti-Bcl-$x_{S/L}$ and anti-Caspase-3 (precursor form and p11–p20 subunits) (TEBU-Santa Cruz), and rabbit anti-Bad (Calbiochem). Anti-mouse and anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (Amersham Pharmacia Biotech) were used as a secondary antibody.

Western blot analysis

Total extracts were performed as follows: macrophages were directly lysed in culture wells with lysis buffer (0.2% Triton X-100, 500 mM NaCl, 500 mM Sucrose, 1 mM EDTA, 0.15 mM Spermine, 0.5 mM Spermidine, 10 mM HEPES pH 8, 200 μM Phenylmethylsulfonylfluoride, 4 μg/ml Pepstatin A, Aprotinine, Chymostatin and Antipain, 110 mM β−mercaptoethanol, 1 mM Sodium-Orthovanadate, 50 mM Sodium Fluoride). Twenty micrograms of protein, measured with Bradford reagent (Bio-rad Laboratories, Ivry sur Seine, France), were subjected to SDS-PAGE and transferred to nitrocellulose for immunodetection. This was performed with the primary and secondary HRP-conjugated antibodies described above. The antigen–antibody complexes were revealed by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, PIERCE).

Electrophoretic mobility shift assay

Total cell extracts were prepared as described above (see Western blot), and EMS was performed using the following NF-κB binding sequence: 5′ GACAGA**GGGGGACTTTC**CGAGAGG 3′ 3′GTCT**CCCCCTGAAAG**GGCTCTCCCT 5′ in which the NF-κB consensus binding sequence is indicated in bold. To identify the subunits constituting NF-κB complexes, specific antibodies against p50, p65, and Rel B were used in supershifting experiments.

Densitometric analysis

Densitometric analysis of the autoradiograms (under conditions of nonsaturating signals in the linear range of measure) for both EMSA and Western blot was carried out with a Gene Genius GG system imager (Ozyme-Syngene). Changes in the expression of factors of interest were determined by densitometric analysis for each kinetic time point as a ratio equal to factor level in infected cells/factor level in control cells. In this ratio analysis, factor levels (for Bcl-2, Bcl-x_L, Bax, or Bad) were previously divided for standardization with the corresponding β-Actin level as a protein whose expression does not change.

Measurement of apoptosis and cytotoxicity

For measurement of apoptosis, macrophages were put in culture in Lab-Tek chamber slides (Nalge Nunc International). Apoptosis was assessed by TUNEL analysis (Terminal dUTP Nick End Labeled) with Fluorescein-labeled dUTP (kit from Boehringer Mannheim). Macrophages treated with Dnase I type IV (Sigma) were used as a positive control of DNA fragmentation and all cells were counterstained with Hoechst (Sigma) for visualization of total nuclei. Counting of apoptotic cells was carried out with a Fluorescence Microscope Nikon-Eclipse E600. Cytotoxicity was assessed by titration of Lactate Deshydrogenase release in the cell supernatants (CytoTox 96 Non-Radioactive Cytotoxicity Assay kit, Promega, Madison, WI, USA).

ELISA for TNF

TNF production was quantified in cell supernatants by ELISA using a Quantikine High Sensitivity kit from R&D Systems (France).

Recombinant TNF and inhibitors of TNF or NF-κB activities

Recombinant TNF was provided by TEBU-Peprotech (Le Perray en Yvelines, France). For inhibition of TNF activity, a polyclonal rabbit antihuman TNF serum kindly provided by Dr. J.-M Cavaillon (Pasteur Institute, Paris, France) was used at 1/300 final dilution. RP55778 was also used at 1 mM and was kindly provided by Dr. Y. Hénin (Aventis, Paris, France). This compound was shown to act as an inhibitor of TNF synthesis at a post-transcriptional level (Le Naour et al., 1994a). Sesquiterpene Lactone was used as an inhibitor of NF-κB activity (Hehner et al., 1998). Each compound was added to the MDM culture medium at the time of infection and every 3–4 days throughout the time of culture.

Statistical analysis

Statistical analyses were performed using the nonparametric test of Mann–Whitney. The level of significance was set at $P < 0.05$.

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