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IL-2 and IL-7 but not IL-12 protect natural killer cells from death by apoptosis and up-regulate bcl-2 expression

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SUMMARY

Human natural killer cells (NK) respond to interleukin-2 (IL-2) with augmented cytolytic activity, cytokine secretion and cell proliferation. Here we show that IL-2 protects NK cells from death by apoptosis (programmed cell death; PCD). Highly purified NK cells ($CD3^-$ $CD56^+$) were isolated from peripheral blood lymphocytes (PBL) of either control donors or of an asymptomatic donor with 60% NK cells. Glucocorticosteroids (GCS) induced PCD in NK cells, as shown by nuclear condensation and DNA fragmentation. IL-2 completely prevented GCS-induced PCD in a dose-dependent manner without overcoming GCS-induced inhibition of NK cell proliferation. The IL-2 protective effect was mediated through the p75 β chain of the IL-2R, as neutralizing monoclonal antibody (mAb) to the p75 β chain but not to the p55 α chain completely abolished the IL-2 anti-apoptotic activity. In addition to IL-2, the cytokines IL-7 and IL-12 have been reported to regulate NK cell functions. Our present data showed that IL-7 but not IL-12 rescued NK cells from apoptosis, but to a lesser extent than IL-2. Although IL-4 had a marginal protective effect, IL-1, IL-3, IL-6, IL-8, interferon-γ (IFN-γ) and IFN-α, tumour necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β) and granulocyte–macrophage colony-stimulating factor (GM-CSF) displayed no significant activity. Finally, we report that IL-2 and IL-7 enhanced bcl-2 expression in NK cells, suggesting the existence of a bcl-2-dependent survival pathway. In addition to regulating various functions, it is concluded that IL-2 and IL-7 have the ability to prevent PCD in NK cells.

INTRODUCTION

Physiological cell death in multicellular organisms enables the elimination of unwanted cells. This genetically controlled process of self-destruction, termed apoptosis or programmed cell death (PCD), is characterized morphologically by chromatin condensation and DNA fragmentation.[1,2] Apoptosis is involved in the process of negative selection in the thymus and positive selection in germinal centres of secondary lymphoid organs.[3,4] This cell-committed suicide occurs in response to various inductive stimuli, such as exposure to glucocorticoids,[5] irradiation[6] or triggering through FAS/APO-1 antigen.[7] Apoptosis can also be initiated by withdrawal of specific 'trophic' factors that are required for the survival of the respective cell species.[8,9] Finally, tumour necrosis factor-α (TNF-α) produced by cytotoxic effector cells (i.e. cytotoxic T lymphocytes and natural killer cells) directly triggers apoptosis in target cells.[10]

Human natural killer (NK) cells are a discrete population of non-T, non-B lymphocytes defined functionally by their ability to lyse target cells without prior sensitization and without major histocompatibility complex (MHC) restriction.[11,12] The proposed role for NK cells in immune surveillance against neoplasia has now been extended to protective immunity against various pathogenic micro-organisms.[13] The recently identified NK cell stimulatory factor (interleukin-12; IL-12), produced by macrophages infected with intracellular organisms, strongly induces interferon-γ (IFN-γ) production by NK cells, which in turn activates macrophages to a microbicidal state.[14] In addition to IL-2, IL-12 is a potent stimulator of IFN-γ production by NK cells.[15] NK cells therefore may be considered as important immunoregulatory cells, inasmuch as IFN-γ displays a pivotal role in the differentiation of T-helper type-1 (Th1) cells.[16] In that regard, it was recently reported that IL-12 induces the maturation of human naive T cells into Th1 cells.[17] In addition to IL-2 and IL-12, the cytokines IL-4 and IL-7 have been shown to stimulate lymphokine-activated killer (LAK) activity, proliferative activity and cytokine production.[18] The present report shows that glucocorticosteroids (GCS) trigger PCD in NK cells and that IL-2 and IL-7, but not IL-12, prevent their entry into apoptosis and up-regulate bcl-2 expression.

