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Target-cell-induced anergy in natural killer cells: suppression of cytotoxic function

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Abstract Our earlier studies have demonstrated that natural killer (NK) cells are the effectors that participate during the spontaneous regression of AK-5 tumour in syngeneic hosts. We have shown that the tumour cells are killed by necrosis and apoptosis. In this study, we have examined the induction of functional anergy in NK cells following coculture with fixed AK-5 tumour cells at high ratio. NK cells, upon coculture with fixed AK-5 cells (1:1 ratio), showed loss of cytotoxic function against both AK-5 (antibody-dependent cell cytotoxicity) as well as YAC-1 targets. The response of these cells to the activation by recombinant interleukin-2 and recombinant interferon γ was poor. Induction of tumour necrosis factor α (TNFα) secretion was observed after coculture of NK cells with fixed AK-5 cells. The cocultured cell supernatant inhibited the cytotoxic activity of NK cells, which was partially restored with anti-TNFα antibody. In addition, NK cells, after treatment with fixed tumour cells showed overexpression of the Fas receptor. We have also observed induction of apoptosis in cocultured NK cells. These studies suggest that the fixed tumour cells (antigen) at high ratio are able to suppress NK cell function as well as induce death in NK cells.

Key words NK cell anergy

Introduction

Natural killer (NK) cells have been shown to participate in the regulation and development of an effective immune response against infection and cancer. Patients with poor NK activity possess lower resistance to infection and increased cancer metastases [3, 22]. NK cells can be activated by interleukin-2 (IL-2), IL-12 or interferon γ (IFNγ) to mediate potent cytotoxic activity against a variety of tumour cells and virally infected cells. However, NK cells become inactive and lose their cytotoxic function following a short exposure to sensitive target cells [1, 10]. One mechanism of inactivation proposed is the lack of production of cytolytic factors by NK cells following exposure to target cells. NK cells that have been inactivated by target cells exhibit down-regulation and/or shedding of the FcγRIII CD-16 receptor. This observation suggested that CD-16 might be playing an active role in the target-cell-induced NK cell anergy and apoptosis [8, 11].

AK-5 is a highly immunogenic rat histiocytic tumour [4, 13, 14], which regresses spontaneously in syngeneic animals when transplanted s.c., whereas it kills 100% animals when transplanted i.p. The death of AK-5 cells is achieved through necrosis [12] and apoptosis [5]. Our present studies demonstrate that fixed AK-5 cells in vitro are able to activate or inactivate NK cell cytotoxic function depending on their ratio in coculture. When the ratio of the fixed cells to the splenocytes is high in coculture experiments, the NK cells are inactivated. In addition, there is secretion of tumour necrosis factor α (TNFα) into the culture medium, which seems to down-regulate NK cell function. There is also up-regulation of Fas receptor (Fas-R)expression on the NK cells that have been inactivated.

Thus, in this present study, the following questions were examined: (a) whether coculture of normal and immune NK cells (NK cells obtained from an AK-5-tumour-rejecting rat) with a high concentration of fixed AK-5 cells results in reduced cytotoxic function of NK cells; (b) whether the inactivated NK cells can be activated by recombinant IL-12 and IFNγ; (c) whether selective induction of TNFα secretion by splenocytes after coculture with fixed tumour cells is responsible for the inactivation of NK cell function; (d) whether there is any change of the NK cell surface markers after coculture with fixed AK-5 cells; (e) whether the treatment of NK

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cells with fixed AK-5 cells results in the induction of apoptosis in NK cells.

Materials and methods

Animals and tumours

Wistar rats from the inbred colony of this laboratory were used in this study. The AK-5 tumour was maintained as ascites in 6- to 8-week-old rats by injecting 5×10^6 cells intraperitoneally. The murine lymphoma cell line YAC-1 was maintained in vitro in RPMI-1640 medium supplemented with 10% fetal calf serum. Solid AK-5 tumours were obtained by injecting 5×10^6 AK-5 cells s.c. into 6-week-old rats.

