Interleukin-15 is a potent survival factor in the prevention of spontaneous but not CD95-induced apoptosis in CD4 and CD8 T lymphocytes of HIV-infected individuals. Correlation with its ability to increase BCL-2 expression

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Abstract

IL-15 shares many biological properties with IL-2, a cytokine whose administration to HIV-infected individuals has been effective in enhancing depleted CD4 T lymphocyte numbers. The present study examined whether exogenous IL-15 could protect lymphocytes of HIV-infected individuals from spontaneous apoptosis, associated with growth factor deprivation, and CD95-induced apoptosis, which is believed to play a major role in T lymphocyte loss and HIV pathogenesis. Although IL-15, like IL-2, failed to inhibit CD95-induced lymphocyte apoptosis *in vitro*, IL-15 was found to act as a potent survival factor in the prevention of spontaneous apoptosis. The greater enhancement of lymphocyte survival, promoted by IL-15 as compared with IL-2 when used at an equivalent concentration, was associated with higher up-regulation of bcl-2 expression. In addition, IL-15 was more potent than IL-2 in stimulating lymphocyte proliferation. Despite the strong ability of IL-15 to promote both lymphocyte survival and proliferation, the increases in representation and total numbers of viable cells induced by IL-15 were not higher than those induced by IL-2. This appears to be associated with the greater ability of IL-15 to activate lymphocytes and increase their apoptosis-susceptibility. Therefore, lymphocyte loss occurring by growth factor deprivation in HIV infection may be potentially prevented by IL-15, although its benefits for survival need to be closely assessed against its ability to augment lymphocyte activation.

Keywords: interleukin-15; HIV; apoptosis; CD4$^+$ and CD8$^+$ T lymphocytes; bcl-2

Abbreviations: 7-AAD: 7-amino actinomycin D; mAbs: monoclonal antibodies; MFI: mean fluorescence intensity; PCNA: proliferating cell nuclear antigen; PE: phycoerythrin

Introduction

IL-15 exhibits many activities in common with IL-2, including stimulating T cell proliferation, induction of cytolytic effector cells and NK cell activity.[1–3] The overlapping biological activities of IL-15 and IL-2 are attributable to the utilization by these cytokines of two common receptor chains β and γ.[4] The two cytokines differ in their cellular sources. IL-2 is primarily produced by activated T cells, whereas IL-5 is expressed by a wide variety of cells and tissues such as activated monocytes, placenta, skeletal muscle, kidney, lung and heart.[1,2]

Administration of IL-2 to HIV-infected individuals has been shown to significantly enhance CD4$^+$ T cell numbers.[5] Although disadvantages of IL-2 therapy such as toxicity and enhanced HIV replication have been addressed, at least in part, by improved regimes involving lower IL-2 dosages and the combined use of anti-retroviral agents, recent attention has turned towards IL-15 as a possible alternative immunotherapy. Various reports indicate that IL-15 has less of a stimulatory influence on HIV expression than IL-2.[6,7] IL-15 reportedly stimulates expansion of HIV-specific CTL[8] and enhances activities of NK and LAK cells which are impaired in HIV-infected individuals.[6,7] In addition, studies in mouse models indicate that IL-15 has a therapeutic index superior to IL-2 when toxicity of these cytokines was assessed against their efficacy in inducing cytolytic activity.[9] To date, most studies regarding the immunotherapeutic potential of IL-15 have focused on its ability to enhance cytolytic activities. However, little is known regarding the potential of IL-15 to control lymphocyte apoptosis in HIV infection. While apoptosis plays essential roles in immune regulation, it can be pathogenic where it interferes with the generation and renewal of effector cells, as appears to be the case in HIV infection.[10,11] The causes of lymphocyte apoptosis in HIV-infected individuals are multi-factorial and are believed to include TCR/CD3 triggering in the absence of appropriate co-stimulation, CD95-CD95 ligand interactions and gp120-CD4 interactions.[10,11] In addition, alterations in the profile of cytokine production and deprivation of growth factors arising from deletion of lymphocyte populations can contribute to further lymphocyte loss.[11,12]

Freshly isolated lymphocytes of HIV-infected individuals are highly susceptible to apoptosis following short-term culture in the absence of exogenous cytokines, commonly referred to as spontaneous apoptosis, and to apoptosis induced *in vitro* by mitogens, recall antigens and monoclonal antibodies (mAbs) directed against the CD3 and CD95 molecules.[13–15] This marked apoptosis-susceptibility is believed to be associated with the

chronically activated state of lymphocytes of HIV-infected individuals.[15] In contrast, lymphocytes of healthy, uninfected individuals do not exhibit a highly activated state *ex vivo*, and exhibit little or no spontaneous apoptosis and CD3- and CD95-induced apoptosis following culture.[13–15] Various cytokines appear to influence apoptosis susceptibility. Exogenous IL-12 reportedly inhibits CD3- and CD95-induced death of lymphocytes of HIV-infected individuals *in vitro*,[16,17] whereas IL-2 protects against spontaneous apoptosis.[18,19] In addition, recent studies found IL-15 to be more effective than IL-2 in protecting against lethal CD95-induced hepatic failure in mice.[20] In view of the detrimental role which lymphocyte apoptosis plays in HIV pathogenesis, it was of interest in the present study to examine the effects of exogenous IL-15 on apoptotic death of lymphocytes of HIV-infected individuals. Although IL-15 appears to act as a potent survival factor in the prevention of spontaneous apoptosis, and also promotes lymphocyte proliferation, IL-15 failed to block CD3- and CD95-induced death, and its beneficial effects on survival and proliferation need to be considered against its ability to further increase the lymphocyte activation state.

Results

Effect of exogenous IL-15 on apoptotic death of lymphocytes of HIV-infected individuals induced by anti-CD95 mAbs

Our laboratory and other investigators have observed that freshly isolated lymphocytes of HIV-infected individuals undergo marked apoptosis when cultured overnight with mAbs against the CD95 molecule.[14,22,25] In contrast, little or no anti-CD95-induced apoptosis has been observed in lymphocytes of uninfected persons.[14,22,25] In preliminary experiments we observed that the level of apoptosis induced in PBMC of HIV-infected individuals with immobilized anti-CD95 mAbs was stable from day 1–3. In contrast, the proportion of apoptotic lymphocytes in cultures incubated in medium alone markedly accelerated after day 1, as previously found,[15] and approached the level observed in anti-CD95-stimulated cultures by day 3. Effect of exogenous IL-15 on anti-CD95-induced apoptosis was therefore examined at day 1, since perceived reductions in apoptosis in cultures incubated beyond day 1 would become increasingly likely to be attributable to cytokine-mediated rescue from spontaneous apoptosis.