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Abbreviations: GCS, glucocorticosteroids; HC, hydrocortisone; NK, natural killer; (NKSF/IL-2), NK cell stimulatory factor; PBMC, peripheral blood mononuclear cells; PCD, programmed cell death; PI, propidium iodide.

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MATERIALS AND METHODS

Antibodies

Purified anti-CD14 and anti-CD56 monoclonal antibodies (mAb) were obtained from Becton Dickinson (Mountain View, CA). Anti-IL-2R-p75 was purchased from Cedarlane Laboratories (Ontario, Canada). Neutralizing anti-IL-2R-p55 (H41) was kindly provided by Dr Uchiyama (Tokyo University, Tokyo, Japan). Anti-bcl-2 mAb was a gift from Dr Mason (John Radcliffe Hospital, Headington, Oxford, UK). Class-matched negative control mAb was prepared in our laboratory.

Cell preparation and culture conditions

Peripheral blood mononuclear cells (PBMC) from control donors or from one normal asymptomatic donor with 60% $CD3^-$ $CD56^+$ $CD16^+$ cells (LZ donor) were isolated with Lymphoprep (Cederlane Laboratories). Highly purified NK cells from LZ donor (>95% $CD56^+$ cells) were obtained by negative selection using anti-CD19-coated Dynabeads and purified anti-CD3- (CRL 8001; ATCC, Rockville, MD) or anti-CD14-treated goat anti-mouse IgG Dynabeads (Dynal, Oslo, Norway). Highly purified NK cells from control donors were obtained by positive selection using purified anti-CD56 mAb and goat anti-mouse IgG Dynabeads. PBMC or purified NK cells were incubated at 10^6/ml (48-well flat-bottomed plate; Costar, Cambridge, MA) in RPMI-1640 culture medium (Gibco Laboratories, Grand Island, NY) supplemented with 1% heat-inactivated fetal calf serum (FCS; HyClone Laboratories, Logan, UT), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamin (2 mM), for the indicated culture period. Hydrocortisone (HC) was purchased from Sigma (St Louis, MO). IL-2 (50 U/ml), IL-3 (10 ng/ml), granulocyte–macrophage colony-stimulating factor (GM-CSF) (10 ng/ml), IL-6 (300 U/ml) and IFN-γ (500 U/ml) were obtained from Genzyme Corp. (Boston, MA). Recombinant IL-12 was a generous gift from Dr M. Gately (Hoffmann-La Roche, Nutley, NJ) and was used at 40 pM. Recombinant IFN-α (500 U/ml) and transforming growth factor-β (TGF-β); (2 ng/ml) were obtained from CIBA-GEIGY (Basel, Switzerland). IL-4 (10 ng/ml) and IL-7 (1000 U/ml) were kindly provided by Immunex (Seattle, WA). IL-8 (5 ng/ml) was obtained from Sandoz (Vienna, Austria) and TNF-α (25 ng/ml) was a gift from Dr Tavernier (Fiers, Ghent, Belgium).

Cell viability and cell proliferation

Cell viability was determined by staining the cells with propidium iodide (PI; 2 μg/ml) and analysis by flow cytometry (FACscan; Becton Dickinson Co., Mountain View, CA). The contour plots show forward scatter (FSC; linear) on the *x*-axis and (FL3; log) on the *y*-axis. Results are expressed as the percentage of viable cells, representing the number of cells in the lower right quadrant. For the cell proliferation assay, cells were seeded in quadruplicate (10^6/ml) in 96-well flat-bottomed plates for 2 days. Cells were pulsed with 0·5 μCi [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL) during the last 8 hr of the culture period. Results are expressed as the mean c.p.m. ± SD.