Fixation of AK-5 cells

Formaldehyde-fixed AK-5 cells were obtained by suspending washed tumour cells in 3% formaldehyde for 30 min at room temperature. Fixed cells were washed four times with phosphate-buffered saline (PBS), counted and used in the coculture experiments.

Antibodies and reagents

Recombinant rat IFNγ was obtained from Genzyme and recombinant murine IL-12 was kindly provided by Dr. U. Gübler, Hoffmann-La-Roche. Antibody against Fas-R was procured from Santa-Cruz Biotechnology, anti-CD-16 and anti-CD25 were from Serotec (UK), fluorescein-isothiocyanate(FITC)-conjugated goat anti-(mouse IgG) and rabbit anti-(mouse IgG) were from Amersham Pharmacia and anti-TNFα antibody was from Genzyme.

Preparation of splenocytes

Spleens from both normal and tumour-bearing rats were removed aseptically, teased in RPMI-1640 medium and passed through a 20-gauge needle to obtain a single-cell suspension. Mononuclear cells were obtained after fractionation on a Ficoll-Hypaque gradient. The cells were washed twice with the medium and used in different experiments.

Preparation of NK cells

NK cells from the cocultured splenocytes and AK-5 cells were isolated after their adherence to Dynal magnetic beads coated with mAb 3.2.3 (anti-NKR-P1, Endogen Inc.). Preparations with more than 95% positive NK cells were used in these experiments.

Enzyme-linked immunofiltration assay (ELIFA)

The cytokine levels in the coculture supernatants were quantified by ELIFA. The ligand solution was filtered through the nitrocellulose membrane, allowing it to bind to the membrane. The membrane was blocked with PBS/bovine serum albumin (BSA) solution and treated with primary antibody. The membrane was washed and treated with horseradish-peroxidase-conjugated secondary antibody. The free antibody was removed from the membrane by washing and the signal was generated after developing for peroxidase.

Analysis of NK cell markers

The paraformaldehyde-fixed NK cells were washed twice with PBS containing 1% BSA. Predetermined optimal concentrations of specific mAb were added to 5×10^4 cells in 50 µl cold PBS/BSA and the cells were kept at 4 °C for 60 min. The cells were washed twice and treated with FITC-conjugated secondary antibody (1:100) for 30 min. The cells were washed and analysed by flow cytometry.

Anti AK-5 antibody

Serum collected from rats that had previously rejected AK-5 tumour and were positive in a complement-fixation assay was used as anti-AK-5 antiserum. The serum was partially purified by precipitation with 18% and 12% sodium sulfate followed by dialysis against PBS. Anti-AK-5 antibody free from cytokines like IL-2, IFNγ and IL-12, as checked by ELIFA, was pooled, filter-sterilised, divided into aliquots and stored at −20 °C with 0.1% NaN_3.

Cytotoxicity assays

Cytotoxicity was measured by ^{51}Cr-release assay. Purified NK cells were isolated from the cocultured cell mixture by Dynal magnetic beads coated with mAb 3.2.3 and incubated with ^{51}Cr-labelled AK-5 cells in the presence of anti-AK-5 antibody and YAC-1 cells at an E:T ratio of 100:1 for 4 h. Chromium released in the supernatant was counted in a Packard gamma counter and the percentage cytotoxicity was calculated.

Pretreatment of splenocytes with fixed AK-5 cells

Freshly isolated normal and immune (AK-5-tumour-rejecting animals) splenocytes were cocultured with fixed AK-5 cells at different ratios for 20–24 h. The NK cells were then separated on mAb-3.2.3-coated Dynal beads, detached from the beads, washed and used in different experiments.

