Immunology 1998 **93** 296–305

Signalling through NK1.1 triggers NK cells to die but induces NK T cells to produce interleukin-4

A. ASEA* & J. STEIN-STREILEIN*† *Division of Pulmonary and Critical Care, Department of Medicine, and †Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, FL USA

SUMMARY

In vivo inoculation of specific antibody is an accepted protocol for elimination of specific cell populations. Except for anti-CD3 and anti-CD4, it is not known if the depleted cells are eliminated by signalling through the target molecule or through a more non-specific mechanism. C57BL/6 mice were inoculated with anti-natural killer (NK1.1) monoclonal antibody (mAb). Thereafter spleen cells were harvested, stained for both surface and intracellular markers, and analysed by flow cytometry. As early as 2 hr post inoculation, NK cells were signalled to become apoptotic while signalling through the NK1.1 molecule activated NK1.1$^+$ T-cell receptor (TCR)$^+$ (NK T) cells to increase in number, and produce interleukin-4 (IL-4). Anti NK1.1 mAb was less efficient at signalling apoptosis in NK cells when NK T-cell deficient [β2-microglobulin β2m-deficient] mice were used compared with wild type mice. Efficient apoptotic signalling was restored when β2m-deficient mice were reconstituted with NK T cells. NK-specific antibody best signals the apoptotic process in susceptible NK cells when resistant NK T cells are present, activated, and secrete IL-4.

INTRODUCTION

Programmed cell death, a form of apoptosis, is proposed as the principal mechanism for eradicating the immune repertoire of anti-self specificities in naive T lymphocytes during development and the generation of somatic mutations in germinal centres.[1] Recognition of antigen or antigenic peptides results in either activation and proliferation, or induction of apoptotic cell death of those lymphocytes expressing specific receptors.[2,3] Activation-induced cell death, another form of cell death is a property of mature T and natural killer (NK) cells induced by repeated antigenic stimuli and involves up-regulation of Fas and FasL.[4,5] In addition, apoptosis can occur in mature lymphocytes in the absence of Fas/FasL up-regulation.[6] Thus, there are several pathways which when activated may lead to apoptosis by mature lymphocytes as well as developing cells.

NK cells make up a population of lymphocytes that mediate spontaneous cytotoxicity of virus-infected cells, certain tumour cells and bone-marrow cells without prior sensitization.[7,8] It is now widely accepted that although NK cell-mediated lysis is not major histocompatibility complex (MHC) restricted, it is under the regulation of class I MHC molecules.[9,10] NK cells are known to play an important role in immune regulation by the production of cytokines. Interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and granulocyte–macrophage colony-stimulating factor (GM-CSF) are important in antigen-independent activation of phagocytic cells early in infection and for biasing the development of antigen-specific T-helper type-1 (Th1) cells to produce IFN-γ and interleukin-12 (IL-12).[11,12] The NK1.1 molecule, identified by the mAb PK136,[13] displays allelic polymorphism, and is expressed by C57BL/6 (B6) mice but not BALB/c mice.[14,15] NK1.1 antigen is also expressed by a small fraction of T cells (CD4$^+$ or CD4$^-$ CD8$^-$ T-cell receptor (TCR)αβ$^+$) in the spleen, thymus and liver.[16,17] Unlike classic TCRα/β$^+$ T cells, NK1.1$^+$ T cells are able to spontaneously lyse several kinds of tumour cells *in vitro*[18,19] and to promptly produce large quantities of IL-4 upon primary stimulation of the TCR *in vivo*.[18–21] The latter functional characteristic implicates NK T cells in regulation and polarization of immune responses towards Th2-type responses.[20,22,23]

Our studies were designed to study the effects of signalling through NK1.1 surface molecules *in vivo*. It is known that one effect of the *in vivo* inoculation of specific antibody is to deplete unwanted lymphocyte populations. It is not known if the depletion occurs through signalling through the target

Received 23 September 1997; revised 2 November 1997; accepted 2 November 1997.

Abbreviations: AsGM1, asialo-ganglio-*n*-tetraosylceramide; β2m$^-$, β2-microglobulin deficient mice; FSC, forward scatter; LGL, large granular lymphocyte; mAb, monoclonal antibody; NK, natural killer; PI, propidium iodide; SSC, side scatter; TdT, terminal deoxynucleotidyl transferase.

Present address: *The Schepens Eye Research Institute, Harvard Medical School, 20 Staniford Street, 2 West, Boston, MA 02114–2500, USA.

