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Differential cytokine regulation of natural killer cell-mediated necrotic and apoptotic cytotoxicity

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SUMMARY

Natural killer (NK) cells can kill target cells by either necrotic or apoptotic mechanisms. Using the ^{51}Cr-release assay to measure necrotic death of target cells, neonatal NK cells had low NK activity (K562 targets) and high lymphokine-activated killer (LAK) activity (Daudi targets) compared with adult cells, as has been previously reported. Using a ^{125}I-deoxyuridine (^{125}I-UdR) release assay, cord cells were shown to also have higher apoptotic LAK activity against YAC-1 target cells. Interleukin-4 (IL-4) inhibited interleukin-2 (IL-2)-induced necrotic killing of target cells by adult effectors but had no such inhibitory effect on cord cells. In contrast, IL-4 inhibited both adult and cord LAK cytotoxicity of YAC-1 target cells by apoptotic mechanisms with higher suppression observed in cord cell preparations. Using a colorimetric substrate conversion assay, IL-2 induced higher, and IL-4 had a more significant suppressive effect on, cord cell granzyme B enzyme activity compared with adult cells, paralleling apoptosis cytotoxicity data. Co-culture of either adult or cord LAK cells with IL-4 had a similar inhibitory effect on granzyme B protein expression, as detected by Western blotting. In contrast, IL-4 did not inhibit perforin expression, thereby defining IL-4 as a cytokine that can differentially regulate the NK cell-mediated cytotoxicity processes of apoptosis and necrosis. The differential sensitivity of cord cells to cytokine regulation of cytotoxicity may also have implications for cord blood transplantations, as NK cells are known to function as an effector cell in both graft-versus-host disease and in the graft-versus-leukaemia phenomena.

INTRODUCTION

Cell-mediated cytotoxicity is a fundamental process used by the immune system to destroy a range of endogenous target cells, such as transformed cells or those infected by intracellular pathogens. Among lymphocytes, cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells have been identified as key effector cells which mediate this process. Cytotoxic cells can kill their target cells by at least two recognized mechanisms: necrosis and apoptosis.[1] Necrosis is mediated by perforin, found in cytotoxic granules, which primarily causes membrane damage.[2] Apoptosis of target cells can be triggered by a number of effector mechanisms including tumour necrosis factor-α (TNF-α),[3] fas/fas-ligand interactions[4] or via granzymes.[5] Data from knock-out mice indicate that granzyme B is essential for the rapid induction of apoptosis in susceptible target cells, although its mechanism of action is unknown.[6]

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Abbreviations: CM, complete medium; E : T, effector : target; GVH, graft-versus-host-disease; GVL, graft-versus-leukemia; LAK, lymphokine-activated killer.

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One of the primary differences between granzyme B or fas/fas-ligand-mediated apoptosis, and that induced by TNF-α, are the apparent kinetics of action. Apoptosis induced by TNF-α is relatively slow,[3] while both granzyme B[6]- and fas/fas-ligand[4]-mediated apoptosis occur rapidly and can be measured in short-term assays (4 h). Historically, cytotoxicity research almost exclusively employed a necrosis-based cytotoxicity model system (^{51}Cr-release assay). The recognition of apoptosis as the physiological form of cell death *in vivo*[7] challenges this paradigm and demands investigation of NK cell-induced apoptosis of target cells as a more relevant model system.

The neonatal population has a high rate of morbidity and mortality associated with infection,[8] presumably a reflection of their relatively immature immune status. Immune responses of many individual cord blood cell types are altered relative to adult responses,[9] with differences at both effector and regulatory levels. NK cell cytotoxic activity of cord blood against the standard K562 target cell line is reduced[10] while interleukin-2 (IL-2)-induced lymphokine-activated killer (LAK) activity against Daudi target cells is increased.[11,12] Cord blood is now being used successfully as a source of stem cells for haematological reconstitution with reduced graft-versus-host-disease (GVH) observed in patients.[13] As NK cells

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have been implicated in GVH[14] and the graft-versus-leukaemia (GVL) effect,[15] differences in cord and adult NK cell responses help to explain clinical observations while increasing our potential to beneficially exploit the immune system in transplantation situations.