PBMC were incubated for 1 day in the absence or presence of anti-CD95 mAbs, and apoptosis assessed by immunofluorescence analysis of 7-AAD-staining within CD4+ and CD8+ populations. Responses to exogenous IL-15 were compared with those to IL-2, IL-12, and IL-10 at a final concentration of 10 ng/ml, which have been previously studied.[16,20,26] Although the level of apoptosis occurring spontaneously in medium alone at day 1 was much lower than that induced by anti-CD95 mAbs, a statistically significant reduction in spontaneous apoptosis was observed in both the CD4+ (Figure 1) and CD8+ (not shown) subsets by the addition of IL-15. Spontaneous
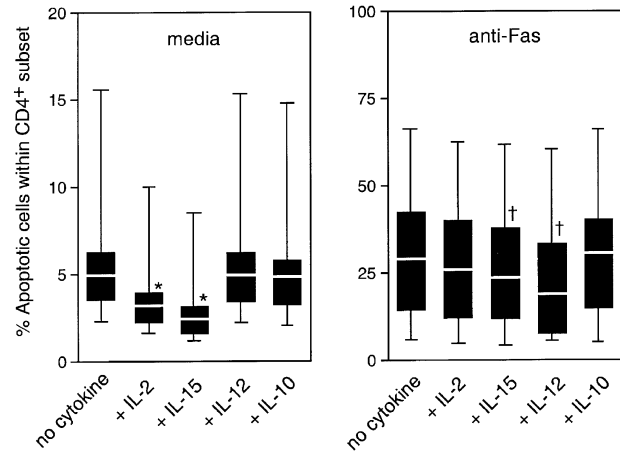
Figure 1 Differential effects of IL-15 and other exogenous cytokines on CD95-induced apoptosis. PBMCs of HIV-infected individuals were incubated for 1 day in the absence or presence of exogenous IL-2, IL-15, IL-12 and IL-10 at a final concentration of 10 ng/ml in uncoated wells, or in wells coated with immobilized anti-CD95 mAbs. Cells were stained with 7-AAD and either anti-CD4 or anti-CD8 mAbs, and the percentage of 7-AAD-stained cells in each subset was determined by dual-colour immunofluorescence analysis. Shown are the median (25th-75th percentiles) values of apoptotic 7-AAD-stained cells within the CD4+ and CD8+ subsets of the patient group (n=18). Statistical significance was assessed by the Wilcoxon's signed rank test for paired data. *P < 0.0005, †P < 0.05

apoptosis was likewise reduced by IL-2 addition (Figure 1), as previously observed.[18,19] IL-12 partially inhibited CD4+ T cell apoptosis induced by CD95-triggering, as reported by others.[16,17] Significant, but only modest, inhibition by IL-10 of anti-CD95-induced CD8+ T cell death was also observed (not shown), which could explain the partial inhibition found in one study[27] and the weakness reported by others.[26] Modest decreases in the proportion of apoptotic T cells were detected in response to IL-15 and to IL-2 in anti-CD95-stimulated cultures. However, such diminution was not consistently observed for all patients, and was only marked in lymphocytes of patients which also exhibited an appreciable reduction in spontaneous apoptosis in response to IL-15 and to IL-2 (Figure 1). This suggests that the perceived reduction in apoptosis in anti-CD95-stimulated cultures could be largely attributable to prevention by these cytokines of spontaneous death of lymphocytes which were otherwise not induced to die by CD95-triggering.

### Inhibitory effect of exogenous IL-15 on spontaneous lymphocyte apoptosis

The above findings indicated that exogenous IL-15 neither directly nor strongly inhibits CD95-induced death of lymphocytes of HIV-infected individuals, but could potentially protect lymphocytes from spontaneous apoptosis. Although the reduction in spontaneous apoptosis by IL-15 was statistically significant, the level of spontaneous apoptosis seen in the absence of exogenous cytokines at day 1 was not appreciably high. Addition of IL-15 at 10 ng/ml clearly reduced the level of spontaneous apoptosis within the CD8+ subset, as indicated by the reduced

percentage of 7-AAD-stained cells (Figure 2B) and the reduced pool of cells exhibiting small size and high granularity (Figure 2C). Exogenous IL-15 was more effective in reducing the percentage of apoptotic cells within the CD8⁺ subset than within the CD4⁺ subset, as was likewise observed for IL-2 (Figure 2A,B). Effects of cytokines were also compared at different concentrations ranging from 1 to 100 ng/ml. IL-15 was more effective than IL-2 in reducing spontaneous apoptosis , particularly within the CD8 subset at lower concentrations of the range tested, and less effective at higher concentrations (Figure 3A,B). Co-stimulation with IL-15 and IL-2 at equivalent concentrations failed to further reduce the percentage of apoptotic cells observed with stimulation with either cytokine alone (data not shown). IL-12 failed to block spontaneous apoptosis in both subsets when used at 10 ng/ml (Figure 2A,B) and at other concentrations (1, 3, 30 and 100 ng/ml) (data not shown). IL-10 was ineffective for CD4⁺ cells and weakly effective for CD8⁺ cells (Figure

2A,B). IL-4 and IL-7, which utilize the common γ receptor chain but not the β chain,[28] were less effective than IL-15 and IL-2, particularly for CD8⁺ cells (Figure 3A,B).

