Excess apoptosis of mononuclear cells contributes to the depressed cytomegalovirus-specific immunity in HIV-infected patients on HAART

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Abstract

HIV-infected patients on highly active antiretroviral therapy (HAART) have persistently decreased cytomegalovirus (CMV)-specific proliferative responses [lymphocyte proliferation assay (LPA)] in spite of increases in CD4+ T cell counts. Here we demonstrate an association between apoptosis of unstimulated peripheral blood mononuclear cells (uPBMC) and decreased CMV-LPA. HAART recipients had more apoptosis of uPBMC than controls when measured by caspases 3, 8, and 9 activities and by annexin V binding. Patients with undetectable HIV replication maintained significantly higher apoptosis of CD4+ and CD14+ cells compared to controls. CMV-LPA decreased with higher apoptosis of uPBMC in patients only. This association was independent of CD4+ cell counts or HIV replication. Furthermore, rescuing PBMC from apoptosis with crmA, but not with TRAIL- or Fas-pathway blocking agents or with other caspase inhibitors, increased CMV-LPA in HAART recipients. This effect was not observed in uninfected controls, further indicating that the down regulatory effect of apoptosis on cell-mediated immunity (CMI) was specifically associated with the HIV-infected status.

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Introduction

Human immunodeficiency virus (HIV) preferentially destroys CD4+ T lymphocytes and interferes with other regulatory mechanisms of the immune system, thereby weakening defenses against infectious agents (Fauci et al., 1991). Highly active antiretroviral therapy (HAART) restores CD4+ T cell numbers and decreases the incidence of opportunistic infections including cytomegalovirus (CMV) (Austran et al., 1997; Hammer et al., 1997; Palella et al., 1998). Although anti-CMV therapy can be generally discontinued in patients who achieve CD4+ counts ≥100 cells/µl after ≥3 months of HAART (Jabs et al., 1998; Macdonald et al., 1998; Whitcup et al., 1999), CMV eye lesions occasionally occur in these patients despite high CD4+ cell numbers, usually in association with abnormal CMV-specific cell-mediated immunity (CMI) (Jacobson et al., 2000; Karavellas et al., 1998, 1999; Zegans et al., 1998). Other findings also indicate that immune reconstitution in HAART recipients is incomplete. These include observations such as incomplete recovery of CMI to varicella–zoster virus (Weinberg et al., 2004b) and to candida (Weinberg et al., 2004a) and decreased immune responses to immunizations (Lederman et al., 2003; Valdez et al., 2002; Weinberg et al., 2003a). Taken together, these findings suggest that HAART recipients might benefit from additional interventions to boost their immune responses.

We previously showed that CMV-specific proliferative responses [lymphocyte proliferation assay (LPA)] are down regulated in HIV-infected patients on HAART to a greater extent than other CMI components, such as IFN-γ production (Weinberg et al., 2001). We and others have shown that the down regulation of CMV-LPA in HIV-infected patients...
on HAART is associated with deficient IL2 production (Iyasere et al., 2003; Weinberg et al., 2003b). In vitro CD8+ T cell depletion partially restored CMV-specific proliferation, which led to the hypothesis that nonspecific regulatory mechanisms associated with chronic HIV infection contributed to the LPA deficit.

In this study, we examine the role of apoptosis in the down regulation of CMI in HAART recipients and the effect of interventions that rescue peripheral blood mononuclear cells (PBMC) from programmed cell death. Apoptosis, which remains elevated in HIV-infected patients on HAART (de Oliveira Pinto et al., 2002), is used by the host to modulate immune responses and avoid excessive activation (Bouillet et al., 2002; Janssens et al., 2003; Khaled and Durum, 2002). This involves both the death receptor and the mitochondrial pathways of apoptosis (Bouillet et al., 2002; Janssens et al., 2003). Furthermore, TRAIL and FAS receptor–ligand interactions, which commonly mediate the activity of regulatory CD4+ T cells, were shown to play an important role in spontaneous PBMC apoptosis in HIV-infected patients (Badley et al., 1998; Lum et al., 2001). To assess the role of apoptosis in the residual defect of CMI in HAART recipients, we examined the correlations between caspase activity and CMV-LPA and the effect of caspase inhibitors on CMV-specific proliferation in HIV-infected patients on HAART and uninfected controls.