Cytokines play an important role in the regulation of cytotoxicity. IL-2 provides sufficient signal to activate LAK cell cytotoxicity,[16] induce NK cell proliferation[17] and stimulate increased production of granule contents.[18] Interleukin-4 (IL-4)[19] and transforming growth factor-β (TGF-β)[20] are reported to be down-regulatory cytokines of IL-2-induced LAK responses. Given the dearth of information regarding cytokine regulation of NK cell-mediated apoptosis as an independent mechanism of killing, this study was undertaken to examine the role of IL-4 in IL-2-induced cytotoxicity, in both necrotic and apoptotic assay systems. In addition, adult and cord blood cells were evaluated for their NK cell responses at both end-effector and mechanistic levels.

MATERIALS AND METHODS

Effector cells

Adult peripheral blood was obtained by venepuncture from healthy adult volunteers. Cord blood samples were obtained from full term, normal delivery, healthy infants into 0·01 M EDTA in phosphate-buffered saline (PBS), pH 7.4. Mononuclear cells were isolated by lymphoprep (Nycomed, Oslo, Norway) density-gradient centrifugation.[21] Cells cultured *in vitro* were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco, Paisley, UK), 20 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 20 mM HEPES, referred to as complete medium (CM). Cytokines were added at the start of culture; IL-2 (Cetus Corporation, Emeryville, CA) was used at 500 U/ml and IL-4 (R&D Systems, Abingdon, UK) was used at 100 U/ml.

^{51}Cr-release assay

A standard 4-h ^{51}Cr-release assay was used to measure necrotic death of target cells.[22] Briefly, 10^6 Daudi target cells (American Type Culture Collection, Rockville, MD) were labelled with 50 μCi $Na_2{}^{51}CrO_4$ for 1 h at 37°. Cells were washed twice with PBS, resuspended in CM and 1×10^4 cells were dispensed into each well in 100 μl volumes. Effector cells in an equal volume were added, in quadruplicate, to give a desired effector : target (E : T) ratio. The plate was centrifuged at 150 *g* for 5 min at room temperature to initiate cell contact, incubated at 37° for 4 h, and then recentrifuged at 350 *g* for 10 min at room temperature. Decanted supernatants were counted using a γ-counter (Wallac-LKB, Milton Keynes, UK). CM alone or HCl (1 M), added to labelled targets, allowed calculation of spontaneous or maximum release respectively. Percentage kill was calculated using the following equation:

% kill = (experimental − spontaneous)/(maximum − spontaneous) × 100%.

^{125}I-UdR release assay

This assay was used to measure apoptosis of target cells induced by LAK effectors.[23] YAC-1 target cells (5×10^5) (American Type Culture Collection, Rockville, MD) were labelled with 10 μCi ^{125}I-deoxyuridine (^{125}I-UdR), in a 200 μl volume, for 2 h at 37°. Cells were washed twice with PBS, resuspended in CM at 10^6 cells/ml and aliquoted in 0·5 ml volumes into 1·5 ml eppendorf tubes in triplicate. Effector cells, in CM, were added to give the desired E : T ratios. Tubes were centrifuged at 600 g in a minifuge (Heraeus, Hannau, Germany) at room temperature for 5 min to initiate cell contact. After 4 h, tubes were centrifuged at 3500 **g** at room temperature in a minifuge for 5 min. Supernatants were collected and the cell pellets resuspended in 1 ml of a lysis buffer (5 mM Tris/HCl, pH 8·0; 0·1 M EDTA, pH 8·0; 0·5% Triton-X-100). After a brief vortex, tubes were centrifuged at 8000 *g* in a minifuge for 20 min at room temperature to separate fragmented from bulk DNA. These supernatants were combined with those collected previously and counted on a γ-counter for 1 min each. The spontaneous count was obtained from tubes in which CM alone was added to the radiolabelled target cells. The maximum release was found by adding the radioactivity remaining in the spontaneous pellets to the supernatant counts, and the percentage kill calculated using the equation defined above.