Potent stimulation of proliferation by IL-15

Forward *versus* side scatter profiles revealed substantial induction of large blastic cells in cultures stimulated with IL-15 and to a lesser extent with IL-2 (Figure 2C). The potent ability of IL-15 to stimulate proliferation was verified by measuring [³H]thymidine incorporation following 3 days stimulation. Proliferation of PBMCs was induced by IL-15 and IL-2 in a dose-dependent manner (Figure 3C). IL-15 was more effective than IL-2 at equivalent concentrations above 1 ng/ml, with differential potency being particularly apparent at 10 ng/ml. Only low levels of [³H]thymidine incorporation were detected in IL-12- and IL-10-stimulated cultures, confirming the poor induction by these cytokines of blastic cells distinguished by light scatter properties (Figure 2C), while
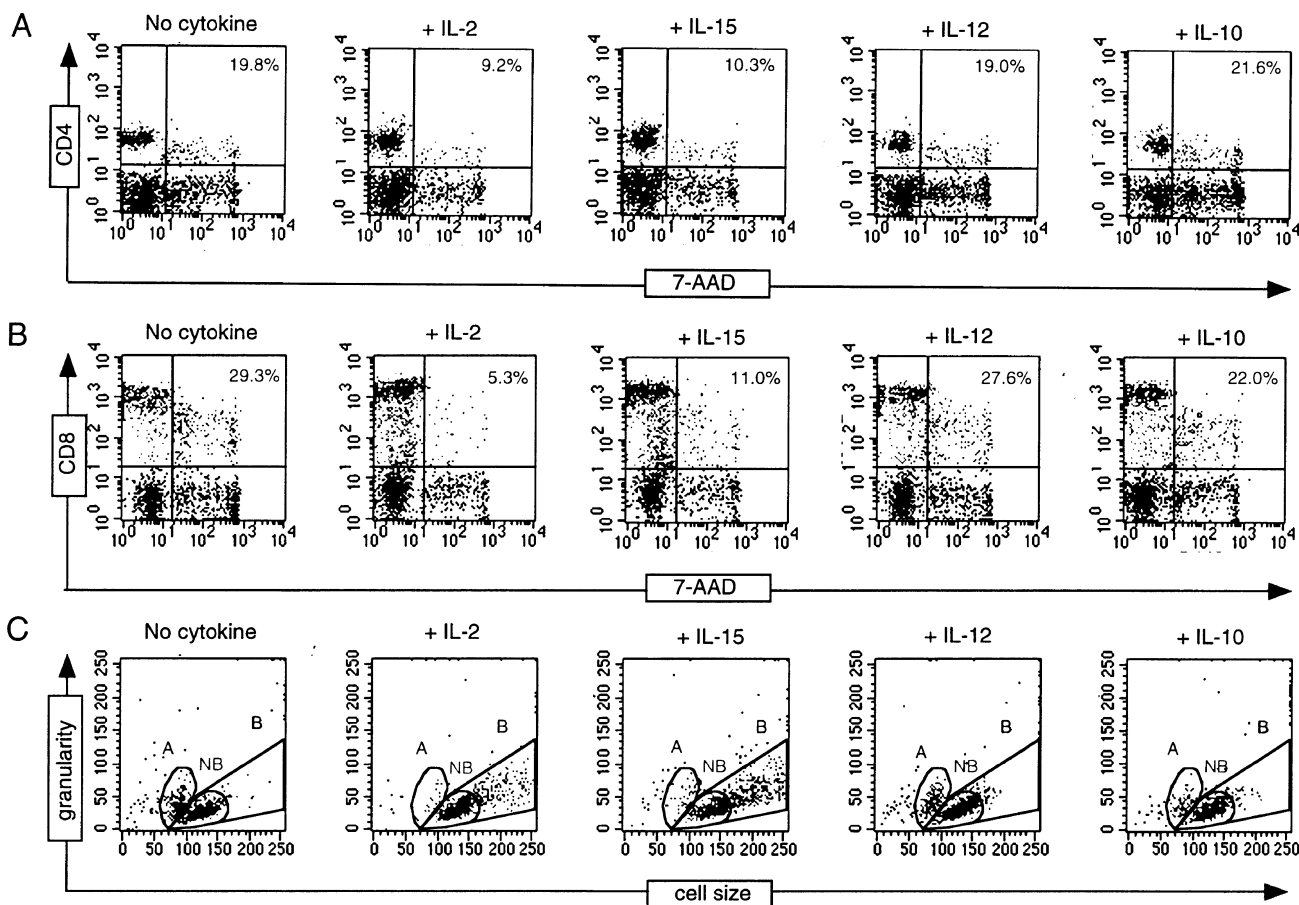
Figure 2 Differential effects of IL-15 and other exogenous cytokines on spontaneous apoptosis. PBMCs of HIV-infected individuals were incubated for 3 days in the absence of exogenous cytokines or with the addition of IL-2, IL-15, IL-12 or IL-10 at a final concentration of 10 ng/ml, followed by dual-colour immunofluorescence analysis of 7-AAD-staining within the CD4⁺ and CD8⁺ subsets. Shown are representative FACScan profiles indicating the percentage of 7-AAD-stained cells within the CD4⁺ (**A**) and CD8⁺ (**B**) populations of cultures of PBMCs derived from the same patient. The assessment of apoptosis by 7-AAD incorporation was verified by comparing proportions of apoptotic and living cell populations as distinguished by their distinct light scatter properties. Shown in **C** are typical forward *versus* side scatter profiles of cells gated on the CD8⁺ subset. Apoptotic cells (**A**), characterized by their small size and high granularity, almost totally comprised the population of 7-AAD-stained CD8⁺ cells shown in (**B**) whereas cells excluding 7-AAD primarily consisted of living non-blastic (NB) and larger blastic (**B**) cells
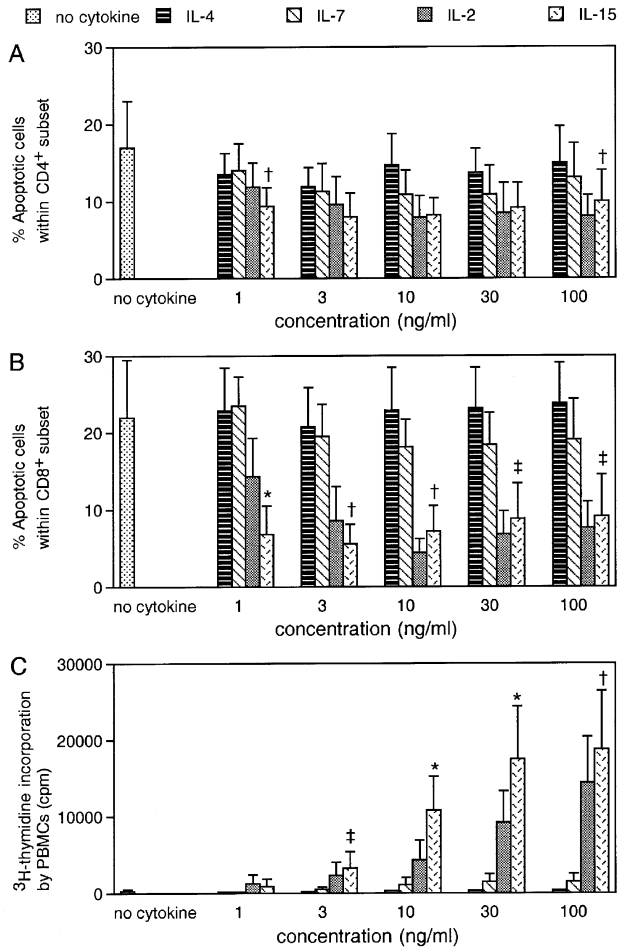
Figure 3 Concentration dependent-effects of exogenous cytokines on spontaneous apoptosis and on proliferation. PBMCs of HIV-infected individuals (n=15) were incubated for 3 days in the absence of exogenous cytokines, or with the addition of the indicated cytokines at final concentrations of 1, 3, 10, 30 and 100 ng/ml. Dual-colour immunofluorescence analysis was performed to determine the percentages of 7-AAD-stained cells within the CD4⁺ (**A**) and CD8⁺ (**B**) subsets. PBMCs of the same patients were also incubated for 3 days as described above and pulsed for the final 16 h with 1 μCi of [³H]thymidine. (**C**) proliferative responses are expressed in terms of [³H]thymidine incorporation (c.p.m.). Statistical significance between responses observed with IL-2 and with IL-15 at a given concentration are as follows: †$P<0.02$ *$P<0.01$, ‡$P<0.05$

proliferative responses induced by IL-4 and IL-7 were similarly weak (Figure 3C).