Correspondence: Dr J. Stein-Streilein, Schepens Eye Research Institute, 20 Staniford Street, Boston, MA 02114–2500, USA.

molecule or by mechanisms such as complement activation or antibody-mediated cellular cytotoxicity and phagocytosis that do not require signals within the targeted cells. Recently it was reported that inoculation of anti-CD4 mAb signals target cells to induce apoptosis.[24] This report shows that inoculation of antibodies specific for NK1.1 and other NK cell surface markers signal apoptosis in NK cells, but signal proliferation and production of IL-4 in T cells that co-express the same NK markers. Moreover antibodies inoculated in β2m deficient mice are less able to induce apoptotic signals than in wild type mice and NK cells appear to be removed by necrosis when there is a NK T-cell deficit. Reconstitution of the β2-microglobulin (β2m) deficient mice with NK T cells restores both IL-4 production and apoptosis. Thus signalling through the NK1.1 molecule to induce apoptosis may require second signals, partially supplied by the NK T cell in this model.

MATERIALS AND METHODS

Mice

Eight to 12-week-old female BALB/c and C57BL/6 mice were obtained from the Institute's vivarium. Breeding stock animals were purchased from Taconic Farms, Inc. (Germantown, NY). β_2-microglobulin deficient mice ($\beta_2m^{-/-}$), C57BL/6J-β_2m ⟨tm1Unc⟩, which bear a homozygous inactivation of their gene encoding β_2m[25] and IL-4 deficient mice (IL-$4^{-/-}$) [C57BL/6J-IL-4 ⟨tm 1Cgr⟩[26]] were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were maintained on food and water *ad libitum* and screened regularly for the presence of pathogens and consistently tested negative. All animals were treated humanely and in accordance with NIH guidelines and the University of Miami School of Medicine, Division of Veterinary Resources.

Antibodies and reagents

The following hybridomas were purchased from American Type Culture Collection (Rockville, MD) and thereafter maintained in our laboratory: PK.136 (anti-mouse NK1.1) mouse immunoglobulin G2a (IgG2a); GK1.5 (anti-mouse CD4) rat IgG2b; 3.155 (anti-mouse Lyr 2, CD8) rat IgG2a. Purified antibodies that were used are as follows: 4D11 (anti-LGL-1) rat IgG2a purified ascites was a gift from Dr J. R. Ortaldo, NCI, Frederick, MD; J11d.2 (anti-heat stable antigen (HSA)) rat IgM; mouse IgG2a k (anti-trinitrophenyl, TNP); GK1.5 (anti-CD4) rat IgG2b; isotype control rat IgG2b, were purchased from PharMingen (San Diego, CA).

The following fluoroscein isothiocyanate (FITC) -conjugated antibodies were purchased from PharMingen unless indicated otherwise: anti-mouse 2B4 (mouse IgG2b); anti-mouse IL-4 (BD4-1D11, rat IgG1); anti-IFN-γ (XMG1.2, rat IgG1); Extra Avidin® FITC conjugate was purchased from Sigma Chemical Company (St. Louis, MO). Phycoerythrin (PE)-conjugated antibodies used were: anti-mouse αβTCR (hamster IgG) and anti-mouse CD3ε (2C11, hamster IgG) was purchased from PharMingen; R-PE-conjugated streptavidin was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Cy-chrome-conjugated anti-mouse CD4 (GK1.5, rat IgG2a) was purchased from PharMingen. Biotinylated antibodies used were: anti-5E6 (LY-49C, mouse IgG2a); anti-mouse αβTCR (H57-597, hamster IgG); anti-mouse NK1.1(PK 136, mouse IgG2a) were purchased from PharMingen. Polyclonal rabbit anti-asialo-GM1 was purchased from Wako Chemicals (Richmond, VA).

Baby rabbit complement, Normal rabbit serum and Lympholyte-M were purchased from Cedarlane (Horny, Ontario, Canada). Acridine orange, Ethidium bromide, Propidium iodide, Brefeldin A, and cobra venom factor (*Naja naja*) were purchased from Sigma Chemical Company. Biotinylated-16-dUTP (uridine triphosphate) and terminal transferase (TdT) were purchased from Boehringer Mannheim (Indianapolis, IN). PermeaFix was a gift from Dr T. Mercolino (Ortho Diagnostic, Raritan, NJ).

In vivo *antibody treatment*

Individual mice were inoculated via a lateral tail vein with anti-NK1.1 (PK136, IgG purified, ascites; 50 μg/mouse), anti-CD4 (GK1.5, IgG purified, ascites: phosphate-buffered saline (PBS), 1:4, 200 μl per mouse); RAsGM1 (25 μl/mouse); anti-LGL-1 (100 μg/mouse, IgG purified ascites). In some experiments isotype controls for anti-NK1.1 (mouse IgG2a), anti-CD4 (rat IgG2b) and RAsGM1 (normal rabbit serum) were used at equivalent concentrations and found to have similar effects to PBS.

Isolation of lymphocytes

Inoculated animals were killed by cervical dislocation, the spleens were aseptically removed and gently tapped through a flamed stainless steel screen. Debris were allowed to sediment for 3–5 min at 23–25° before transferring the cell suspension and collecting cells after pelleting at 250 *g* for 10 min. Viable cell counts were accomplished by trypan blue exclusion and phase microscopy of the single cell suspension.