Asp-ase assay for granzyme B enzyme activity[24]

Granzyme B has a rare enzyme substrate specificity for aspartic acid,[5] which forms the basis for this assay.[24] Briefly, effector cell lysates were prepared by resuspending cultured cells at 5×10^6 ml or freshly isolated cells at 1×10^7/ml, in 1 ml of lysis buffer (0·1 M HEPES, 0·05 M $CaCl_2$, 0·5% NP40, pH 7·5). Cells were freeze-thawed twice and centrifuged in a minifuge at 8000 *g* for 15 min at room temperature. The resulting supernatants were diluted (0·1 M HEPES, 0·05 M $CaCl_2$, pH 7·5) and aliquoted, in duplicate, into a 96-well flat-bottomed plate in 50 μl volumes. Fifty microlitres of 2 mM *n*-boc-ala-ala-asp-pNA (Bachem, Switzerland) substrate was added per well. Controls consisted of sample and diluent alone, and substrate and diluent alone. Substrate conversion was calculated by measuring the change in OD at 405 nm after 3 days of incubation at 37°. YT cell line extract[24] was used as a positive control.

Western blotting[25]

Cells were extracted at 5×10^7/ml into a lysis buffer (10 mM Tris/HCl, pH 8·0, 100 mM NaCl, 1% NP40, 1 mM PMSF, 1 μg/ml aprotinin, 0·001 M EDTA, 50 μM leupeptin, 1 μg/ml pepstatin, 1 μg/ml antipain). The YT cell line was treated similarly and used as a positive control for granzyme B and perforin expression. Cell extracts (30 μl) were diluted with a non-reducing loading buffer (62·5 mM Tris, 3% w/v SDS, 10% w/v glycerol, 0·01% w/v bromophenol blue) and electrophoresed on a 15% non-reducing SDS–PAGE gel. Samples were electroblotted onto nitrocellulose paper and probed with either an anti-granzyme B monoclonal Ab (Pharmacell, Paris, France) or an anti-perforin monoclonal Ab (from Gillian Griffith, Basel, Switzerland). AEC was used to visualize band staining. Immunoblots were densitometrically analysed using a video-imaging gel documentation system (UVP, GDS 8000, Cambridge, UK). Band density was expressed as an arbitrary pixel intensity scale. The values obtained were analysed statistically to determine the effect of cytokine treatment on granzyme B and perforin expression.

Statistics

A two-tailed Student's *t*-test was used to compare adult and cord blood data, and the effect of cytokines on adult and cord

blood-mediated LAK activity, granzyme B and perforin expression.

RESULTS

Cord LAK cells have higher LAK activity and faster kinetics of killing compared with adult cells

Freshly isolated cells, from either adult or cord blood, did not induce cytotoxicity of either Daudi or YAC-1 cell lines (data not shown) thereby defining the effector cells as LAK cells.[26] Cord and adult cells were compared for their LAK activity in assays that measured either necrotic or apoptotic death of target cells. As previously reported,[11,12] cord LAK cells had higher necrotic killing activity against the Daudi cell line than adult cells (Fig. 1a). This difference was significant ($P<0.05$) at all E:T ratios except 25:1. The Daudi cell line does not undergo double-stranded DNA fragmentation and therefore YAC-1 cells were used to measure apoptosis using the ^{125}I-UdR release assay (Fig. 1b). Cord LAK cells were also found to have significantly higher cytotoxicity against YAC-1 cells ($P<0.05$). The enhanced cytotoxicity of cord LAK cells was reflected also in the faster kinetics of killing, seen in both assay systems.

IL-4 differentially regulates necrotic killing of Daudi cells by adult and cord LAK effector cells

IL-4 has been reported to inhibit IL-2-induced LAK activity of adult cells,[19,20] and this was found to be the case for IL-2-induced killing of adult cells against Daudi target cells (Fig. 2a). By contrast, IL-4 had no such inhibitory effect on cord cells (Fig. 2b) over a range of E:T ratios. The differential IL-4 effect on adult compared with cord LAK effector cells was statistically significant ($P<0.05$) at all E:T ratios with the exception of 25:1.