DNA fragmentation assay

Cells were washed twice with phosphate-buffered (PBS) and pelleted by centrifugation at 200 *g* for 5 min at room temperature. Cell pellets ($2·5 \times 10^6$) were resuspended in cell lysis buffer [10 mM EDTA, 50 mM Tris, Ph 8, containing 0·5% (w/v) *N*-laurylsarcosine and 0·5 μg/ml proteinase K] and incubated for 1 hr in a 50° water-bath. Rnase A was then added to a concentration of 0·25 mg/ml and incubation was prolonged for 1 hr at 50°. The DNA preparations were then extracted (twice) with buffered phenol (Sigma), followed by two chloroform/isoamyl alcohol (24 : 1) extractions for removal of protein and residual traces of phenol. DNA preparations were then brought to 1·5 volumes by the addition of 10 mM Tris–HCl, pH 8, and 1 mM EDTA (TE buffer), and were centrifuged at 14 000 *g* to separate intact from fragmented chromatin. The supernatants, containing fragmented DNA, were placed in separate tubes and DNA was precipitated with 0·5-volume ammonium acetate (2 M) and 2 volumes of ethanol, at −70° for 24 hr. The DNA precipitates were recovered by centrifugation

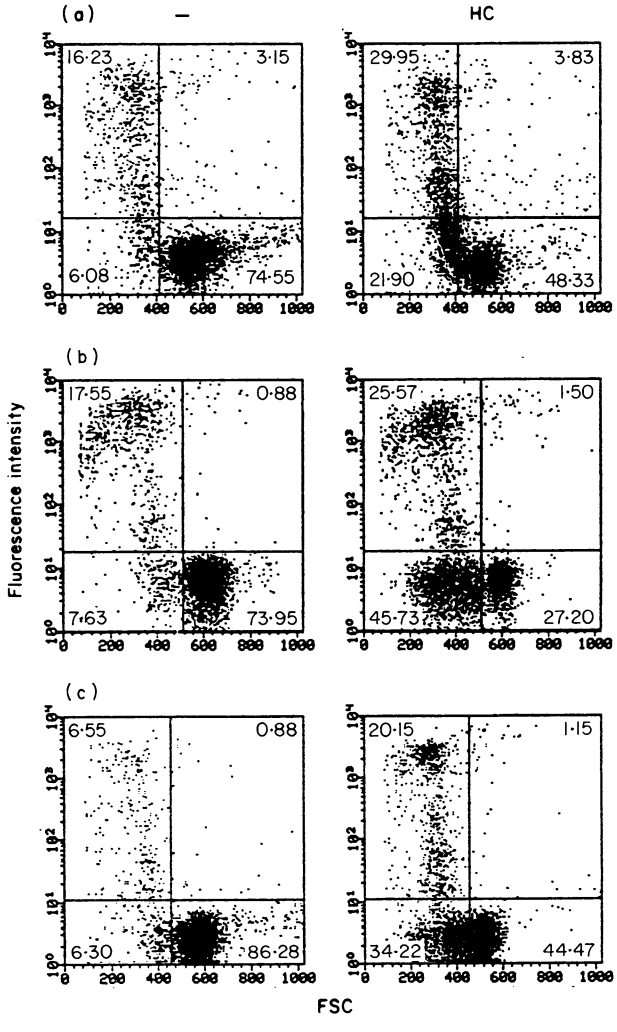

Figure 1. HC induces cell death of NK cells. (a) Highly purified $CD56^+$ cells from the control donor were cultured for 2 days with or without HC (10^{-5} M). (b) Highly purified $CD56^+$ cells or (c) PBMC from the LZ donor were cultured for 3 days with or without HC (5×10^{-4} M). Cells were then stained with PI and analysed by flow cytometry.

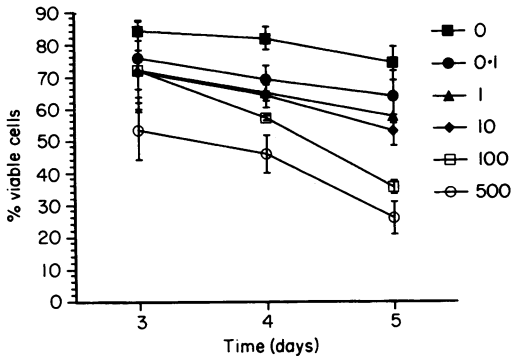

Figure 2. Kinetics and dose–response of HC-induced cell death. PBMC from the LZ donor were cultured for 3, 4 or 5 days with or without titrated concentrations (μM) of HC, and cell viability was assessed by PI staining and flow cytometry.