Adoptive transfer

C57Bl/6.$\beta2m^{-/-}$ mice received a sublethal dose of irradiation (5 Gray). Twenty-four hours later the mice were inoculated i.v. with $1{\cdot}2 \times 10^7$ spleen cells recovered from C57BL/6 $\beta_2m^{+/+}$ (wild type) mice and treated as described elsewhere.[21] In brief, in order to deplete B cells and CD8 T lymphocytes, thymus recovered from wild type mice were made into a single cell suspension and treated with anti-HSA (J11d.2) and anti-CD8 (3.155), for 20 min on ice. Similarly, in order to enrich for antigen-presenting cells, spleen cells were depleted of T lymphocytes by treatment with anti-CD8, anti-CD4 and anti-Thy1.1, for 20 min on ice. Thereafter thymus and spleen cells were washed once and incubated with baby rabbit complement at 37° for 1 hr. Dead cells were removed by centrifugation over a layer of lympholyte-M (Cedarlane). Five days post cell transfer, the animals were inoculated i.v. with anti-NK1.1 (50 μg/mouse; 0·5 ml/mouse). Two hours later the spleens were removed and stained simultaneously for phenotype and intracellular cytokine production, as well as for morphological features characteristic of apoptosis or necrosis.

Morphological analysis of apoptosis and necrosis

Apoptotic and necrotic morphology were monitored in spleen cells stained with a dye mix containing acridine orange (10 μg/ml) and ethidium bromide (10 μg/ml) in PBS. One microlitre of the dye mix was added to 25 μl of cells ($1–2 \times 10^6$/ml). Thereafter, 10 μl of the cell suspension was placed on a glass slide and the normal, apoptotic or necrotic cells were immediately counted using a fluorescence microscope

(Nikon HB-1010AF) under 600× magnification.[27] Briefly, acridine orange intercalates into DNA and makes it appear green thus a viable cell with apoptotic nuclei will be bright green (VA). Acridine orange also binds to RNA, but because it cannot intercalate, the RNA stains red–orange. Therefore a viable cell with normal nuclei (VN) will have bright green chromatin in its nucleus and could have red cytoplasm from the stained RNA. In contrast, ethidium bromide is only taken up by non-viable cells, which may appear slightly red. A necrotic (NVN), cell will have a bright orange chromatin because the ethidium bromide overwhelms the green of the acridine orange. A non-viable apoptotic cell (NVA) is also orange. Thus, live and dead cells are distinguished by green and orange nuclei. Apoptosis and necrosis are distinguished by the structure of the nuclei. Chromatin in apoptotic cells is condensed, fragmented, and lobated. Necrotic cells would have normal appearance, with smooth and round nuclei. Approximately 100 events were counted in five different zones from each of five slides, thus ≈2500 events were counted per mouse. The percentage of apoptotic and necrotic lymphocytes was calculated using the following formula:

$$\%\ \text{apoptotic lymphocytes} = \frac{VA + NVA}{VN + VA + NVN + NVA} \times 100$$

$$\%\ \text{necrotic lymphocytes} = \frac{NVN}{VN + VA + NVN + NVA} \times 100$$

Simultaneous measurement of phenotype and DNA changes in apoptotic cells

Spleen cells were stained simultaneously for surface antigens and DNA changes characteristic of apoptotic cells.[28,29] Briefly, spleen cells were recovered from mice and stained with FITC-conjugated 2B4, -NK1.1 or -5E6 mAb, for 40 min at room temperature in the dark. Cells were then washed twice and resuspended in PBS buffer containing 250 μg/ml propidium iodide and 0·01% RNase for 20 min in the dark followed by flow cytometric analysis. Fluorescence-activated flow cytometric analysis was performed on a FACScan with Lysis II software program (Becton Dickinson, Mountain View, CA), individual cells were gated on the basis of forward (FSC) and orthogonal side scatter (SSC). Cell debris were excluded by raising the FSC-height photomultilier tube (PMT) threshold. The flow rate was adjusted to 200 cells/s and at least 10^4 cells were analysed for each sample.

Terminal deoxyneucleotidyl transferase (TdT) assay

Analysis of DNA strand breaks was performed as previously described.[30] Briefly, 2×10^6 spleen cells that had been simultaneously fixed and permeabilized[28] were incubated with 50 μl of terminal transferase (TdT) buffer containing; 1 mM $CoCl_2$, 0·1 mM Na-cacodylate (pH 7·0), 0·1 mM dithiothreitol, bovine serum albumin (BSA) (0·05 mg/ml), 10 U terminal transferase and 0·5 nmol biotinylated-16-dUTP at 37° for 1 hr. The cells were washed and incubated with streptavidin-conjugated FITC, for 30 min. on ice. The cells were washed twice in PBS before being analysed by flow cytometry. Using a FACScan with a Lysis II software program, individual cells were gated on the basis of FSC and SSC. The biotin–16-dUTP/avidin–FITC complex was registered on the FL1-height in logarithmic scale. Cell debris were excluded by raising the FL2-height threshold. The flow rate was adjusted to 200 nuclei/s and at least 10^4 cells were analysed for each sample.