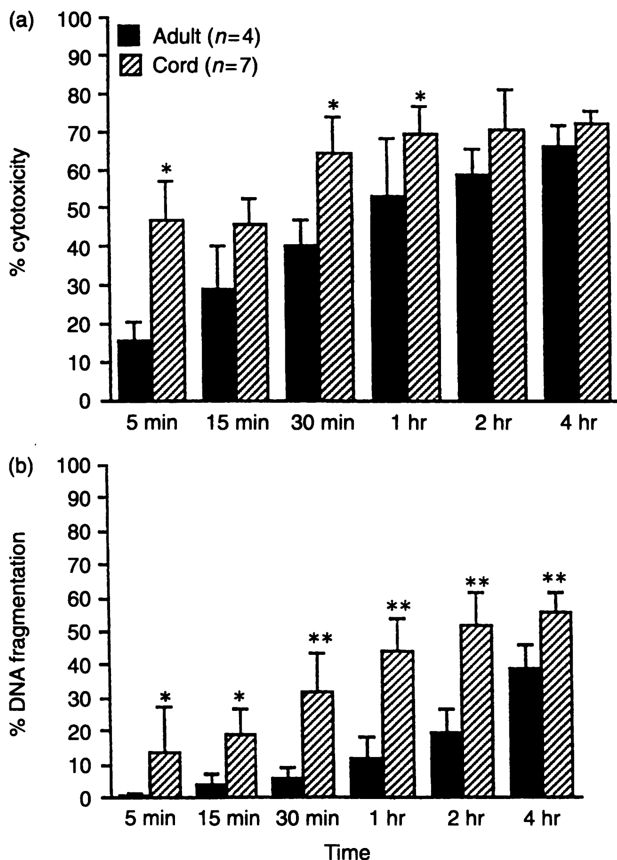

Figure 1. Kinetics of killing by adult and cord LAK effectors. Adult ($n=4$) and cord ($n=7$) mononuclear cells were cultured for 3 days in CM supplemented with 500 U/ml IL-2. Cells were washed twice and their cytotoxicity against Daudi cells (necrosis assay, 1a) or YAC-1 cells (apoptosis assay, 1b) measured. Assays were harvested at the time points indicated. Figures show percentage kill and the error bars show 1 SD. A constant E:T ratio of 25:1 was used throughout. Spontaneous/maximum values were less than 20% in all experiments using Daudi cells and less than 12% in all YAC-1 cell experiments. The Student's t-test was used to compare adult and cord blood data. $^{*}P<0.05$, $^{**}P<0.02$.

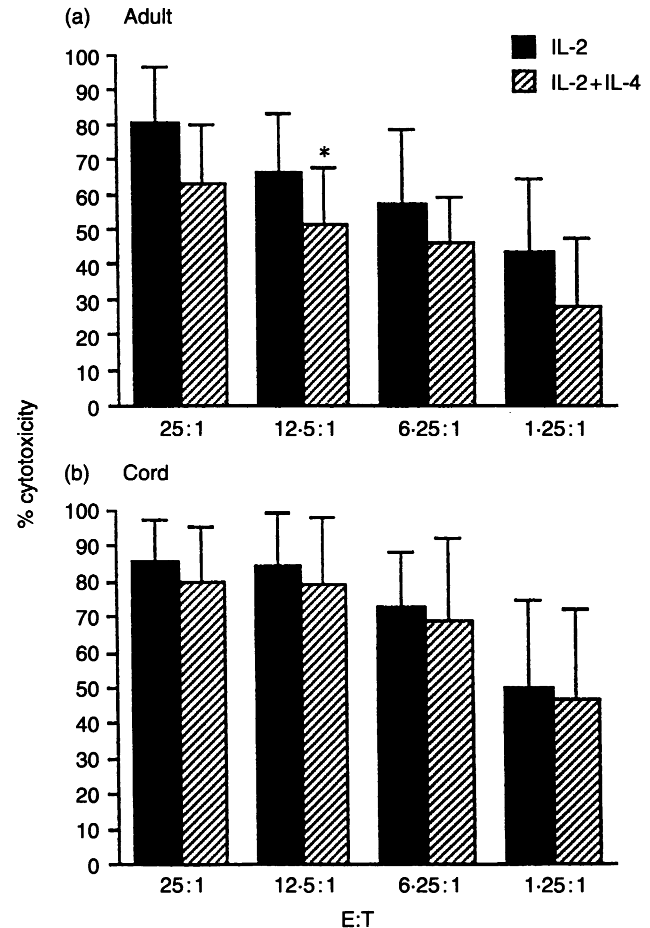

Figure 2. Effect of IL-4 on IL-2-induced LAK activity against Daudi target cells. Isolated adult ($n=7$) and cord blood effector cells ($n=8$) were cultured in IL-2 (500 U/ml), in the presence or absence of IL-4 (100 U/ml). After 3 days at 37°, the cells were washed and their cytotoxicity measured against the Daudi cell line. The figures show the percentage kill + 1 SD, at the E:T ratio indicated. The average spontaneous/maximum value was 17.1%. (a) adult, (b) cord. The student's t-test was used to compare adult and cord blood data. $^{*}P<0.02$.