Western blotting

Culture supernatants concentrated by ultrafiltration were dissolved in sample buffer and boiled for 5 min. The samples were electrophoresed on 15% sodium dodecyl sulfate/polyacrylamide gels and transferred to nitrocellulose membranes. The blots were treated with appropriate dilutions of primary and secondary antibodies, washed and developed for alkaline phosphatase using 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium as substrates.

DNA staining and apoptosis of NK cells

Staining of DNA was performed by labelling the NK cells with propidium iodide. Briefly, 2×10^6 cells were washed twice with PBS and incubated in 80% ethanol on ice. The cells were washed twice with PBS and incubated with 100 µl RNase (1 mg/ml) for 30 min before the addition of 500 µl propidium iodide (1 mg/ml) to each sample. After 1 h of incubation in the dark, the DNA analysis was performed in a flow cytometer.

Statistical analysis

Student's *t*-test was used to analyse the significance of the difference between control and experimental groups. Differences were considered to be significant at $P < 0.01$.

Results

NK cell cytotoxicity after co-culture with fixed tumour cells

Antigen concentration may be a major regulator of effector cell functions. Recent experiments in vitro have

implicated antigen dose as an important factor in immune response. We have studied the effect of fixed AK-5 cells (antigen) on normal rat splenocytes. A fixed number of splenocytes were cocultured with fixed AK-5 cells at different E:T ratios. After 20 h incubation, NK cells were isolated from the cocultured cell suspension by mAb-3.2.3-coated Dynal beads and were tested for their cytotoxic activity against ^{51}Cr-labelled AK-5 and YAC-1 target cells. At higher antigen concentration (E:T = 1:1), the NK cell cytotoxicity was markedly lower than that of untreated NK cells, whereas at lower antigen concentration (E:T = 100:1), NK cells showed a significant augmentation in their cytotoxic activity (Table 1). The augmentation in the cytotoxic activity of NK cells is not due to the binding of mAb 3.2.3, which is a known activator of NK cell function, because the control cells had also been isolated by the same procedure. Since AK-5 is a macrophage-like cell, we also cocultured splenocytes with formaldehyde-fixed normal macrophages at a 1:1 ratio. There was no significant difference in the cytotoxic activity of NK cells (data not shown). These observations suggest that the interaction of splenocytes with fixed tumour cells can either activate or suppress the NK cell cytotoxic function, which is regulated by the concentration of the antigen present during coculture. We have also confirmed the inactivation of NK cell function after coculture of fixed AK-5 cells with Dynal-bead-purified NK cells at 1:1 ratio. There was a significant reduction in the cytotoxic activity of NK cells (data not shown).

Table 1 Effect of fixed AK-5 tumor cells on the cytotoxicity of NK cells. Cytotoxicity of natural killer (NK) cells, isolated from normal rat spleens following coculture with fixed AK-5 cells for 20 h at different splenocytes to fixed tumour cell ratios, on AK-5 and YAC-1 target cells by a 4-h ^{51}Cr-release assay. Cytotoxicity to AK-5 cells was through antibody-dependent cellular cytotoxicity (*ADCC*) in the presence of anti AK-5 antibody

Splenocytes: fixed target cell ratio	Cytotoxicity (%) against	
	AK-5 (ADCC)	YAC-1
Control	14 ± 4	22 ± 5
100:100	2 ± 1	6 ± 2
100:50	3 ± 1	9 ± 2
100:25	5 ± 2	10 ± 4
100:10	12 ± 4	21 ± 6
100:1	45 ± 6	56 ± 8
100:0.5	32 ± 8	48 ± 1
100:0.1	21 ± 4	35 ± 4

Effect of fixed AK-5 cells on the cytotoxic activity of immune NK cells

We examined the cytotoxic properties of immune NK cells obtained from animals that rejected the AK-5 tumour, following their treatment with fixed AK-5 cells at a high tumour cell to splenocyte ratio (1:1). NK cells after coculture were separated by mAb-3.2.3-coated Dynal beads and tested for cytotoxicity against YAC-1 and AK-5 target cells. As compared to the untreated splenocytes, the NK cells isolated after coculture exhibited markedly less cytotoxicity (Table 2). Thus the interaction between immune splenocytes and fixed AK-5 cells for 20 h resulted in the inactivation of NK cell cytotoxic function. We have also done control experiments with formaldehyde-fixed macrophages, which did not show any suppression of NK cell cytotoxicity.