Simultaneous measurement of phenotype and intracellular cytokine production

Lymphocytes were stained simultaneously for surface antigens and intracellular cytokine production by a method developed in our laboratory. Briefly, to prevent the intracellular cytokine protein being secreted, cells were immediately treated with Hank's balanced salt solution (HBSS) supplemented with 5 μg/ml Brefeldin A, a compound known to disrupt the Golgi apparatus, thus inhibiting protein secretion.[31,32] Thereafter $2{\cdot}5 \times 10^6$ spleen cells were stained with PE- or Cy-chrome-conjugated mAb to surface receptors (1 μl mAb/10^6 cells) for 40 min at room temperature. Cells were then washed twice in PBS. The pellet was resuspended in 2 ml PermeaFix buffer and incubated at room temperature for 40 min. The cells were washed twice and stained with FITC-conjugated mAb against IL-4 or IFN-γ (1 μl mAb/10^6 cells), for a further 40 min at room temperature, in the dark. The cells were then washed twice in PBS and analysed by flow cytometry. Flow cytometric analysis was performed on a FACScan with a Lysis II software program, individual cells were gated on the basis of FSC and SSC. Cell debris were excluded by raising the FSC-height PMT threshold. The flow rate was adjusted to <200 cells/s and at least 10^4 cells were analysed for each sample.

Statistical analysis

The double-tailed Students's *t*-test (Nycomed, As, Oslo, Norway) was used to analyse the significance of the difference between control and experimental groups. Differences were considered significant when $P \leq 0{\cdot}05$.

RESULTS

Differential effect of *in vivo* antibody treatment on NK1.1$^+$ cells

The NK1.1 molecule is a C-type lectin of the NKR-P1 family[33] which is expressed as an allele on the majority of NK cells on B6 mice. Others have shown that inoculation of B6 mice with anti-NK1.1 mAb (PK136 hybridoma) depletes NK cells.[13,34,35] In order to study the mechanism of PK136 induced depletion of cells we chose a time point post-inoculation of specific mAb when target cells would be apoptotic but not yet phagocytized by neighbouring macrophages. This time point is dependent on the 'washout rate,'[24] and is an ideal time to analyse apoptotic cells by flow cytometry and fluorescence microscopy.

B6 mice that were inoculated with anti-NK1.1 mAb, showed significant depletion of splenic NK cells as early 2 hr post i.v. inoculation, as compared to the isotype control (Fig. 1). Interestingly, there was a subset of NK1.1$^+$ cells (2–4%) of spleen cells that was resistant to depletion with PK136. Phenotypic analysis showed that these resistant cells were NK1.1$^+$ CD4$^+$ and co-expressed 5E6 (Fig. 1a). The 5E6 molecule is a disulfide-linked homodimer expressed on NK cell subsets with a cDNA nucleotide sequence identical to that of Ly-49C. Ly-49C molecules are NK cell receptors for class 1 MHC and closely associated to NKR-P1 on the distal segment of chromosome 6.[36–38] In contrast, the susceptible