Greater proliferative responses to IL-15 fail to promote higher cell viability as compared with IL-2-mediated responses

While IL-15 potently stimulated proliferation in a dose-dependent manner (Figure 3C), the representation of apoptotic cells within lymphocyte populations of IL-15-stimulated cultures was observed not to decrease in a correspondingly dose-dependent manner (Figure 3A,B). This suggested that high cell loss occurs when cells actively proliferate in response to IL-15. This possibility was examined by assessing the total numbers of viable trypan blue dye-

excluding cells in unstimulated and cytokine-stimulated PBMC cultures at day 3. Despite their ability to stimulate proliferation, both IL-15 and IL-2 (10 ng/ml) failed to induce net increases in viable cell numbers during a 3-day culture period as compared with the initial cell count, although viable cell numbers were higher than observed in cultures incubated in the absence of exogenous cytokines (55 ± 18.10^4 with IL-15 vs 74 ± 18.10^4 with IL-2 ($P<0.05$) vs 36 ± 10.10^4 without cytokine compared to 10^6 cells initially put in culture, n=17). Furthermore, the total numbers of viable cells in IL-15-stimulated cultures at day 3 were lower than those in IL-2-stimulated cultures. The greater potency of IL-15 to stimulate proliferation, as compared with IL-2, is therefore not reflected by a corresponding higher net increase in viable cell numbers. In contrast to 3-day cultures, the total number of viable cells was higher in day-1 cultures stimulated with IL-15- than in IL-2-stimulated cultures (110 ± 16.10^4 with IL-15 vs 94 ± 10.10^4 with IL-2 ($P<0.05$) vs 80 ± 11.10^4 without cytokine), when the representation of blastic cells in these cultures was very small and barely exceeded that observed in cultures without cytokines.

Increased activation and apoptosis-susceptibility of IL-15-stimulated lymphocytes

The above findings indicated that more cell loss occurs when PBMCs were induced to proliferate by IL-15 than when stimulated with IL-2. This suggested that IL-15 could 'prime' lymphocytes for apoptosis, and that such increased apoptosis-susceptibility could be associated with its potent ability to activate lymphocytes and induce blastogenesis. To initially explore these possibilities, we examined more closely the relative effects of IL-15 and IL-2 on activation and blastogenesis within individual lymphocyte populations, since proliferative responses had been previously measured in the total PBMC population. Both CD4⁺ and CD8⁺ lymphocytes which had been stimulated with IL-15 or IL-2 for 3 days were more highly activated than lymphocytes incubated in the absence of exogenous cytokines, as evidenced by the increased representation of cells expressing HLA-DR (Figure 4A) and CD38 (data not shown). Both IL-15 and IL-2 induced higher representation of HLA-DR-positive cells and of blastic cells, as defined by their light scatter properties, within the CD8⁺ subset than within the CD4⁺ subset (Figure 4A,B). CD4⁺ and CD8⁺ populations in IL-15-stimulated cultures were more highly activated than those in IL-2-stimulated cultures (Figure 4A), and contained a higher representation of blastic cells (Figure 4B). The greater potency of IL-15, as compared with IL-2, to induce blastogenesis within the CD4⁺ and CD8⁺ subsets was confirmed by its ability to more highly up-regulate expression of the cell cycle-associated antigens PCNA and Ki-67, and also to induce surface expression of the early activation marker CD69 (data not shown) which strongly correlates with the degree of proliferative activity.[29]

To examine whether lymphocytes proliferating in response to IL-15 were more apoptosis-susceptible than lymphocytes induced to proliferate by IL-2, PBMCs pre-incubated without exogenous cytokines, or pre-activated with either IL-2 or IL-15 for 3 days, were subsequently washed in medium and incubated for a further period of 3
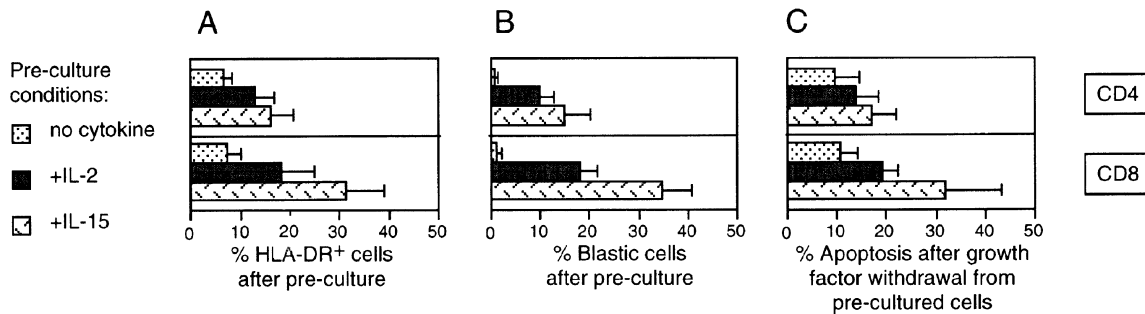
Figure 4 Effects of IL-15 and IL-2 on lymphocyte activation and blastogenesis, and susceptibility of cytokine-pre-activated lymphocytes to apoptosis. PBMCs of HIV-infected individuals (n=14) were pre-incubated for 3 days in the absence of exogenous cytokines or with the addition of IL-2 or IL-15 (10 ng/ml). In (**A**) the percentages of HLA-DR-positive cells within the CD4+ and CD8+ subsets following the pre-incubation period were determined by dual-colour immunofluorescence analysis. Shown in (**B**) are the percentages of blastic cells within each subset, as determined by their distinct scatter patterns as shown in Figure 2C. Following pre-incubation without exogenous cytokines or with IL-2 or IL-15, cells were washed twice in medium and incubated for a further period of 3 days in the absence of exogenous cytokines, at the end of which the percentages of apoptotic 7-AAD-stained cells within the CD4+ and CD8+ subsets were determined (shown in **C**)