TNFα secretion by splenocytes after coculture with fixed AK-5 cells

The effect of fixed AK-5 cell treatment on splenocytes for the secretion of cytokines was investigated. Splenocytes were cocultured overnight with fixed AK-5 cells. The supernatants were harvested and analysed by ELIFA for the presence of cytokines. Addition of fixed AK-5 cells to splenocytes induced significant secretion of TNFα. TNFα was secreted in the coculture supernatants by both immune and normal splenocytes following overnight incubation with fixed AK-5 cells at a 1:1 ratio. No TNFα production was observed at lower antigen concentration, where we have observed activation of NK cell function. TNFα levels secreted by immune splenocytes were higher than the TNFα levels secreted by normal splenocytes (Fig. 1). In the case of untreated splenocytes, low levels of TNFα secretion were observed. Secretion of TNFα in the culture supernatant by cocultured splenocytes was further confirmed by Western blot analysis (Fig. 2). These findings demonstrate that the

Table 2 Functional inactivation of immune NK cells after treatment with fixed AK-5 cells. Cytotoxicity of NK cells, isolated from spleens of AK-5-tumour-rejecting rats, after coculture with fixed AK-5 cells for 20 h, at high fixed cells to splenocytes ratio (1:1), on AK-5 and YAC-1 target cells by a 4-h ^{51}Cr-release assay. Cytotoxicity against AK-5 cells was through ADCC in the presence of anti AK-5 antibody

NK cells	Cytotoxicity (%)					
	AK-5 cells			YAC-1 cells		
	Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 3
Control	28 ± 4	17 ± 5	22 ± 4	56 ± 9	52 ± 9	48 ± 7
Control + anti-AK-5	52 ± 7	47 ± 3	45 ± 6	–	–	–
Treated	11 ± 5	9 ± 2	12 ± 4	18 ± 7	22 ± 8	25 ± 4
Treated + anti-AK-5	17 ± 7	20 ± 5	19 ± 4	–	–	–

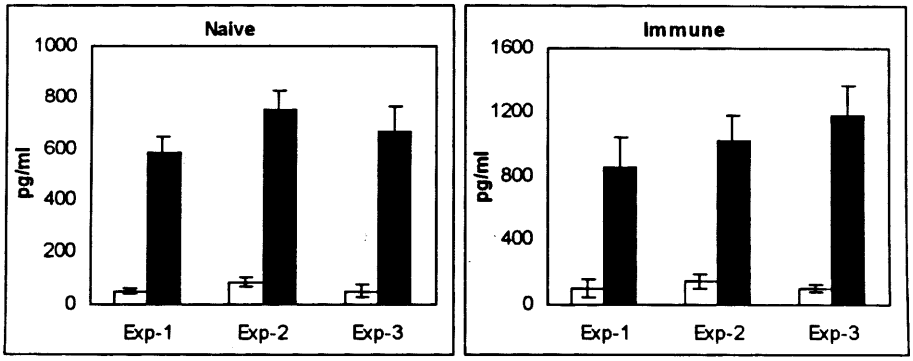


Fig. 1 Induction of tumour necrosis factor α (TNFα) secretion by naïve and immune splenocytes after coculture with fixed AK-5 cells (1:1). Supernatants were harvested from untreated splenocytes (*white bar*) and cocultured splenocytes (*black bar*) after 24 h and analysed for the presence of TNFα by enzyme-linked immunofiltration assay. Data are the means ± SD from three different experiments in triplicate. $P < 0.01$, experimental group versus control

treatment of naïve or immune splenocytes with fixed AK-5 cells selectively triggered the secretion of TNFα.