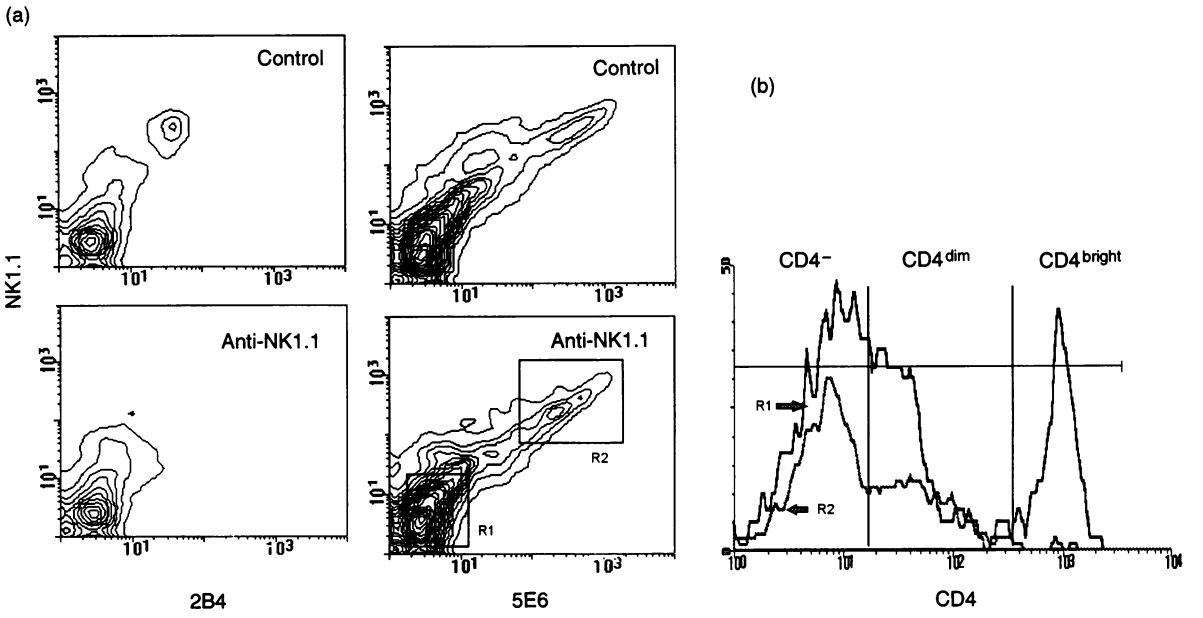

Figure 1. Characterization of target cells after anti-NK1.1 treatment. B6 mice were inoculated i.v. with anti-NK1.1 as indicated, or PBS. Two hours post inoculation, lymphocytes were dissociated from the spleen and triple staining for surface antigens was performed and analysed by flow cytometry. (a) The contour plots represent triple staining of spleen cells stained for NK1.1 (ordinate) and 2B4 (left; abscissa) and 5E6 (right; abscissa). (b) The histogram represents the relative number of cells (ordinate) expressing the CD4 molecule (abscissa). Lines designated R1 and R2 represent the gated population shown in A, lower right contour plot in relation to their expression of CD4. Cells in the R1 population are NK1.1$^-$ 5E6$^-$; cells in the R2 population are NK1.1$^+$ 5E6$^+$ cells. Five mice were used per treatment group. Similar results were obtained in seven separate experiments.

NK cells included all NK1.1$^+$ 2B4$^+$ cells (Fig. 1a). The 2B4 molecule belongs to the immunoglobulin superfamily and shares homology to CD58 (LFA-3), which is also a ligand for CD2[39] and is expressed on a subset of NK cells.[40] Thus, the ligation of NK1.1 antigen on NK1.1$^+$ 2B4$^+$ cells resulted in deletion of the majority of these cells, whereas ligation of the same surface receptor on cells that also express 5E6 did not induce depletion.

Further analysis of the depletion-resistant NK1.1$^+$ 5E6$^+$ T cell by triple staining showed that the majority of resistant subset co-expressed CD4 at an intermediate intensity (CD4^{dim}) as compared to characteristic T helper (Th) cells that express CD4 at high intensity (CD4^{bright}) (Fig. 1b). The NK1.1$^+$ 5E6$^+$ T cell also co-expressed α/β TCR and asialo-GM1 (AsGM1) at an intermediate intensity compared to conventional T and NK cells, respectively (data not shown). Similar to NK1.1$^+$ T cells in the thymus, spleen and liver described by others,[17,41,42] the depletion-resistant NK1.1$^+$ 5E6 T cells were observed to be large granular lymphocytes (LGL) with a higher scatter value than resting B and T cells, but smaller than monocytes or macrophages when analysed by flow cytometry and fluorescence microscopy (data not shown).

To determine if the differential effects were uniquely induced by PK136, we analysed other known depleting NK cell antibodies for their ability to eliminate the NK ag$^+$ cell population *in vivo*. Animals were treated with either anti LGL-1 (4D11), a mAb that targets a C-type lectin of Ly-49G2 subgroup that is known to be expressed on 50% of splenic NK cells in the mouse,[43] or rabbit anti-asialo-GM1(RAsGM1), a polyclonal antibody recognizing a glycolipid expressed on the vast majority of mouse NK cells, some T cells, and macrophages.[44] Interestingly, the two antibodies used *in vivo* depleted the NK cells, and like PK136, induced the resistant NK T cells to increase in number and to produce IL-4 (Fig. 2).

Kinetics studies showed that NK1.1$^+$ 5E6$^+$ T cells produced IL-4 as early as 90 min, continued production through 8 hr post treatment and returned to baseline levels by 24 hr post antibody treatment (data not shown). Not unexpectedly, depletion of CD4$^+$ cells prior to anti-NK1.1 treatment down-regulated IL-4 production (Fig. 2b). These findings show that cross-linking of molecules other than those in the T cell receptor (TCR) complex[20,21] can signal the production of cytokines by NK T cells.

Mechanism of antibody-induced NK cell depletion

Using a novel method that allows for the simultaneous measurement of surface antigens and DNA changes that are characteristic of apoptotic lymphocytes,[28,29] it was shown that anti-NK1.1 treatment *in vivo* induced cellular depletion of NK cells via apoptosis. Whereas greater than 90% of NK1.1$^+$ 2B4$^+$ cells were apoptotic, only 58% of NK1.1$^+$ 5E6$^+$ cells were apoptotic 2 hr post-treatment with anti-NK1.1mAb *in vivo* (Fig. 3). This observation is consistent with the results illustrated by contour plot (Fig. 1) that shows the NK1.1$^+$ 5E6$^+$ cells to be resistant to depletion.