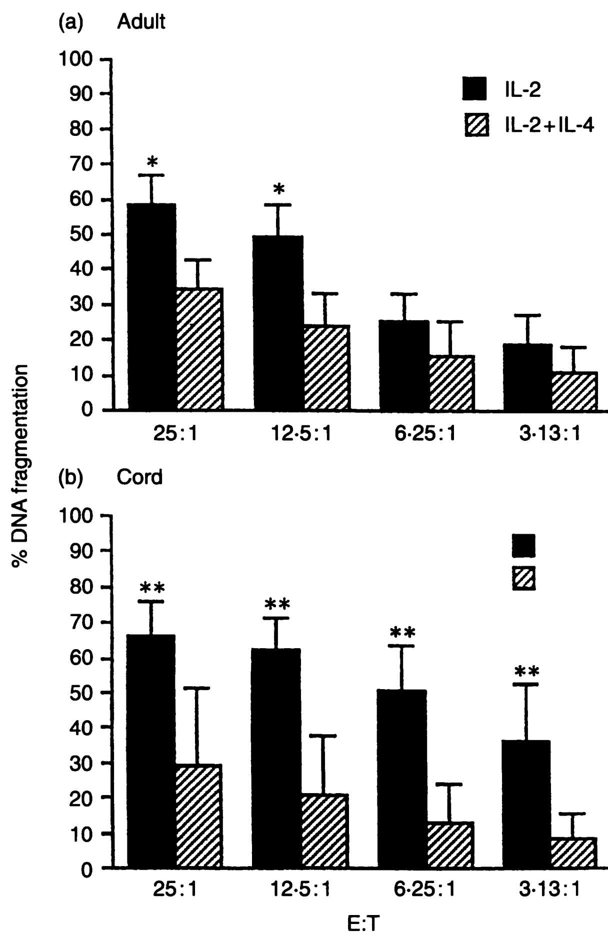

Figure 3. Effect of IL-4 on IL-2-induced LAK activity against YAC-1 target cells. Isolated adult ($n=5$) and cord blood effector cells ($n=8$) were cultured in IL-2 (500 U/ml), in the presence or absence of IL-4 (100 U/ml). After 3 days at 37°, the cells were washed and their cytotoxicity measured against the YAC-1 cell line. The figures show the percentage kill + 1 SD, at the E:T ratio indicated. The average spontaneous/maximum value was 9.4%. (a) adult, (b) cord. The student's t-test was used to compare adult and cord blood data. *$P<0.02$, **$P<0.001$.

IL-4 inhibits IL-2-induced apoptotic killing of YAC-1 target cells by adult and cord LAK effector cells

Co-culture of both adult and cord effector cells in IL-4 significantly inhibited their cytotoxic activity ($P<0.05$) against YAC-1 target cells over a range of E:T ratios. This inhibition was more significant for cord cells (Fig. 3; $P<0.02$).

IL-2 and IL-4 regulation of granzyme B enzyme activity

Cell extracts, prepared from freshly isolated cells, contained low comparable amounts of granzyme B activity: adult ($n=7$) 0.205 ± 0.119 ÆOD units versus cord ($n=8$) 0.183 ± 0.080 Æ OD units. Culture of cells in IL-2, for 3 days, induced granzyme B activity as illustrated in Table 1. Paralleling cytotoxicity results, IL-2 induced higher granzyme B activity in cord blood cells compared with adult cells. Co-culture of cells, from either source, in IL-4, inhibited the IL-2-induced granzyme B activity.