at 13 000 *g* for 15 min, air dried for 1 hr at room temperature, and resuspended in TE buffer. Samples were supplemented with loading buffer [0·25% bromophenol blue (w/v), 50% glycerol (w/v) and 10 mM EDTA] at a 1 : 5 ratio and then heated at 65° for 10 min. Electrophoresis was carried out on a 1% agarose gel at 6 V/cm of gel using 2 mM EDTA, pH 8, 89 mM Tris and 89 mM boric acid (TBE buffer). A *Bgl*I and *Hinf*1 digest of pBR328 DNA (Boehringher Mannheim, Mannheim, Germany) was applied to each gel to provide size markers. After electrophoresis, DNA was visualized by soaking the gel for 30 min in TBE containing 1 μg/ml ethidium bromide and destained for 30 min in TBE.

Light microscopy

For the morphological studies, cytospin preparations of purified NK cells (10^5 cells), cultured with or without HC (5×10^{-4} M) for 17 hr, were stained with Wright (Volu-Sol, Las Vegas, NA) and analysed by light microscopy.

Immunofluroescence for bcl-2 detection

After culture, cells were fixed and permeabilized using 90% ethanol. Cells were stained with anti-bcl-2 mAb or class-matched control mAb, followed by fluorescein isothiocyanate (FITC)-labelled goat $F(ab')_2$ anti-mouse IgG (Southern Biotechnology, Birmingham, AL).

RESULTS

GCS induces apoptosis in NK cells

Several reports indicate that NK cells may kill by inducing apoptosis in their targets.[10] In the present study, we investigate whether NK cells themselves may undergo PCD for self-elimination. Purified $CD56^+$ cells from a control donor (Fig. 1a) and from LZ donor (Fig. 1b) were treated with GCS, a common agent used to induce PCD in various cell populations. The cells were subsequently stained with PI and analysed by flow cytometry to quantify PCD. This method allows a direct monitoring of the transition from viable cells (lower right quadrant), which exclude PI, to dead cells (upper left quadrant), which exhibit a smaller size (as shown by the low FSC) and no longer exclude PI. The intermediate state is