Apoptosis was confirmed by a TUNEL assay that revealed a higher percentage of dUTP incorporation in spleen cells of PK136-treated mice as compared to controls, correlating with increased fragmentation of genomic DNA, a feature characteristic of apoptosis (Fig. 4). To further exclude the possibility that anti-NK1.1-induced depletion of NK cells occurred via complement fixation and necrosis, another group of mice was

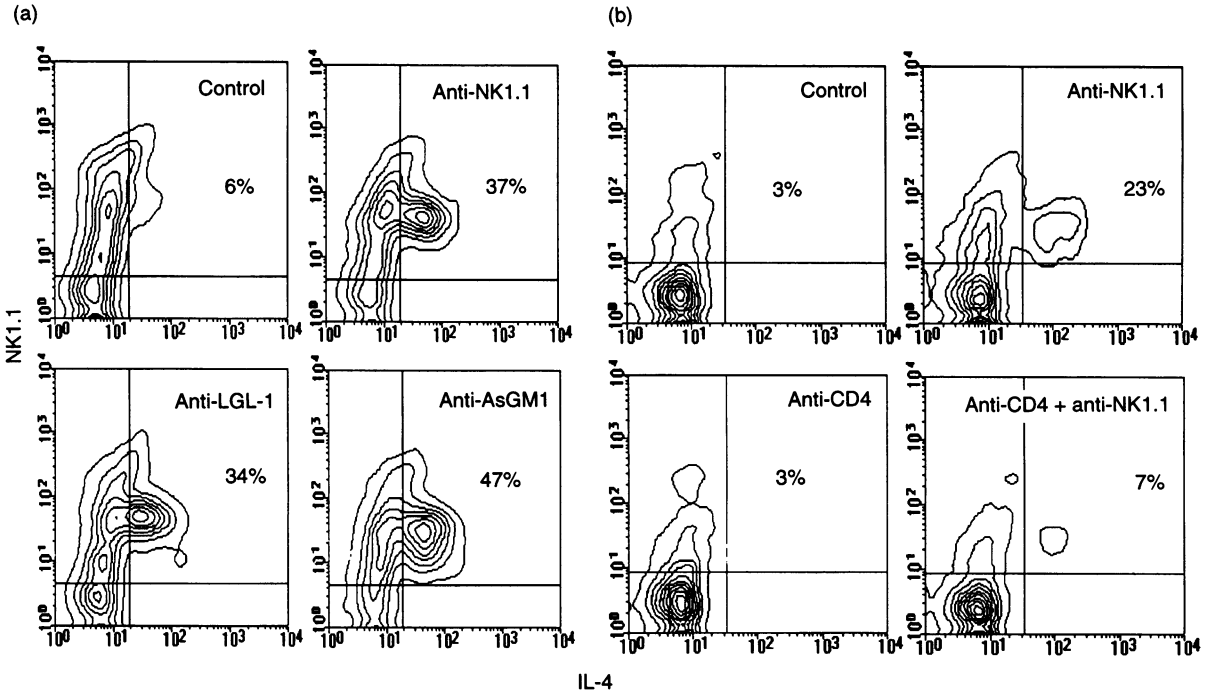

Figure 2. *In vivo* antibody induction of cytokine production in NK1.1$^+$ T cells. B6 mice were inoculated i.v. with anti-NK1.1 (PK136) or PBS. Two hours later lymphocytes were dissociated from the spleen, stained for surface and intracellular antigens and assessed by flow cytometry. (a) Induction of cytokine synthesis by antibodies. Contour plots of analysed cells after mice were treated with depleting antibodies to NK cell surface antigens as indicated in blocks. Percentage of NK1.1$^+$ IL-4 producing cells within selected quadrants is indicated. (b) Role of CD4$^+$ T cells in IL-4 production by NK1.1$^+$ T cells. B6 mice were depleted of CD4$^+$ cells by i.v. inoculation of anti-CD4 (GK1.5) 24 hr before the mice were treated with PK136 or PBS. Spleen cells of six mice were pooled in each treatment group. Percent of NK1.1,$^+$ IL-4-producing cells within selected quadrants is indicated.