days in the absence of exogenous cytokines at the end of which the percentages of apoptotic cells within lymphocyte populations were assessed by 7-AAD incorporation. Higher percentages of 7-AAD-stained apoptotic cells were found in lymphocyte populations which had been previously activated with either IL-15 or IL-2 than in populations which had not been pre-activated with cytokines, and IL-15-pre-activated lymphocytes demonstrated a higher propensity to undergo apoptosis following growth factor withdrawal than lymphocytes pre-activated with IL-2 (Figure 4C). It therefore appears that IL-15 stimulation can render lymphocytes more susceptible to apoptosis than IL-2 stimulation, and that such increased apoptosis-susceptibility appears to be associated with the greater ability of IL-15 to activate lymphocytes and induce blastogenesis.

IL-15 is a potent survival factor for lymphocytes. Influence of etoposide

Although total viable cell numbers in IL-15-stimulated PBMC cultures were lower than those in IL-2-stimulated cultures at day 3, the converse was observed at day 1 when little proliferation was detected in response to either cytokine, as discussed above. Furthermore, IL-15 was more effective than IL-2 in reducing the representation of apoptotic cells at day 3 when used at low concentrations at which only weak proliferative responses were induced by these cytokines (Figures 3A,B,C). These observations together suggested that IL-15 could be more effective than IL-2 in enhancing lymphocyte survival *per se*. The possibility that IL-15 could be more potent than IL-2 as a lymphocyte survival factor was confirmed by the abilities of IL-15 and IL-2 to decrease the pool of apoptotic cells within lymphocyte populations in 3-day PBMC cultures treated with etoposide. This topoisomerase II inhibitor causes a block in the S/G2 phase of the cell cycle and has been used to dissociate cytokine-induced survival from proliferation.[30] As shown in Figure 5, the effectiveness of etoposide in blocking CD4+ and CD8+ lymphocyte proliferation induced by IL-2 and IL-15 was clearly evident by the absence of large blastic cells as visualized on forward *versus* side scatter profiles. IL-15 was markedly more effective than

IL-2, when used at the equivalent concentration of 10 ng/ml, in reducing the percentage of apoptotic cells within the CD4+ subset, and particularly within the CD8+ subset, of etoposide-treated cultures (Figure 5).

Greater survival potency of IL-15 is associated with higher bcl-2 up-regulation

It is well-established that the bcl-2 molecule plays a crucial role in regulating lymphocyte survival.[31–33] Down-regulated expression of bcl-2 has been observed in lymphocytes of HIV-infected individuals undergoing spontaneous apoptosis[22] and the ability of exogenous IL-2 to inhibit such apoptosis is associated with up-regulation of bcl-2 expression.[19] We therefore investigated whether the greater enhancement of CD4+ and CD8+ lymphocyte survival induced by IL-15, as compared with IL-2, was associated with higher up-regulation of bcl-2 expression. This was assessed by examining changes in mean fluorescence intensity (MFI) of intracellular Bcl-2 staining, a method commonly used for heterogeneous cell populations.[12,22,23] As shown in Figure 6A, IL-15 stimulation led to increases in bcl-2 MFI within the gated CD4+ and CD8+ populations of etoposide-treated cultures, which were markedly higher than increases observed with IL-2 stimulation. Since etoposide itself further augmented apoptosis (Figure 5) leading to decreased bcl-2 expression, we confirmed the ability of IL-15 to more highly upregulate bcl-2 expression than IL-2 in cultures incubated without etoposide: MFI within CD4+ subset 135 ± 38 with IL-15 vs 122 ± 36 with IL-2 ($P < 0.05$) vs 91 ± 25 without cytokine, and MFI within CD8+ subset 165 ± 44 with IL-15 vs 138 ± 44 with IL-2 ($P < 0.05$) vs 76 ± 26 without cytokine. The relative reduction in levels of apoptosis within the CD4+ and CD8+ subsets in etoposide-treated cultures stimulated with IL-15 and IL-2 was found to correlate strongly with the relative increases in bcl-2 MFI in the gated living cell populations of each subset (Figure 6B). The correlation factors were very similar for IL-15 and IL-2 for a given subset, and were stronger for the CD8+ subset. The increases in bcl-2 expression in living CD4+ cells, and particularly in living CD8+ cells, were markedly higher in IL-15-stimulated cultures than in IL-2-stimulated cultures, support-
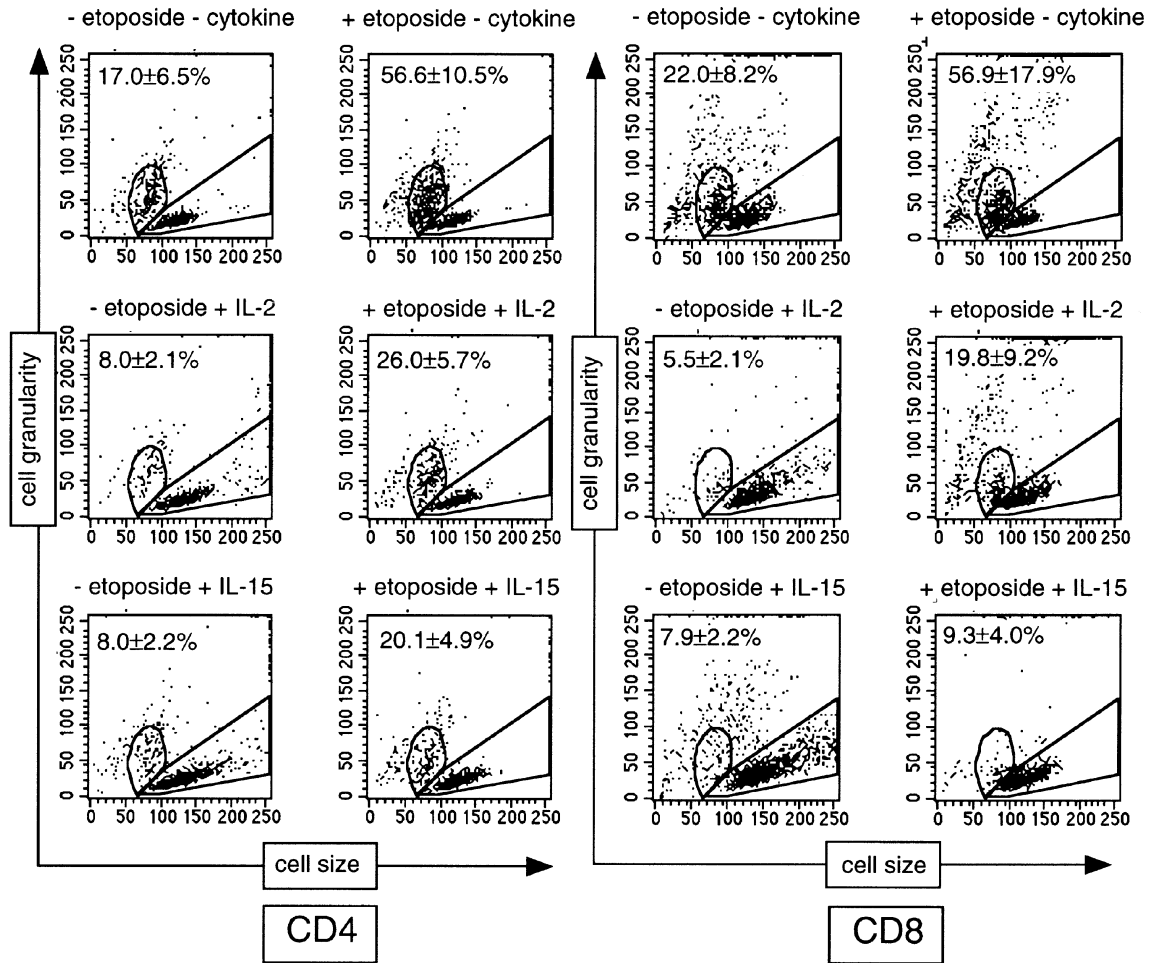
Figure 5 Relative effects of IL-15 and IL-2 on lymphocyte survival. PBMCs of HIV-infected individuals (n=14) were incubated for 3 days in the absence or presence of etoposide (20 μM), either without exogenous cytokines or with the addition of IL-2 or IL-15 (10 ng/ml). Cells were stained with either anti-CD4 or anti-CD8 mAbs. Shown are forward *versus* side scatter profiles gated on CD4⁺ and CD8⁺ cells of cultures of PBMCs derived from the same patient. The cell populations circled correspond to apoptotic cells (low FSC) or living cells (higher FSC). The percentages shown on each panel correspond to the representation of apoptotic cells (mean ± S.D.) within CD4 or CD8 T cell subsets