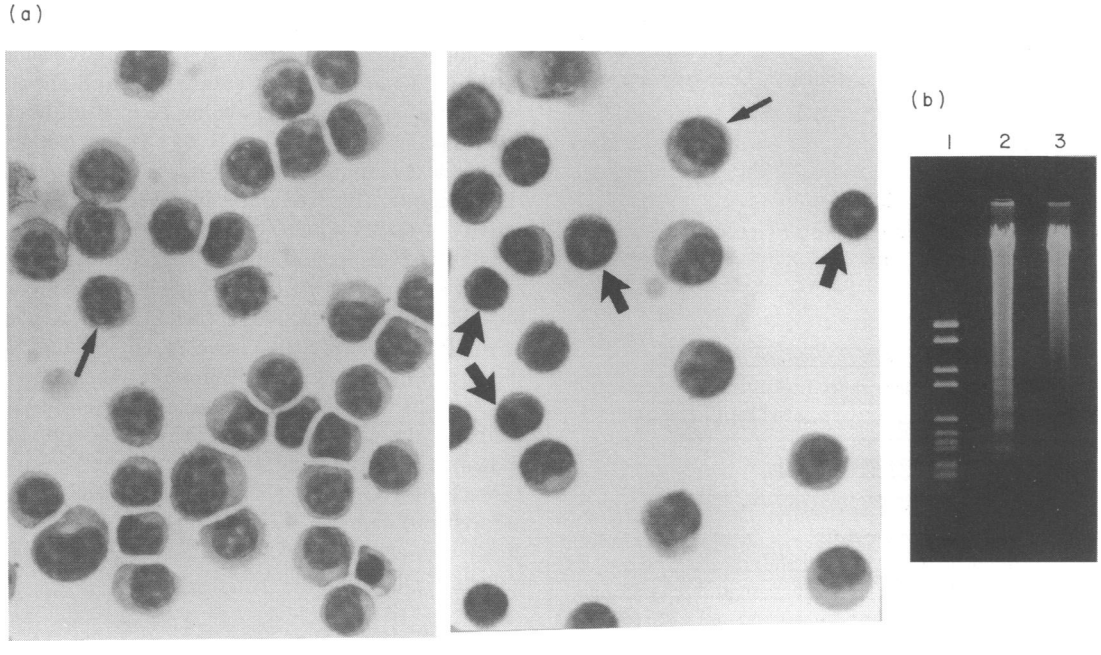

Figure 3. HC-treated NK cells show morphological and biochemical signs of apoptosis. (a) Light microscopy analysis of highly purified $CD56^+$ cultured for 17 hr in the absence (left panel) or presence of HC (5×10^{-4} M) (right panel) showing the presence of normal cells (thin arrows) together with apoptotic cells (large arrows). Magnification ×400. (b) Gel analysis of DNA extracted from highly purified $CD56^+$ cells cultured for 17 hr in the presence (lane 2) or absence (lane 3) of HC. Lane 1 represents molecular weight markers. One representative experiment out of three.

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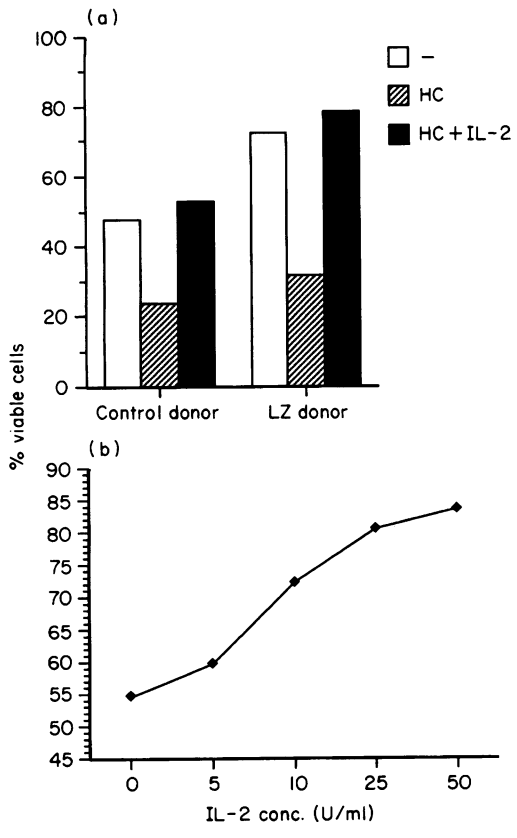

Figure 4. IL-2 rescues NK cells in a dose-dependent manner. (a) Highly purified $CD56^+$ cells were cultured in the absence or presence of 10^{-5} M HC for 2 days (control donor) or 5×10^{-4} M HC for 4 days (LZ donor) with or without IL-2 (50 U/ml). (b) Highly purified $CD56^+$ cells from the LZ donor were cultured for 3 days in the presence of HC (5×10^{-4} M) and various concentrations of IL-2. Cell viability was assessed by PI staining and flow cytometry. One representative experiment out of three.

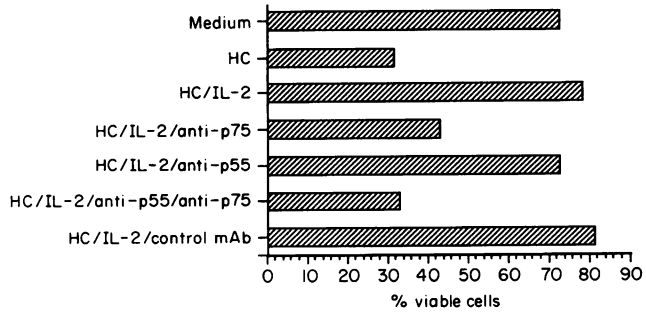

Figure 5. Effect of anti-IL-2R mAb on IL-2-mediated rescue and IL-2-induced proliferation. Highly purified $CD56^+$ cells were cultured for 3 days in the absence or presence of HC (5×10^{-4} M) with or without IL-2 (50 U/ml) in the presence or absence of mAb (10 μg/ml). Cell viability was assessed by PI staining. One representative experiment out of two.