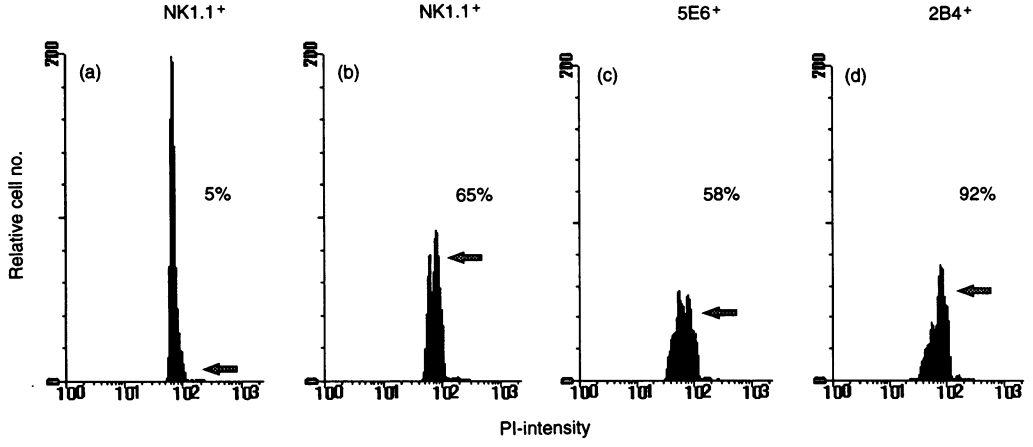

Figure 3. Induction of apoptosis by *in vivo* treatment with anti-NK1.1 mAb. B6 mice were inoculated i.v. with anti-NK1.1 or PBS. Two hours later, lymphocytes were dissociated from the spleen, stained for surface markers and DNA for simultaneous measurement of phenotype and apoptosis (see Materials and Methods). NK cells from B6 mice (five mice per treatment group) inoculated with PBS (block a) or PK136; (blocks b, c and d) are identified by antibodies indicated at top of block. Histograms represent the DNA content for the gated cells expressing surface markers indicated at top of each block. The percentage of cells with fragmented DNA (indicated by arrow) is shown in block. Similar results were obtained in six different experiments.

treated with cobra venom factor (CVF). CVF acts *in vivo* by providing a stable C3b molecule, allowing uninhibited complement activation which results in decomplementation.[45] CVF treatment of mice prior to mAb treatment had no significant effect on apoptosis induced by anti-NK1.1 as observed by fluorescence microscopy (Table 1). As a control for the possibility that deletion was caused by non-specific activation FcR binding, NK1.1 negative Balb/c mice[15,46] were treated with PK136; there was no significant deletion of splenic NK cells by PK 136 mAb in BALB/c mice. Thus, it is concluded that the deletion effect of anti-NK1.1 was specific for cells with the target antigen (Table 1).

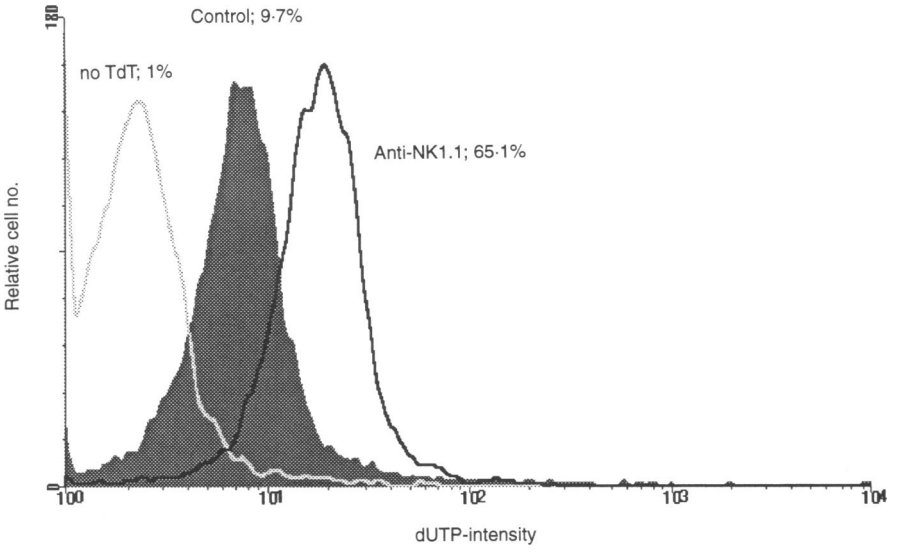

Figure 4. TUNEL assay. DNA strand breaks in NK1.1$^+$ NK cells were labelled with biotinylated-dUTP. Mice were inoculated with PK136. Two hours later lymphocytes were dissociated from the spleen and assayed for DNA strand breaks is the TdT assay. Percentage of strand breaks are indicated. The histogram shows biotinylated-dUTP intensity (abscissa) and relative cell number (ordinate) and is a representative of three separate experiments.

Table 1. Role of complement or antigen specificity in anti-NK1.1mAb mediated apoptosis *in vivo*

		Treatment* %				Specific events ± SD		
Exp no	Strain	CVF	anti-NK1.1	PBS	*n*	Nr	Ap	Nc
1	B6	−	−	+	6	96·0 ± 1	< 5	< 5
		−	+	−	6	46·3 ± 3†	52·3 ± 6†	< 5
		+	−	−	6	95·3 ± 3	< 5	< 5
		+	+	−	6	49·3 ± 3†	52·7 ± 7†	< 5
2	BALB/c	−	−	+	5	94·0 ± 5	< 5	< 5
		−	+	−	5	92·0 ± 6	< 5	< 5

*Mice were inoculated i.p. with cobra venom factor (CVF) (*Naja naja* venom; 20 μg/mouse), in three equal doses at 8 hr intervals or PBS, on day −1. On day 0, anti-NK1.1 (PK136, IgG purified ascites; 50 μg/mouse), or PBS was inoculated i.v. Data are percentage specific events ± SD of three different experiment. Characteristics of normal (Nr), apoptosis (Ap) or necrosis (Nc) cells were determined by fluorescence microscopy.