Results

**HIV-infected patients on HAART have persistently high levels of unstimulated PBMC apoptosis**

The caspase 3 activity of unstimulated PBMC obtained from 12 HAART recipients was significantly higher compared with that of 11 uninfected controls (mean ± SE of 292 ± 72 and 87 ± 19 nmol/min/mg, respectively; P = 0.02, unpaired t test) (Fig. 1). Furthermore, caspases 8 and 9 activities in unstimulated PBMC from six HIV-infected patients had mean ± SE of 16 ± 10 and 30 ± 17 nmol/min/mg, respectively, whereas caspase 8 or 9 activity was not detected in unstimulated PBMC obtained from four healthy controls (Fig. 1).

To characterize the cells responsible for the excess apoptosis, we used annexin V binding assays. There was a highly significant linear relationship between caspase activity and annexin V binding of unstimulated PBMC (P = 0.004, r² = 0.88), indicating that both assays measured the same biologic phenomenon. Furthermore, the percentage of total PBMC that bound annexin V was significantly higher in seven HIV-infected patients with HIV viral load (VL) <200 RNA copies/ml compared with six uninfected controls (mean ± SE of 40 ± 5% vs. 18 ± 2%, P = 0.005, unpaired t test; Fig. 2). The excess apoptosis in patients was due to increased percentage of apoptotic CD4+ T lymphocytes (15 ± 1% vs. 3 ± 1%, P = 0.0008) and CD14+ monocytes (35 ± 2% vs. 16 ± 2%, P = 0.002), but not of CD8+ T lymphocytes (19 ± 2% vs. 16 ± 3%, P = 0.41) or CD19+ B lymphocytes (13 ± 3% vs. 13 ± 2%, P = 0.79). Taken together, these data indicated that in spite of HAART and undetectable viral replication, HIV-infected patients had persistently increased levels of apoptosis of CD4+ and CD14+ unstimulated PBMC.

**Increased spontaneous apoptosis correlates with decreased CMV-specific proliferation in HIV-infected patients on HAART**

Increased caspase 3 activity of unstimulated PBMC was significantly associated with lower CMV-specific proliferative responses, measured by ³H-Thy incorporation, in 12 HIV-infected patients (P = 0.04; r² = 0.37; Fig. 3A). In contrast, an association between spontaneous apoptosis and CMV LPA could not be demonstrated in eight uninfected controls (P = 0.64; r² = 0.03; Fig 3B).

**Immunologic and virologic correlates of apoptosis and of CMV-LPA**

To determine if the association between CMV-specific proliferation and apoptosis of unstimulated PBMC was independent of HIV replication and CD4+ T cell depletion, we examined the correlations of CMV-LPA and apoptosis with HIV VL and CD4+ T cell counts in 12 HIV-infected patients (Fig. 4). The data showed a lack of association between caspase 3 activity and CD4 counts (P = 0.27; r² = 0.12) and a significant increase of caspase 3 activity with higher HIV VL (P = 0.008; r² = 0.52). However, the caspase 3 activity in unstimulated PBMC from seven HIV-infected patients with undetectable HIV VL (<200 RNA copies/ml) remained significantly higher when compared with uninfected controls (mean ± SE of 422 ± 90 vs. 250 ± 100, P = 0.002) and CD8+ T lymphocytes (19 ± 2% vs. 16 ± 3%, P = 0.41) or CD19+ B lymphocytes (13 ± 3% vs. 13 ± 2%, P = 0.79). Taken together, these data indicated that in spite of HAART and undetectable viral replication, HIV-infected patients had persistently increased levels of apoptosis of CD4+ and CD14+ unstimulated PBMC.
We could not demonstrate an association between caspase 3 activity and duration of HAART (linear correlation \( P = 0.33, r^2 = 0.14 \)) or specific drugs included in the ART. With respect to lamivudine, which has been previously implicated in increased PBMC apoptosis (de Oliveira Pinto et al., 2002), the difference of caspase 3 activity in PBMC from three patients on lamivudine-containing HAART compared with nine patients on lamivudine-free HAART was not significantly different between groups.

In contrast to apoptosis, CMV-specific proliferation was significantly associated with CD4+ T cell counts (\( P = 0.03; r^2 = 0.41 \)), but not with HIV VL (\( P = 0.21; r^2 = 0.15 \)). Hence, proapoptotic factors other than HIV replication probably contribute to the down regulatory effect of spontaneous apoptosis on CMV LPA. Similarly, since there was no significant association between CD4+ T cell counts and spontaneous apoptosis, we conclude that the down regulatory effect of apoptosis on CMV LPA is not due to CMV-specific T cell depletion, but rather involves a deficit of T cell function.