defined by cells which have become shrunken (lower FSC) but still do not take-up PI (lower left quadrant) and most probably represent apoptotic cells. As depicted in Fig. 1, HC reduced the percentage of viable NK cells in control and LZ donors by 35% and 63%, respectively. Unfractioned mononuclear cells from LZ donor displayed similar sensitivity to HC as purified NK cells (Fig. 1c). However, NK cells from the LZ donor died at a slower rate or required higher concentrations of HC than control donor cells. As shown in Fig. 2, the concentrations of HC and the kinetics used in this study were selected on the basis of dose–response curves and time–course analyses. As depicted in Fig. 3, HC-treated purified NK cells from LZ donor showed rapid morphological and biochemical signs of apoptosis. After overnight incubation with HC, NK cells demonstrated nuclear condensation and loss of cell volume (Fig. 3a) typically observed during the process of PCD. At the same time, characteristic cleavage of DNA into nucleosomal fragments was visualized using a qualitative gel electrophoresis assay (Fig. 3b), further demonstrating that HC-treated NK cells were in apoptosis. Because LZ donor cells were a convenient source of functional NK cells, as they secreted cytokine (e.g. IFN-γ) and exhibited cytolytic activity (data not shown), they were used to examine regulation of apoptosis in NK cells.

IL-2 prevents HC-induced PCD in NK cells

We next investigated whether IL-2, a cytokine known to modulate NK functions, may prevent HC-induced PCD. Purified $CD56^+$ NK cells from the LZ donor were treated with HC in the absence or presence of IL-2. As shown in Fig. 4a, IL-2 prevented the HC-induced decrease in the percentage of viable cells, suggesting that it rescues NK cells from death by apoptosis. Of note, IL-2 had the same protective effect on NK cells from the control donor. The effect of IL-2 was dose-dependent, already detectable at 5 U/ml and reaching a plateau between 25 and 50 U/ml (Fig. 4b). The anti-apoptotic activity of IL-2 was specific, since it could be blocked by mAb to IL-2 and was effective from day 2 to day 5 (data not shown).

We next examined which of the p55 α or p75 β chains of the IL-2 receptor (R) would be involved in the anti-apoptotic activity of IL-2 following the observations that the β chain was constitutively expressed on NK cells and that the α chain was rapidly induced by IL-2 (data not shown). We therefore studied the ability of mAb reactive to the p55 and p75 chains of IL-2R to inhibit IL-2-mediated rescue of HC-treated NK cells. As shown in Fig. 5, only anti-p75 mAb could effectively abrogate IL-2-mediated protection. Of note, the two mAb completely inhibited the IL-2-induced [^{3}H]thymidine uptake (data not shown). The neutralizing activity of these mAb was tested in the absence of HC, since HC alone completely inhibited IL-2-induced proliferation. It was concluded that IL-2 can exert a direct protective effect on HC-treated NK cells without inducing cell proliferation.

IL-7 prevents HC-induced PCD on NK cells

We next determined whether other cytokines known to regulate NK cell functions might influence their survival. As summarized in Table 1, IL-7 significantly and consistently prevented HC-induced cell death. IL-12, a potent NK-stimulating factor,

Table 1. Effect of IL-7, IL-12 and IL-4 on HC-induced PCD of NK cells

Group	Added to cultures	% viable cells*	
I	—	79·9 ± 2·5	
	HC	54·6 ± 1·8	$n=3$ $P<0·001$
	HC + IL-7	73·3 ± 4	
II	—	71·7 ± 8	
	HC	42·2 ± 7·8	$n=3$ NS
	HC + IL-12	46·7 ± 13·5	
III	—	69·3 ± 6·3	
	HC	36·1 ± 6	$n=5$ $P<0·5$
	HC + IL-4	47·7 ± 8·2	

Purified NK cells from LZ donors were cultured for 3 days with or without HC (5×10^{-4} M) and in the absence or presence of IL-4 (10 ng/ml), IL-7 (1000 U/ml) or IL-12 (40 pM). Cell viability was assessed by PI staining.