Role of endogenous TNFα secretion in the suppression of NK cell function

The above findings demonstrated selective secretion of TNFα following treatment of either naïve or immune splenocytes with fixed AK-5 cells, suggesting a possible role for TNFα in various manifestations observed in the fixed AK-5-cell-treated NK cells. Accordingly, it was presumed that the neutralization of TNFα activity would reverse the induction of anergy in NK cells. The addition of anti- TNFα antibodies to the splenocytes cocultured with fixed AK-5 cells resulted in partial inhibition of NK cell anergy (Fig. 3). Normal and immune splenocytes, when treated with fixed AK-5 cells, showed a decrease in NK cell cytotoxicity, as tested against YAC-1 and AK-5 cells (Fig. 3, bars A and D). However, the suppression of cytotoxicity was partially recovered after the addition of anti-TNFα antibody during coculture, (Fig. 3, bars B and C). These observations suggest an important role for TNFα in the induction of anergy in NK cells.

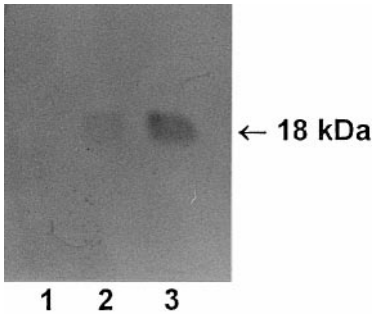


Fig. 2 Western blotting of TNFα secretion by splenocytes after 24 h incubation with fixed AK-5 cells. *Lanes*: *1* normal splenocytes, *2* normal splenocytes + fixed AK-5 cells, *3* immune splenocytes + fixed AK-5 cells

Production of soluble molecules during splenocyte tumour cell coculture

Earlier experiments suggested TNFα to be at least partly responsible for the inactivation of NK cells. In order to study whether the inactivation of NK cells is due to the production of soluble molecules or whether cell–cell interaction is absolutely essential, we tested the effect of culture supernatants obtained from cocultured normal and immune splenocytes on the cytotoxic activity of naïve and immune NK cells. At higher concentrations of culture supernatants the cytotoxicity of NK cells was significantly reduced (Fig. 4). These observations suggest that the interaction between splenocytes and fixed-AK-5 cells triggers the secretion of soluble molecule(s), including TNFα, which are involved in the induction of anergy in NK cells.

Expression of surface molecules by NK cells

We also tested the expression of different molecules like CD-16, CD-25 and perforin, which are generally associated with the activation of NK cell function. There was no significant difference in the expression of CD-16, CD-25 and perforin by NK cells before and after treatment with fixed tumour cells (data not shown). However, the induction of Fas-R in cocultured NK cells was confirmed by immunofluorescence and cytofluorimetric analysis by staining NK cells with a specific anti Fas-R antibody. The number of Fas-R-positive NK cells was higher after coculture with fixed AK-5 cells than in untreated NK cells (Fig. 5).

Apoptosis in NK cells

The death of cocultured NK cells by apoptosis was determined by propidium iodide staining. Significant NK cell apoptosis was observed following 48 h and 72 h incubation of splenocytes with fixed AK-5 cells. A higher percentage of apoptotic cells was seen in immune