**Caspase 3 activity of CMV-stimulated PBMC increases with proliferation**

To further assess the contribution of apoptosis to CMV-LPA down regulation in HIV-infected patients, we measured the caspase 3 activity in CMV-stimulated PBMC cultures from HAART recipients and healthy controls. Contrary to our expectations, the caspase 3 activity increased with proliferation both in HIV-infected patients (\( P = 0.002; r^2 = 0.63 \)) and in uninfected controls (\( P = 0.004; r^2 = 0.46 \)) (Fig. 5). This indicated that proliferation constitutes a very potent stimulus of caspase 3 activity obfuscating any preexisting differences between HIV-infected patients and uninfected controls.

**Effect of Inhibitors of apoptosis on PBMC proliferation**

We studied the effect of biological and chemical compounds, which disrupt the apoptotic cascade at different steps (Table 1). To determine the effect of blocking agents that interfere with signaling through the cell membrane death receptors, CMV- and mock-stimulated PBMC cultures were treated with anti-Fas L or with TRAIL R1/R2. Results were compared with proliferation in untreated cultures. We chose to block the ligands rather than the receptors on the T cell surface in order to avoid partial agonistic effects. The data showed that blocking the in vitro interactions between these cell death receptors and their cognate ligands did not affect proliferation of CMV-stimulated (Table 1) or control-stimulated PBMC (data not shown) of HIV-infected patients and uninfected controls.

The effect of caspase inhibitors on in vitro PBMC proliferation was determined (Table 1). Pan-caspase, caspase 3, and caspase 8 inhibition decreased proliferation of CMV-stimulated PBMC of HIV-infected patients and healthy individuals (\( P = 0.05 \) and 0.007 in HIV+ and HIV− individuals, respectively, for pan-caspase inhibition, ANOVA for repeated measures; \( P < 0.001 \) and \( P = 0.03 \)).
0.005, respectively, for caspase 3 inhibition; and \( P = 0.05 \) and 0.005, respectively, for caspase 8 inhibition). Only the caspase 3 inhibitor significantly decreased proliferation of mock-stimulated PBMC from either group of subjects (data not shown). These data suggest that one or more caspases play active roles in PBMC proliferation and are consistent with the increase of caspase 3 activity associated with antigen-driven PBMC proliferation shown in Fig. 5. The down-regulatory effect of certain caspase inhibitors on PBMC proliferation negates their utility in the effort to unravel the interactions between apoptosis and proliferation.

CrmA had the unique effect of significantly increasing proliferation of CMV-stimulated PBMC from HIV-infected patients, but not from uninfected subjects (\( P \) values of 0.02 and 0.5, respectively). CrmA also increased proliferation in mock-stimulated PBMC from HIV-infected subjects (\( P = 0.03 \)), but not from healthy donors (\( P = 0.97 \)). The increase of CMV-specific proliferation of PBMC from HIV-infected patients generated by in vitro treatment with 10 ng/ml of

![Fig. 5. Caspase 3 activity increases with proliferation of CMV-stimulated PBMC both in HIV-infected patients on HAART (\( P = 0.002; r^2 = 0.63 \)) and in uninfected controls (\( P = 0.004; r^2 = 0.46 \)).](image)

**Table 1**

Differential effect of apoptosis inhibitors on CMV-specific PBMC proliferation in HIV-infected patients and uninfected controls

<table>
<thead>
<tr>
<th>Compound</th>
<th>HIV infected</th>
<th>Uninfected</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td>Anti-Fas L (10 ( \mu )g/ml)</td>
<td>3.0 ± 0.5(^a)</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>TRAIL R1 and R2 (100 ( \mu )g/ml each)</td>
<td>2.0 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Z-VAD.fmk (100 ( \mu )M)</td>
<td>3.2 ± 0.9(^b)</td>
<td>2.9 ± 0.9</td>
</tr>
<tr>
<td>Anti-caspase 3 (30 ( \mu )M)</td>
<td>3.6 ± 0.6</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Anti-caspase 8 (33 ( \mu )M)</td>
<td>2.1 ± 0.4</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>CrmA (10 ng/ml)</td>
<td>2.7 ± 0.4(^c)</td>
<td>3.2 ± 0.3</td>
</tr>
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</table>