IL-4 inhibits IL-2-induced granzyme B expression in adult and cord effector cells

IL-2 induced granzyme B expression in freshly isolated adult and cord mononuclear cells (data not shown). Co-culture of cells with IL-2 and IL-4 resulted in significantly decreased expression of granzyme B protein, as detected by Western blotting, compared with culture in IL-2 alone (Fig. 4; $P<0.001$). This inhibition was seen for both adult and cord blood cells.

IL-4 does not inhibit IL-2-induced perforin expression in adult and cord effector cells

Perforin expression in freshly isolated cells was low but variable (data not shown). However, IL-2 induced strong perforin bands in all samples, as detected by Western blotting (Fig. 5). Co-culture of cells with IL-4 did not significantly inhibit IL-2-induced perforin expression ($P<0.05$). Results were similar for both adult and cord blood cells.

DISCUSSION

Given that apoptosis is the physiological form of cell death[7] and the probability that it is the primary mechanism involved in cell-mediated cytotoxicity, adult and cord blood NK cells were compared for their ability to kill target cells by apoptosis: IL-2 induced higher LAK activity in cord cells compared with adult cells against YAC-1 target cells (Fig. 1b). This was found over a range of E:T ratios (data not shown). Cord cells had faster kinetics of killing YAC-1 target cells than did adult cells. The increased sensitivity to IL-2, of cord compared with adult NK cells, was paralleled in the necrosis-based cytotoxicity assay (Fig. 1a), as previously reported.[11,12] This increased sensitivity may be caused in part by the increased expression of IL-2Rβ, the signalling moiety of the IL-2R involved in NK cell activation, on cord blood NK cells.[27] Kinetics of killing were faster in the necrosis assay, compared with the apoptosis assay, using either adult or cord effector cells. This has been previously reported for NK cell-mediated cytotoxicity[28] although reports to the contrary also exist;[1,23] these discrepancies may be a result of differences in target cell lines and/or assay protocols and remain to be reconciled. The relatively low NK activity of freshly isolated cord cells (data not shown) may contribute to the low GVH seen in cord blood transplant patients, but the readily induced cytotoxicity of cord LAK activity, measured using both necrosis and apoptosis-based assays, as presented in this study, indicates that the potential for a GVL effect remains.

The role of IL-4 in necrosis-mediated cytotoxicity has been extensively examined. In mice, IL-4 can directly generate LAK cells from NK cell precursors.[29] In humans, preactivation of cells with IL-2 facilitates LAK induction by IL-4;[30] however, IL-4 is generally reported to be a suppressive cytokine for NK cell activation.[19,20] In this study, IL-4 was found to have differential regulatory effects on human adult and cord LAK cell killing of Daudi target cells. IL-4 inhibited IL-2-induced LAK activity of adult cells, as has been previously reported; however, IL-4 had no effect on cord LAK activity. In contrast to the necrosis assay system, IL-4 had an inhibitory effect on both adult and cord LAK activity in the apoptosis-based assay and, furthermore, IL-4 inhibition of cord activity was more

Table 1. Cytokine regulation of granzyme B activity†

	Adult ($n=5$)		Cord ($n=8$)	
	IL-2	IL-2+IL-4	IL-2	IL-2+IL-4
Δ OD405 nm	0·478±0·157	0·272*±0·112	0·705±0·401	0·269**±0·130

†Isolated adult ($n=5$) and cord ($n=8$) effectors were cultured for 3 days in IL-2 (500 U/ml), in the presence or absence of IL-4 (100 U/ml). After this time, the cells were washed, lysed and extracts prepared. These extracts were incubated with a substrate containing aspartic acid. A colour change was observed as the substrate was enzymatically converted. Figures shown represent the change in OD at 405 nm±1 SD, after 3 days of incubation. The Student's t-test was used to analyse adult and cord data.

$^*P<0{\cdot}02$, $^{**}P<0{\cdot}01$.

Figure 4. Effect of IL-4 on IL-2-induced granzyme B protein expression. Cord blood ($n=3$) and adult peripheral blood ($n=3$) mononuclear cells were cultured in IL-2 (500 U/ml) and IL-2+IL-4 (500 U/ml, 100 U/ml) for 3 days. Samples were extracted, electrophoresed on 15% non-reducing SDS–PAGE gel and immunoblotted using anti-granzyme B monoclonal antibody. YT cell extract was used as a positive control. AEC was used to visualize staining.