ing the notion that IL-15 can act as a potent lymphocyte survival factor.

Discussion

The present study indicates that exogenous IL-15 can exert a beneficial influence on lymphocytes of HIV-infected individuals by two fundamental mechanisms. Firstly, IL-15 can act as a potent survival factor in the prevention of spontaneous lymphocyte apoptosis. Secondly, IL-15 can expand the pool of viable lymphocytes by its potent ability to induce proliferation. The abilities of exogenous IL-15 to strongly promote both survival and proliferation of lymphocytes of HIV-infected individuals is of critical importance, given the detrimental role which alterations in the profile of cytokine production and cytokine deprivation, and associated cell loss play in HIV pathogenesis. However, the present study also reveals two disadvantages of IL-15 in regard to regulating viability and number of lymphocytes of HIV-infected individuals. One concern relates to its inability to inhibit

CD95-induced apoptosis, which in addition to cytokine deprivation, is believed to be major contributory factor to lymphocyte depletion in HIV-infected individuals. The second disadvantage is the increased lymphocyte activation state and associated increased apoptosis-susceptibility arising from the potent ability of IL-15 to induce proliferation.

The greater survival-enhancing effect of IL-15, as compared with that of IL-2 at an equivalent concentration, is highly likely to be associated with quantitative differences in the strength of the transduced signal, and not with qualitative differences in the signaling pathways triggered by these cytokines. Both IL-15 and IL-2 signal through the β and γ receptor chains, which are coupled to the bcl-2 survival pathway, and also growth regulatory pathways involving c-myc and fos/jun.[4,34,35] The α receptor chains, specific to each cytokine, do not appear to play crucial roles in signal transduction but determine binding affinity.[28,36] The greater ability of IL-15 to enhance survival, and also to induce proliferation, is consistent with studies indicating the greater binding affinity of the IL-15

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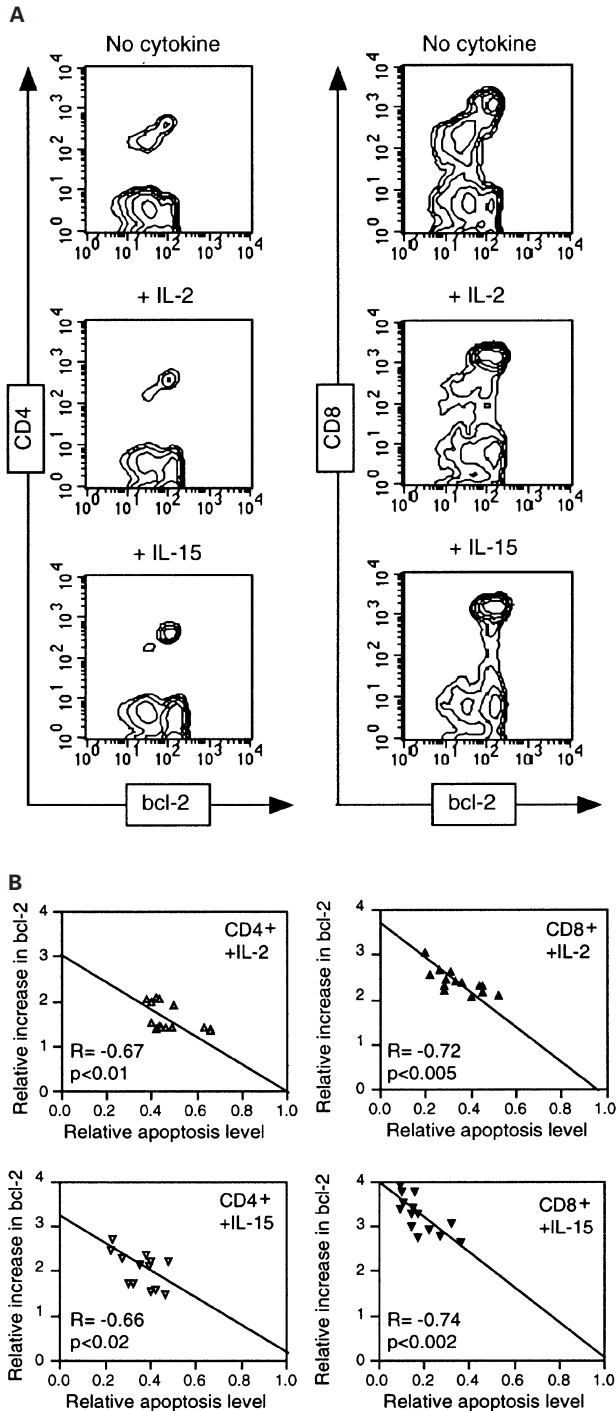
Figure 6 Relative effects of IL-15 and IL-2 on up-regulating bcl-2 expression and correlation with reduced levels of apoptosis. PBMCs of the same group of individuals as described in Figure 5 were incubated for 3 days in the presence of etoposide (20 μM), either without exogenous cytokines or with the addition of IL-2 or IL-15 (10 ng/ml). Cells were stained with 7-AAD and either anti-CD4 or anti-CD8 mAbs, permeabilized and stained intracellularly with anti-bcl-2 mAbs. Shown in (**A**) are FACScan profiles of bcl-2 expression in the CD4⁺ and CD8⁺ subsets of the same patient. Mean MFI values of bcl-2 staining within gated CD4⁺ and CD8⁺ populations of the patient group are the following: 57 ± 16 without cytokine, 93 ± 24 with IL-2 ($P < 0.01$) and 112 ± 22 with IL-15 ($P < 0.01$) for gated CD4⁺ cells, 40 ± 11 without cytokine, 93 ± 29 with IL-2 ($P < 0.01$) and 127 ± 28 with IL-15 ($P < 0.01$) for gated CD8⁺ cells. In (**B**) the relative apoptosis level observed within a given subset in cultures stimulated