\(^a\) Data represent mean ± SE of \( \log_{10} \) CPM of CMV-stimulated PBMC obtained from six donors. Multiple concentrations of the inhibitory compounds were used but only one (indicated in parentheses) is denoted in the table. The following drug concentrations were used in these experiments: anti-Fas L at 1.1, 3.3, and 10 \( \mu \)g/ml; TRAIL R1/R2 mixed at 0/100, 100/0, and 100/100 \( \mu \)g/ml; Z-VAD.fmk pan-caspase inhibitor at 50 and 100 \( \mu \)M; DEVD-CHO caspase 3 inhibitor at 3.3, 10, and 30 \( \mu \)M; Z-IETD.fmk caspase 8 inhibitor at 11, 33, and 100 \( \mu \)M; and crmA at 0.1, 1, and 10 ng/ml.

\(^b\) Bold-faced figures indicate significant differences by ANOVA for repeated measures (\( P \leq 0.05 \)).

\(^c\) Italics indicate significant proliferation increases in treated cultures.
The data showed that death cell receptor blockade did not affect PBMC proliferation of HIV-infected patients, but that crmA inhibition, which blocks the mitochondrial pathway of apoptosis at the caspase 9 level (caspase 8 and others) in addition to the death cell receptor pathway, increased proliferation. Furthermore, it indicated that rescuing the PBMC from apoptosis partially corrected their proliferation deficit.

Discussion

Our data demonstrate that apoptosis of unstimulated PBMC, which remains elevated in HIV-infected patients on HAART, contributes to the persistence of functional immunologic defects in HAART recipients. This conclusion was supported by the observation that apoptosis of unstimulated PBMC from HIV-infected patients was significantly associated with decreased CMV-stimulated proliferation and that crmA, which rescued PBMC from apoptosis, increased proliferation in HAART recipients. Furthermore, the cells responsible for excess PBMC apoptosis in HAART recipients with undetectable HIV VL, CD4+ T lymphocytes and CD14+ monocytes, play critical roles in antigen-driven proliferation, providing a mechanistic explanation for the association between apoptosis and decreased proliferation.

The down regulatory effect of PBMC apoptosis on CMV-specific proliferation in HAART recipients was independent of CD4 depletion. Although CMV LPA increased with higher CD4 counts and decreased with higher spontaneous caspase 3 activity, we did not find an association between caspase 3 activity or CD4-annexin V binding (data not shown) in PBMC from HIV-infected patients and their CD4+ T cell counts. These data suggest that the mechanism by which apoptosis down regulates CMV-specific PBMC proliferation does not involve depletion of CMV-specific memory cells. The alternative explanation is that proapoptotic stimuli limit proliferation of PBMC from HAART recipients but not from uninfected individuals, which is also consistent with the excess apoptosis detected in unstimulated PBMC from HIV-infected patients.

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To gain a better insight into the mechanisms that increase apoptosis and decrease PBMC proliferation in HAART recipients, we used inhibitors of apoptosis in our in vitro experimental model. Our attempts to increase proliferation by blocking the Fas/FasL and TRAIL/cognate receptors interactions were unsuccessful. We chose these cell death pathways because they have been previously shown to contribute to increased apoptosis in HIV-infected patients (Badley et al., 1998; Lum et al., 2001). Since these manipulations were performed in vitro, our data do not exclude the in vivo contributions of Fas and/or TRAIL pathways to PBMC apoptosis. Nevertheless, these cell death receptors seem to play a minor role in the down regulation of antigen-driven PBMC proliferation.

Pan-caspase, as well as caspase-3- and caspase-8-specific inhibition, decreased proliferation. Although this effect seems paradoxical from the standpoint of inhibition of apoptosis, it is consistent with previous observations (Alam...
et al., 1999; Chun et al., 2002) that some caspases actively participate in PBMC proliferation. An alternative explanation consists of the possibility that these caspase inhibitors might have a cross-reactive blocking effect against other cellular proteases involved in proliferation. However, the engagement of caspases in PBMC proliferation was also supported by the fact that caspase 3 activity increased with CMV-specific proliferation in HIV-infected patients and uninfected controls.