* Percentage viable cells ± SEM.

NS, not significant.

was unable to prevent PCD of purified NK cells, while IL-4 had a slight protective effect. Similarly to IL-12, a panel of other cytokines, including IL-1, IL-3, IL-6, IL-8, IFN-γ, IFN-α, TNF-α, TGF-β and GM-CSF, had no effect on the survival of NK cells treated with HC (data not shown).

IL-2 and IL-7 up-regulate bcl-2 expression in NK cells

The above data indicated that IL-2 and IL-7 prevented HC-treated NK cells from their entry into apoptosis. Since rescue of various cell populations is often associated with expression of the proto-oncogene bcl-2,[19,20] we examined, by immunofluorescence, the effect of these cytokines on bcl-2 expression in purified NK cells. As shown in Fig. 6, IL-2- or IL-7-treated NK cells expressed bcl-2 after 2 days culture. The level of bcl-2 protein was maintained in the presence of HC. A small but not significant level of bcl-2 expression was observed in NK cells cultured in medium or HC alone, suggesting that IL-2 and IL-7 up-regulated rather than prevented loss of bcl-2 expression in NK cells. Of note, IL-4 and IL-12 (data not shown) did not up-regulate bcl-2 expression.

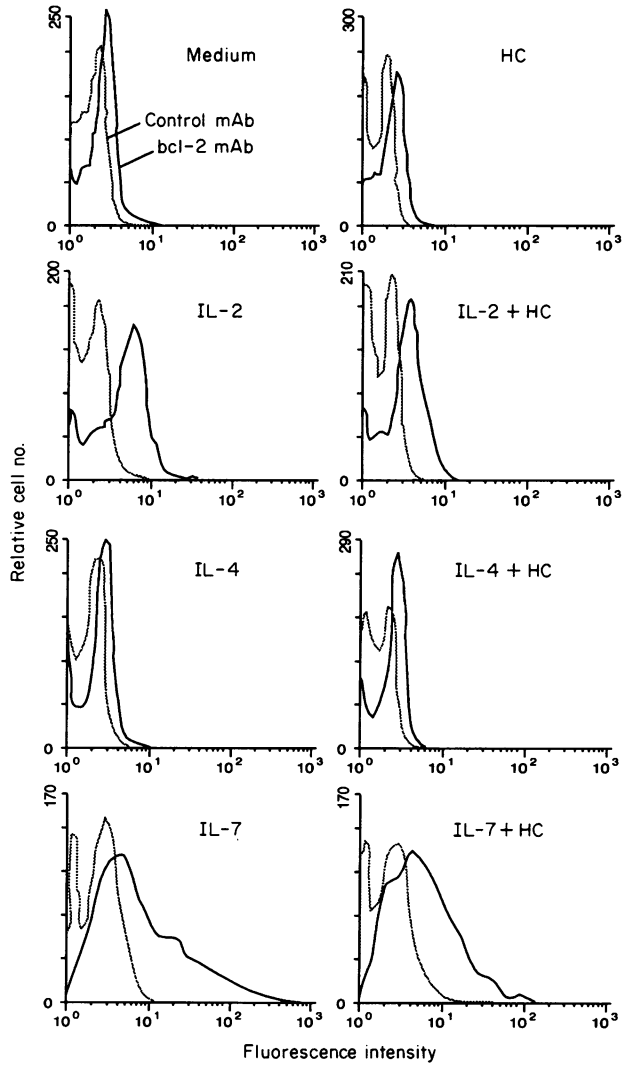

Figure 6. Effect of IL-2, IL-4 and IL-7 on bcl-2 expression. Highly purified $CD56^+$ cells from the LZ donor were cultured for 2 days either alone or in the presence of IL-2 (50 U/ml), IL-4 (10 ng/ml) and IL-7 (1000 U/ml) with or without HC (5×10^{-4} M). Cells were stained as described in the Materials and Methods.