receptor α chain for IL-15, as compared with that of the IL-2 receptor α chain for IL-2.[37] The stronger ability of both IL-15 and IL-2 to promote survival and proliferation of the CD8⁺ subset, as compared with the CD4⁺ subset, is likely to be associated with the higher surface expression of the β chain on CD8⁺ cells,[38] since we have observed little difference between the subsets in their *ex vivo* expression levels of the γ chain.[39] Furthermore, proliferative responses to IL-15 are more efficiently blocked by mAbs directed against the β chain than by mAbs against the γ chain.[40] It should be noted that the correlations between the reduced levels of apoptosis induced by IL-15 and by IL-2 and the up-regulated expression of bcl-2 were less strong in the CD4⁺ subset, as compared to the CD8⁺ subset. This suggests the involvement of other factors in addition to bcl-2 in enhancing survival of CD4⁺ lymphocytes, for example bcl-x$_L$, which is up-regulated by γ chain signaling.[30]

Since spontaneous apoptosis involves decreased bcl-2 expression,[19,22] the protective effect of IL-15 could be explained in terms of promoting a survival pathway whose down-modulation is associated with this particular mode of death. By the same token, findings that signaling through the CD95 molecule does not directly down-regulate bcl-2, and that the bcl-2 survival pathway poorly protects lymphocytes from death triggered by these molecules,[41,42] could simplistically explain the effectiveness of IL-15 (and of IL-2) against death triggered by these molecules. Although the bcl-2 and CD95 molecules apparently regulate distinct, independent pathways in lymphocytes, bcl-2 is able to block apoptosis induced in mouse liver by anti-CD95 antibodies.[43] This could explain the recently reported greater ability of IL-15 fusion proteins, as compared with those of IL-2 at equivalent doses *in vivo*, to inhibit lethal CD95-induced hepatic failure.[20] Up-regulation of bcl-2 expression apparently protects cells from HIV-protease-mediated death[44] and also from apoptosis induced by CD4 cross-linking.[45] While further investigation is required, the high increases in bcl-2 expression associated with IL-15-mediated survival could have important implications in protecting lymphocytes from apoptosis in HIV infection involving the actions of various viral proteins, as well as preventing death from occurring by cytokine deprivation.

Our observations with lymphocytes of HIV-infected individuals contrast with those of a recent study which found IL-15 to inhibit CD95-induced death of T cells derived from uninfected individuals which had been transiently preactivated by mitogens *in vitro*.[20] Given that lymphocytes of HIV-infected individuals have been chronically activated *in vivo*, the differences between the observations could

with IL-2 and with IL-15 were plotted against the relative increases in bcl-2 expression in the living cell population of each subset. The relative apoptosis level within each subset in the presence of a given cytokine is defined as the percentage of 7-AAD-stained cells in cultures incubated with the cytokine expressed as a ratio of the percentage of 7-AAD-stained cells in cultures incubated in the absence of exogenous cytokines. The relative increase in bcl-2 expression within the gated living cell population of each subset is defined as the MFI of anti-bcl-2 mAb staining detected in the presence of the cytokine expressed as a ratio of the MFI detected in the absence of exogenous cytokines. Correlations were determined using the Spearman regression analysis

possibly lie in the quantitative and/or qualitative differences in the nature of lymphocyte activation, and raises the possibility that chronic activation *in vivo* could modify the ability of cytokines to influence susceptibility to CD95-induced death. Although a mechanism for the protective effect of IL-15 against such death was not shown in the earlier study, it could possibly involve bcl-x_L which, when ectopically expressed, prevents loss of mitochondrial membrane potential associated with CD95-induced protease activation.[46] Although up-regulated expression of bcl-x_L in preactivated T lymphoblasts derived from uninfected donors has been observed in response to IL-15 and IL-2,[30,47] the question as to whether the modest level of cytokine-induced bcl-x_L would be sufficient to counter the CD95 death signal is challenged by recent findings.[48]

While the potent ability of exogenous IL-15 to enhance survival of lymphocytes of HIV-infected individuals is clearly advantageous, the present study indicates that its likewise potent ability to induce proliferation can be both beneficial in expanding lymphocyte populations, and also detrimental by virtue of increasing the lymphocyte activation state. The negative effect associated with increased blastogenesis was apparent by the less effective ability of IL-15 to reduce the representation of apoptotic cells in the absence of etoposide as compared to in the presence of etoposide (Figure 5). Furthermore, although IL-15 was more potent than IL-2 at an equivalent concentration in both enhancing survival and in stimulating proliferation, IL-15 failed to induce increases in the representation and total number of viable cells which were higher than those induced by IL-2. Such failure appears to be attributable to greater cell loss occurring when cells are actively proliferating in response to IL-15 than to IL-2, and associated with the ability of IL-15 to more highly activate lymphocytes and increase their apoptosis-susceptibility. In other words, it appears that cell loss associated with the increased activation state counter-balances increases in lymphocyte viability promoted by cytokine-induced survival and proliferation to a greater extent in the case of IL-15 than with IL-2. The mechanisms underlying the increased apoptosis-susceptibility of activated proliferating lymphocytes are as yet unclear. However since IL-15 was more effective than IL-2 in promoting bcl-2 up-regulation both in the presence and absence of etoposide, it would seem that cell loss associated with IL-15 stimulation does not involve a poorer ability of IL-15 to upregulate bcl-2 under conditions where lymphocytes are proliferating. While the increased apoptosis-susceptibility of activated, proliferating lymphocytes plays a crucial, homeostatic role in controlling expansion of lymphocyte populations,[32,49] the detrimental effect of increased activation associated with cytokine stimulation should be closely considered particularly in the case of HIV infection where the immune system is already chronically activated.