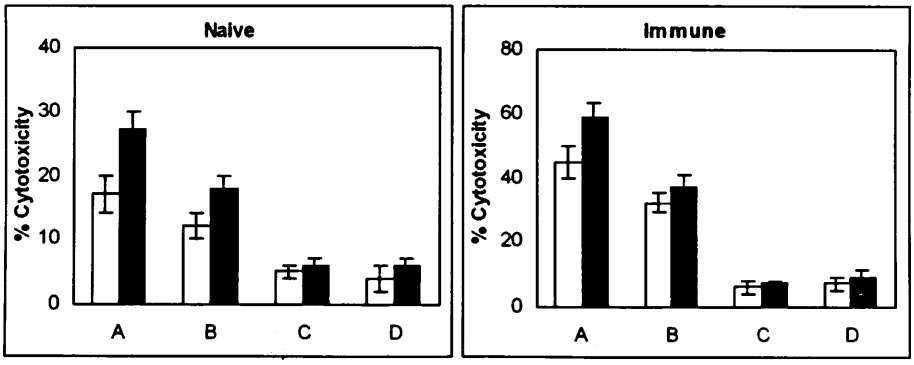


Fig. 3 Cytotoxicity of natural killer (NK) cells to AK-5 (*white bar*) and YAC-1 (*black bar*) cells. Normal and immune splenocytes were cocultured with fixed tumour cells in the presence and absence of anti TNFα antibody; NK cells were then separated on magnetic beads. *A* NK cells without coculture, *B* NK + anti-TNFα antibody + fixed AK-5 cells, *C* NK + control isotype antibody + fixed AK-5 cells, *D* NK + fixed AK-5 cells. Data shown are the means ± SD and are representative of three experiments. $P < 0.01$ between *B* and *C*

NK cells than in the naïve NK cells, following coculture with fixed AK-5 cells. There was a gradual increase in NK cell death with coculture incubation time; after 72 h incubation about 80% NK cells had undergone apoptosis (Fig. 6).

Discussion

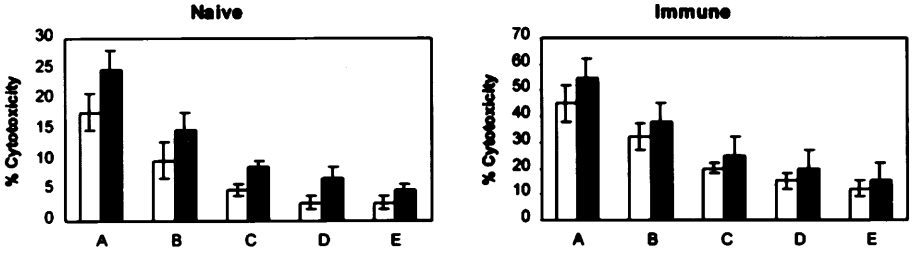
AK-5 tumour, when transplanted s.c., regresses spontaneously in syngeneic rats [4, 13, 14]. We have earlier shown that the killing of tumour cells is mediated by $CD3^-$, $CD8^+$ NK cells through antibody-dependent cellular cytotoxicity [12] and that the effector cell is able to kill the tumour cell through necrosis and apoptosis [5]. We have also shown earlier that the tumour-infiltrating lymphocytes (TIL) were ineffective in a cytotoxicity assay, ascribing it to the depletion of the lytic molecules in the effector cell. The lytic molecules were regenerated by the effector cell during an overnight culture with IL-2 [19].

This study provides evidence for the role of endogenously secreted TNFα in mediating functional inactivation, which ultimately leads to apoptosis in NK cells that have been cocultured with fixed AK-5 cells at 1:1 ratio. In addition, a change in the phenotype from Fas-R^{dim} to Fas-R^{bright} was observed in NK cells cocultured with target cells. The up-regulation of Fas-R expression on the NK cells after coculture with fixed AK-5 cells may play an important role in signalling NK cells for functional anergy and apoptosis [7].