Our data suggest that the mitochondrial pathway might be involved in PBMC apoptosis and CMI down regulation of HIV-infected patients on HAART. Caspase 9, which is recruited into the apoptosis cascade downstream of the mitochondrial membrane depolarization, was active in unstimulated PBMC from HIV-infected patients on HAART but not in uninfected controls. Furthermore, the crmA-mediated inhibition of caspase 9, unlike the death cell receptor blockade, led to modest but significant increases in proliferation and decreases in apoptosis of PBMC from HAART recipients. More importantly, this effect was specific for HIV-infected patients in the sense that it was not reproduced in healthy controls.

Several mechanisms may activate apoptosis in HIV-infected patients. Firstly, HIV replication has been associated with recruitment of the mitochondrial apoptotic pathway via overexpression of p53 (Genini et al., 2001). A direct effect of HIV replication is supported by the association of caspase 3 activity in unstimulated PBMC with the HIV VL. However, CMV-specific proliferation was not significantly associated with HIV VL, suggesting that other proapoptotic factors, independent from HIV replication, may contribute to the down regulation of T cell function. Secondly, apoptosis and decreased proliferation may occur as a consequence of insufficient T cell growth cytokine production, which has been previously described in HIV-infected patients (Adachi et al., 1996; Jaleco et al., 2003; Zaunders et al., 2003). Thirdly, regulatory T cells might be involved in this process. We have previously shown that CD8+ T cell depletion increases CMV-specific proliferation in HIV-infected patients (Weinberg et al., 2001), which would be consistent with a CD8+ T cell regulatory effect on proliferation (Cosmi et al., 2003; Najafian et al., 2003). CD8+ regulatory cells might use granzyme B-mediated cytotoxicity as an effector of their suppressive activity. Granzyme B induces apoptosis primarily through the mitochondrial pathway (Trapani and Smyth, 2002) and its effect can be inhibited by crmA (Quan et al., 1995). This last mechanism is also consistent with our previous observations correlating activated CD8+ T cells with lack of antigen-driven proliferation in HIV-infected children on HAART (Weinberg et al., 2004a). The three potential CMI down regulatory mechanisms outlined above are not mutually exclusive. Further studies are warranted to elucidate their relative importance and measures capable of reverting their effect on CMI of HIV-infected patients.

Subjects and methods

Subjects

This study enrolled 55 CMV-seropositive individuals including 39 HIV-infected patients on HAART and 16 uninfected controls. Inclusion criteria for HIV-infected patients were the following: HAART, defined as ≥3 drug regimens including ≥2 antiretroviral classes for ≥3 months; CD4+ cell count <100 cells/µl before HAART; and ≥100 cells/µl when entered into the study. The median CD4+ cell count of the HIV-infected patients in this study was 259 cells/µl and the maximum was 1504 cells/µl. The HIV VL on study varied between <10^1.3 and >10^5.9 copies/ml with a median of 10^2.6 copies/ml. The mean ± SD time on HAART was 39 ± 22 months.

Monoclonal antibodies (MAb) and reagents

The MAb and reagents used in this study were as follows: anti-human Fas Ligand (R&D Systems), APC-conjugated anti-CD4, CD8, CD14, and CD19 (Pharmingen), caspase inhibitor zVAD (Z-Val-Ala-Asp(OMe)-FMK (Enzyme Systems Products), recombinant human TRAIL R1/Fc Chimera and TRAIL R2/Fc Chimera (R&D Systems), caspase 3 inhibitor I DEVD-CHO (Calbiochem), caspase 8 inhibitor Z-IETD-FMK (R&D Systems), and crmA (Sigma), which inhibits caspase 9 and other caspases.

Specimen processing and cryopreservation

Peripheral blood mononuclear cells from heparinized blood, separated by Ficoll/histopaque density gradient centrifugation (Sigma), were counted and cryopreserved as previously described (Weinberg et al., 2000). In brief, PBMC were resuspended at 10^7 cells/ml in cold fetal bovine serum (FBS, HyClone) with 10% dimethyl sulfoxide (DMSO, Sigma). A Mr. Frosty freezing container (Fisher) gradually brought the cells to −70°C. After 24 h, the cells were transferred to liquid nitrogen for long-term storage. For use in assays, cells were removed from the liquid nitrogen and thawed in 37°C water bath until a small amount of cells remained frozen. The slow addition of RPMI 1640 with glutamine (Gibco) and 10% human type AB serum (Gibco). A Mr. Frosty freezing container (Fisher) gradually brought the cells to −70°C. After 24 h, the cells were washed twice and resuspended 10^7/ml final concentration with RPMI 1640 with glutamine (Gibco).