Figure 5. Effect of IL-4 on IL-2-induced perforin protein expression. Cord blood ($n=2$) and adult peripheral blood ($n=3$) mononuclear cells were cultured in IL-2 (500 U/ml) and IL-2+IL-4 (500 U/ml, 100 U/ml) for 3 days. Samples were extracted, electrophoresed on 10% non-reducing SDS–PAGE gel and immunoblotted using anti-perforin monoclonal antibody. YT cell extract was used as a positive control. AEC was used to visualize staining.

profound. Differential cytokine regulation, by adult and cord blood cells of key effector molecules involved in necrosis and apoptosis, was investigated as a basis for the disparate cytotoxicity data obtained. Fas-mediated cytotoxicity was thought not to play a role in the model systems studied here as YAC-1 cells did not express fas while Daudi cells (necrosis model) expressed it only at a very low level. Furthermore, inclusion of neutralizing anti-TNF-α or TNF-β antibodies had no effect on the data obtained in either assay for the 4-h incubation period studied (data not shown). IL-4 inhibited IL-2-induced

granzyme B activity and expression in both adult and cord cell populations, thereby explaining the IL-4 inhibition of cytotoxicity observed in the apoptosis cytotoxicity assay; the more profound inhibition of granzyme B enzyme activity in cord cells by IL-4 explained the increased sensitivity to IL-4 inhibition of cytotoxicity by cord cells in the apoptosis assay. In contrast to its effect on granzyme B, IL-4 had no apparent regulatory effect on IL-2-induced perforin expression. Although this result correlates with the cord cytotoxicity data, it implies that IL-4 inhibition of adult cytotoxicity of Daudi cells is not at the level of inhibition of perforin and that other mechanisms are involved, which could involve altered production of regulatory cytokines[31,32] and/or cytokine receptors.[20]

The differential effect of IL-4 on LAK cell cytotoxicity, in each of the assay systems, further highlights differences which exists between adult and cord effector cells. Given the similar qualitative regulation of cytotoxic molecules in the two populations, differences appear to be more quantitative with respect to the magnitude and efficiency of down-regulation of responses. Cord cells are reported to have a reduced expression of the IL-4R[33–35] and are generally reported to produce minimal amounts of IL-4.[36] One might therefore expect a reduced responsiveness of cord cells to this cytokine. Although the necrosis assay results support this, the profound inhibition of cytotoxicity observed in the apoptosis assay belies this suggestion. Further differential responses of adult and cord cells to IL-4 have recently been reported by this laboratory and others. Highly purified $CD4^{+}CD45RA^{+}$ cord cells were shown to proliferate directly in response to IL-4, in the absence of a second stimulus, in contrast to a similar population of adult cells.[37] Others have shown that, despite having significantly fewer cells expressing the IL-4R, neonatal B cells are as responsive to IL-4 in up-regulating major histocompatibility complex (MHC) Class II and surface immunoglobulin M (IgM); and as, if not more responsive in their up-regulation of the CD23 antigen.[33] Thus, differential sensitivity of cord cells to IL-4 is likely to account for many of the differences seen between adult and cord cells and may contribute to the reported preferential development of a T helper 2 (Th2) immune response observed *in vivo* in the newborn.[38]

Perforin has traditionally been implicated in the necrosis pathway of cell-mediated cytotoxicity.[2] Recent reports however, suggest a role for perforin in the apoptosis pathway where it may facilitate transfer of cytotoxic granule contents into the target cell.[39,40] It seems likely that apoptosis, the physiological form of cell death, is also the primary mechanism of cell-mediated cytotoxicity *in vivo* with perforin representing just one step, albeit a critical one, in the apoptotic pathway. The tighter regulation by cytokines of granzyme B compared to perforin expression, as presented in this report, lends credence to this hypothesis because key biological effector molecules are tightly regulated to govern the response magnitude. Furthermore, the differential regulation of perforin and granzyme B by IL-4 serve to caution against extrapolating cytokine regulation data from necrosis to apoptosis-mediated cytotoxicity and highlights the requirement for further research on apoptosis as an independent mechanism of cytotoxicity used by LAK effector cells.

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