DISCUSSION

Apoptosis is a normal physiological process of cell death occurring in various cell populations, and is mandatory for homeostasis.[1,2] Previous studies have reported that growth factor deprivation (i.e. IL-2) or antigen clearance-induced entry into apoptosis of antigen-specific effector T cells [freshly isolated cytotoxic T lymphocytes (CTL) or CTL clones].[21] Here we show that GCS-treated NK cells die by apoptosis, as shown by nuclear chromatin condensation and the presence of the typical DNA ladder, and that IL-2 prevents their entry into apoptosis. IL-2 exerts its protective effect via the p75 β chain of IL-2R and it fails to induce NK cell proliferation in the presence of HC. The functional importance of IL-2R β has been described in several studies[22,23] and was recently underlined by the demonstration of long-term elimination of NK cells *in vivo* in mice that had been injected by anti-IL-2R β mAb.[24]

Although IL-2 appears to be the primary stimulus for the generation of LAK activity, three other cytokines, IL-4, IL-7 and IL-12 (NK-cell stimulatory factor) have been reported to influence cytotoxic cell functions and NK cell proliferation.[18] Our data indicate that IL-7 but not IL-12 rescues HC-treated purified NK cells from death by apoptosis. The marginal protective effect of IL-4 may be related to a decreased affinity of HC for its receptor in the presence of cytokines.[25] These results support previous findings in rodents showing that IL-2 and IL-4 inhibited GCS-induced apoptosis of NK and CTL cells.[26] Interestingly, IL-12 augmented prevention of apoptosis of enriched but not purified NK cells in response to IL-2

(M. Armant & M. Sarfati, personal observations). Indeed, IL-12 and IL-2 are directly implicated *in vivo* in the host resistance to viral or bacterial infections because they are potent inducers of IFN-γ production by NK cells, leading to the conclusion that NK cells are active partipants in the prevention of early infection, but that T cells and macrophage-derived products such as IL-2 and IL-12 are required to achieve complete remission.[13]

Besides their ability to prevent PCD of NK cells, IL-2, IL-4 and IL-7 display other common properties. It was recently reported that their respective receptors (IL-2R, IL-4R and IL-7R) share the same γ chain, originally described to be associated with the IL-2R β (p75) chain.[27–29] It is therefore tempting to speculate that the γ chain mediates the cytokine-induced signal of rescue in HC-treated NK cells. In addition, IL-2, IL-7 and IL-12 induce IFN-γ and TNF-α production by NK cells.[18] However, IFN-γ is not involved in the IL-2-mediated protection of GCS-induced death of NK cells, as IFN-γ production could not be detected in HC- and IL-2 treated PBMC, and anti-IFN-γ neutralizing mAb did not abrogate the IL-2 protective effect (data not shown). Moreover, addition of exogenous IFN-γ or TNF-α displayed no protective activity.

Bcl-2 is a proto-oncogene extending cell survival by preventing PCD.[30] In normal tissues, Bcl-2 expression is topographically confined to the zones of surviving B cells in germinal centres or surviving mature thymocytes in the medulla. The present studies indicate that IL-2 and IL-7 up-regulate bcl-2 expression in purified NK cells, suggesting without demonstrating the existence of bcl-2-dependent survival pathways. In that regard, the up-regulation of bcl-2 expression by IL-4 in leukaemic B cells, or by IL-10 in germinal centre B cells, is also associated with protection from apoptosis.[19,20]

It is concluded that PCD, which is a common process of cell death, also occurs in NK cells and that cytokines such as IL-2 and IL-7 exert a pivotal role for these effector cells by preventing cell death, in addition to regulating cell proliferation and cytolytic activity.

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