The findings of the present study highlight the need to closely weigh the positive effects of exogenous cytokines on survival and on proliferation against the disadvantages of increased activation and associated apoptosis-susceptibility when assessing their immunotherapeutic potential particularly in treating chronic viral infections such as that of HIV. The proliferative advantage of IL-15 may need to be more strongly compromised than that of IL-2 in order to minimize cell loss. Although caution must be obviously exercised in interpreting relative effects of cytokines observed *in vitro*, the study raises the possibility that the required dosage of exogenous IL-15 *in vivo* may be considerably less than that of IL-2 to promote comparable maintenance and expansion of lymphocyte populations in HIV-infected individuals. In addition, the required dosage of IL-15 *in vivo* could be possibly less than that of IL-2 in view that IL-15 is endogenously produced by a wide variety of tissues, in contrast to IL-2 whose production is primarily restricted to T cells,[1,2] and that IL-15 can apparently stimulate IL-2 secretion by T lymphoblasts whereas IL-2 is unable to induce IL15 secretion.[50] Lower dosages would be of obvious benefit in reducing toxic side-effects and augmentation of HIV replication. In addition, the observed potency of IL-15 in stimulating expansion of CD8+ lymphocytes should also be considered in assessing dosage in regard to risks associated with augmented virus replication and benefits of enhancing host-mediated anti-viral activities.

Materials and Methods

Human blood samples

Peripheral blood samples were obtained from 58 HIV-1 infected patients at the Service for Infectious and Tropical Diseases (Pr René Roué), Bégin Military Hospital, St. Mandé, France. None of the patients studied were receiving treatment with IL-2 or HIV protease inhibitors. Forty-six patients received therapy consisting of the combination of two HIV reverse transcriptase inhibitors. *Ex vivo* CD4 percentages ranged as follows: CD4 > 28%, n=2; 13% < CD4 $\leqslant$ 28%, n=24; CD4 $\leqslant$ 13%, n=32, according to the revised HIV infection classification of the Centers of Disease Control (Atlanta, GA, USA).[21]

PBMC isolation and stimulation

PBMCs were freshly isolated from heparinized blood by centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and incubated for 1–3 days at 10^6 cells/ml in complete medium, as previously described.[15] Anti-CD95 mAbs (clone UB2) (Immunotech, Marseille, France) were immobilized to wells of 24-well plates by incubating wells with 100 μl of 100 μg/ml mAbs for 1 h, following by rinsing with PBS. Recombinant human IL-2, IL-4, IL-7, IL-10, IL-12 and IL-15 (R&D Systems, Abingdon, UK) were used at concentrations indicated in the text. In specific experiments, etoposide (Sigma) was added at a pre-titrated optimal final concentration of 20 μM.

Proliferation assays

PBMCs were resuspended at an initial concentration of 2×10^5 cells/ 200 μl per well in 96-well flat bottom plates and incubated for 3 days in the absence or presence of exogenous cytokines. Samples were pulsed for the final 16 h with 1 μCi/well of [^{3}H]thymidine (Amersham, Bucks., UK) and harvested on to glass fibre filters. Radioactivity was measured by liquid scintillation counting.

Monoclonal antibodies

The mouse mAbs specific for human surface antigens included: FITC-conjugated anti-CD69 (IgG$_1$, clone L78); anti-CD38 (IgG$_1$, clone HB-7)

and anti-HLA-DR (IgG$_{2a}$, clone L243) conjugated to phycoerythrin (PE); anti-CD4 (IgG$_1$, clone SK3) and anti-CD8 (IgG$_1$, clone SK1) conjugated to either FITC, PE or PerCP (Becton Dickinson, San Jose, CA, USA). FITC-conjugated mAbs against bcl-2 (IgG$_1$, clone 124) (Dako, Glostrup, Denmark), proliferating cell nuclear antigen (PCNA) (IgG$_{2a}$, clone PC10) (Caltag, Burlingame, CA, USA) and Ki-67 (IgG$_1$, clone MIB-1) (Immunotech) were used for intracellular staining. Appropriately conjugated isotype-matched controls (mouse IgG$_1$ and IgG$_{2a}$) were obtained from Dako.

Cell staining and FACScan analysis

5×10^5 PBMCs, either freshly isolated or following culture, were washed in PBS containing 1% BSA and 0.1% sodium azide (PBS-BSA-NaN$_3$) and incubated for 20 min at 4°C with surface antigen-specific mAbs described above (1 : 50 dilution). Stained cells were washed and fixed in 1% paraformaldehyde for 20 min at 4°C. Subsequent intracellular staining was performed as previously described.[22] Briefly, fixed cells were washed, permeabilized in PBS-BSA-NaN$_3$ containing 0.05% saponin (w/v) (Sigma) and stained with mAbs against bcl-2, PCNA or Ki-67 (1 : 50 dilution). Stained cells were washed, fixed and applied to a FACScan flow cytometer (Becton Dickinson). For each sample, 10 000 viable lymphocytes were gated following size and granularity parameters and analyzed with Cell Quest software (Becton Dickinson).

Analysis of apoptotic cells

Quantification of apoptotic cells in short-term cultures was performed by staining with 7-amino-actinomycin D (7-AAD) (Sigma), the degree of fluorescence of which closely corresponds with alterations in membrane permeability and the stage of apoptosis.[23,24] Cultured cells were incubated with mAbs specific for surface antigens together with 7-AAD (20 μg/ml) for 20 min at 4°C. Subsequent washes and cell fixation were carried out with the inclusion of nonfluorescent actinomycin D (20 μg/ml) to prevent further 7-AAD staining within apoptotic cells and non-specific labeling of living cells.[22] Spectral properties of 7-AAD allowed detection of apoptotic cells by fluorescence emission in the red channel FL-3, and simultaneous labeling of surface antigens (FL-1 and FL-2).

Acknowledgements

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