Lymphocyte proliferation assay (LPA)

LPA was performed as previously described (Weinberg et al., 2001). In summary, stimulation medium consisted of RPMI 1640 with glutamine (Gibco), 10% human AB serum (Nabi), and 1% antibiotics (Gibco). PBMC (10^5 cells/well) were added to quadruplicate wells containing CMV antigen, prepared as previously described (Weinberg et al., 1998), at
a 1:200 final concentration. Mock-infected antigen and pokeweed mitogen (Sigma) were used as negative and positive controls at dilutions of 1:200 and 10 μg/ml final, respectively. MAb and compounds used to modulate cell proliferation were added to the stimulation medium in the concentrations specified in the Results section. After 6 days of culture at 37°C in 5% CO₂ atmosphere, cells were pulsed with ³H-thymidine and their DNA was harvested 6 h later onto unifilter plates (Perkin Elmer). Radioactivity gathered on the filters was counted on a microplate scintillation counter (Packard).

**Caspase activity assays**

The activity of caspases 3, 8, and 9 were determined by use of fluorescent substrates as previously described (Melnikov et al., 2001, 2002) with modifications. Two million lymphocytes were mixed with a lysis buffer containing 25 mM Na⁺ Hepes, 2 mM dithiothreitol (DTT), 1 mM EDTA, 0.1% 3-[3-(cholamidopropyl) dimethylammonio]-1-pro-panesulfonate (CHAPS), 10% sucrose, 1 mM phenylmethylsulfonyl fluoride, and 1 μM peptatin A, pH 7.2. The lysates were immediately frozen and stored at −70°C until use. Lysate protein was measured by the Bio-Rad Detergent Compatible (DC) protein assay kit with bovine serum albumin as standards. The caspase assay was performed on lysate as follows: 40 μl of lysate (50–100 μg protein) and 10 μl of the substrate (final concentration, 25 μM) were added to 150 μl caspase assay buffer. The assay buffer for caspase 3 contained 250 mM K⁺ Hepes, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 0.1% CHAPS, pH 7.4. Ac-Asp-Glu-Val-Asp-7-amido-4-methyl coumarin (Ac-DEV-D-AMC) in 10% DMSO was used as a susceptible substrate for caspase 3 (Thornberry et al., 1997). The assay buffer for caspases 8 and 9 contained 75 mM Na⁺ MOPS, 10% glycerol, 1 mM DTT, 1 mM EDTA, pH 7.4. Ac-Ile-GLu-Pro-Asp-7-Amino-4-methylcoumarin (IEPD-AMC) in 10% DMSO was used as a susceptible substrate for caspase 8 (Autran et al., 1997). Ac-Leu-Glu-His-Asp-7-Amino-4-methylcoumarin (LEHD-AMC) in 10% DMSO was used as a susceptible substrate for caspase 9 (Autran et al., 1997). The solution was preincubated for 10 min at 30°C before substrate was added. The reaction was then initiated by addition of substrate. Peptide cleavage was measured over 1 h at 30°C using a Cytofluor 4000 series fluorescent plate reader (Perceptive Biosystems) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. An AMC standard curve was determined for each experiment. Caspase activity was expressed in nmol of AMC released per min of incubation time per mg of lysate protein.

**Annexin V flow cytometry assay**

TSCS annexin V-FITC apoptosis detection kit (R&D Systems) was used for quantification of PBMC apoptosis. Samples containing 1.0 × 10⁵ to 1.0 × 10⁶ PBMC were centrifuged at 500 g for 10 min, washed with cold PBS, centrifuged, and resuspended in 100 μl of Annexin V Incubation Reagent containing Annexin V-FITC (100× final concentration) and propidium iodide (PI). After 15 min of incubation in the dark, 400 μl of 1× binding buffer were added. Samples were analyzed using a FACSScan flow cytometer (Becton Dickinson).

**Statistical analysis**

Statistical analysis was performed using Instat3 and Prism software (Graph Pad) with a level of significance established at P ≤ 0.05. Distribution of results was checked for normality and parametric tests were preferentially applied when the data had a normal distribution.

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