NK cells, when treated with fixed AK-5 cells, lost their cytotoxic function. This was observed for both normal and immune NK cells. Functional inactivation could be due to the depletion of the lytic factors or to the impairment of the activation pathway of the lytic machinery. However, no difference in perforin expression was observed between treated and untreated immune NK cells, suggesting that anergy induction in the tumour-cell-treated NK cells is not due to the depletion of the lytic factors. The other possibility is that the treatment of splenocytes with fixed AK-5 cells caused the impairment of the activation pathway of the lytic machinery, finally leading to the apoptosis of NK cells. Addition of IL-12 and IFNγ to the NK cells, isolated from splenocytes following coculture with fixed AK-5 cells for 20 h, partially restored their cytotoxic function, suggesting their recovery from the anergic state (data not shown). These results indicate that the reduction in cytotoxic function is more likely due to the functional inactivation of NK cells and the possible involvement of IL-2 and IFNγ in the reactivation of inactivated NK cells.

Furthermore, the treatment of splenocytes with fixed AK-5 cells triggered the secretion of TNFα, which was found to play a critical role in the induction of NK cell

Fig. 4 Cytotoxicity of NK cells to AK-5 (*white bar*) and YAC-1 (*black bar*) cells. Normal and immune NK cells were incubated with culture supernatants (at different dilutions with fresh culture medium) obtained from fixed AK-5-cell-treated splenocytes. *A* Control splenocytes, *B* splenocytes + culture supernatant (2:1), *C* splenocytes + culture supernatant (1:1), *D* splenocytes + culture supernatant (0.5:1), *E* splenocytes + fixed AK-5 cells control. Data shown are mean ± SD and are representative of three similar experiments



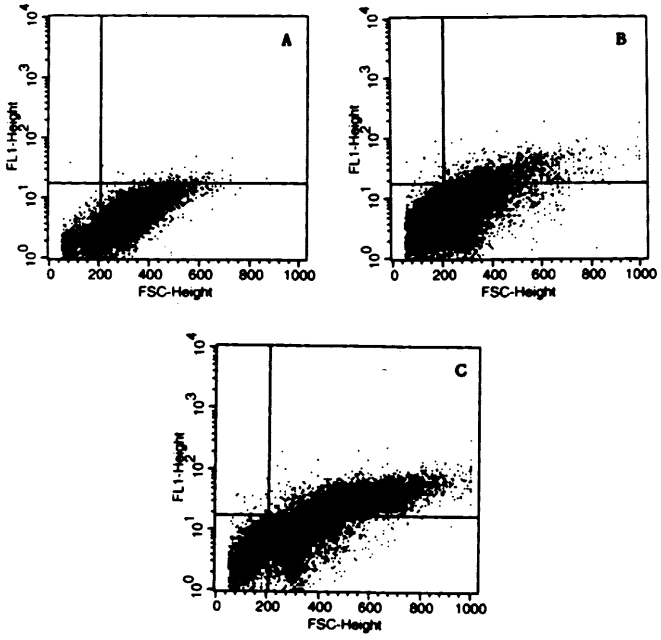


Fig. 5A–C Up-regulation of Fas-R in immune NK cells cocultured with fixed AK-5 cells. **A** Secondary antibody control, **B** untreated immune NK cells, **C** immune NK cells cocultured with fixed AK-5 cells for 24 h. After 24 h, NK cells were isolated and stained for Fas-R expression

inactivation and death. The role of TNFα was corroborated by demonstrating that the blocking of TNFα function by anti-TNFα antibody partially abrogated the AK-5-cell-mediated NK cell inactivation. The addition of anti TNFα resulted in partial inhibition of the induction of anergy in normal and immune NK cells. These observations suggest that TNFα may be one of the molecules involved in the NK cell inactivation. In addition, other molecules and cellular interactions may also participate in NK cell inactivation. Addition of culture supernatants harvested from AK-5-treated splenocytes to the normal and immune splenocytes caused inactivation of NK cells, as was observed for tumour-cell-treated splenocytes. This clearly indicated that the soluble molecules secreted by the splenocytes following coculture with fixed AK-5 cells are responsible for the inactivation of NK cell function.

CD16 has been implicated in signal-transduction pathway of NK cells [16, 20, 21]. Nagler et al. [15] showed that $CD16^{dim}$ cells had significantly lower cytotoxic activity than the $CD16^{bright}$ fraction. In our system, we did not observe any changes in the expression of CD16 levels on the surface of treated and untreated NK cells, which suggested that the lower cytotoxic function of treated NK cells may not be due to the reduced expression of CD16 on these cells. Similarly, a direct correlation between cytotoxic function and decreased levels of CD25 (IL-2R) expression has been suggested previously [6]. No significant differences in the levels of CD25 expression in treated or untreated NK cells were observed in our studies. These observations indicated that the lower cytotoxic function in the tumour-treated NK cells is not due to the down-regulation of either CD-16 or CD-25 expression. However, a change in the phenotype from $Fas\text{-}R^{dim}$ to $Fas\text{-}R^{bright}$ was observed in NK cells treated with fixed tumour cells. The up-regulation of Fas-R may play an important role in the induction of anergy in NK cells. Fas-R is involved in the induction of apoptosis in immune cells to maintain selection and development and to limit lymphoid expansion [2, 17]. We have also observed increased apoptotic death in the NK cells that were cocultured with AK-5 cells. Thus, our results suggest that the up-regulation of Fas-R may be an indication of the anergic state of the NK cells.

This study demonstrates that TNFα plays an important role in the induction of anergy in NK cells. TNFα has been reported to play a pivotal role in the activation of NK cells by IL-2. It is able to activate and inhibit the function of NK cells, depending on the nature of the stimulus used [9, 11, 18]. From our study, it is not clear whether TNFα contributes directly to NK cell inactivation or whether it may trigger other molecules that are involved in NK cell inactivation and death. We have observed up-regulation of Fas-R on the NK cells that were treated with fixed AK-5 cells, and its regulation by TNFα could represent a mechanism by which TNFα contributes towards inactivation of NK cells.

Thus, our findings demonstrate that interaction of NK cells with fixed AK-5 cells at high antigen concen-

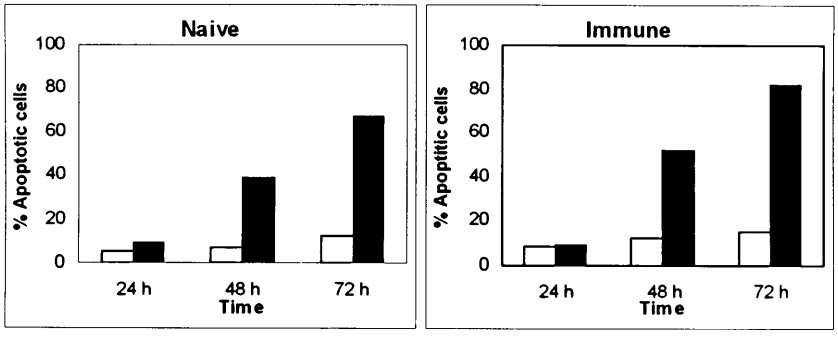


Fig. 6 Fixed AK-5-cell-mediated apoptosis of NK cells. Frequency of apoptotic NK cells was obtained by propidium iodide staining and fluorescence-activated cell sorting analysis. NK cells were isolated after coculture of naïve and immune splenocytes with fixed AK-5 cells for 24, 48 and 72 h. *White bars* the control NK cells, *black bars* the cocultured cells

tration caused a decrease in cytotoxic function of both normal and immune NK cells. The up-regulation of Fas-R expression and induction of TNFα secretion are coupled with negative signalling for cytotoxic function and trigger induction of apoptosis in NK cells. In this context it is conceivable that, when the concentration of target cells is high, the NK cells are constantly interacting with the target cells either directly or indirectly through the T cells. Such interactions might result in the loss of cytotoxic function in NK cells. Further studies directed towards inhibiting the processes leading to NK cell inactivation might help the task of restoring the function of NK cells in immune surveillance against infection and cancer.

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