†indicates significant difference ($P \leq 0{\cdot}05$) comparing anti-NK1.1 vs. control treatment.

Role of NK T cells in mAb-induced apoptosis

To test the role of the depletion-resistant NK T cell in the antibody-induced apoptosis of the NK cells, the protocol was repeated in NK T-cell depleted mice. First all CD4$^+$ cells, including CD4$^+$ NK T cells, were depleted in B6 mice with anti-CD4 mAb prior to deletion of NK cells with PK136 (Fig. 5). Following successful depletion of CD4$^+$ splenic lymphocytes, animals were inoculated with anti-NK1.1 mAb and apoptosis was analysed by flow cytometry. Anti-NK1.1 mAb treatment in the absence of all CD4$^+$ lymphocytes (including NK1.1$^+$ CD4$^+$ cells), depleted NK cells but the relative number of NK1.1$^+$ cells that showed apoptosis characteristics was one-third less than apoptotic cells seen in PK136 treated mice with CD4$^+$ cells (Fig. 5). Morphological analysis with acridine orange/ethidium bromide staining confirmed the flow cytometry data and showed a reduction in the relative number of apoptotic cells and a concomitant increase in the number of necrotic cells (Table 2, experiment 1). It was concluded that the CD4$^+$ T cells influence the mechanism of depletion and favours enhancement of apoptosis over necrosis for the mechanism of *in vivo* mAb deletion of NK cells.

In the next experiments only the NK T-cell subpopulation of CD4$^+$ cells was absent in the mice treated with PK136. β_2-microglobulin 'knock-out' ($\beta_2m^{-/-}$) mice do not express MHC Class I proteins, and consequently are deficient in CD8$^+$ T cells[25] and have markedly diminished NK1.1$^+$ T cells.[20,21] Treatment of $\beta_2m^{-/-}$ mice with anti-NK1.1 resulted in a significant reduction of the number of apoptotic NK cells quantified and a concomitant fourfold increase in necrotic cells (Table 2, experiment 2). Apoptosis was fully restored in PK136-treated $\beta_2m^{-/-}$ mice after sub-lethal irradiation and reconstitution with thymus and spleen cells from $\beta_2m^{+/+}$ mice

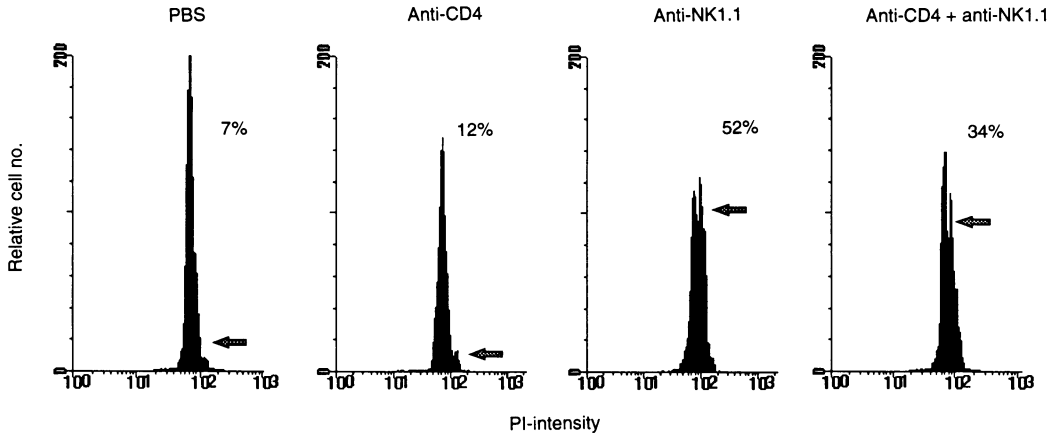

Figure 5. Role of $CD4^+$ T cells in anti-NK1.1 mAb-induced apoptosis of NK cells *in vivo*. B6 mice were inoculated i.v. with GK1.4, 24 hr before animals were inoculated i.v. with PK136. Two hours later lymphocytes were dissociated from the spleen and simultaneous measurement of phenotype and apoptosis. The histograms represent the DNA content for gated ($NK1.1^+$, splenic) lymphocytes, from mice treated as follows: (a) PBS; (b) GK1.5; (c) PK136; (d) GK1.5 given 24 hr prior to PK136. Apoptosis of $NK1.1^+$ cells was measured 2 hr post inoculation. The percentage of apoptotic cells (arrow) is indicated within the blocks. Spleen cells were pooled from seven mice per group. The histogram is a representative of three separate experiments.

Table 2. Role of $CD4^+$ and NK T cells in mAb-mediated apoptosis and IL-4 production

		Treatment*				% Specific events† ± SD			
Exp no	Strain‡	anti-CD4	anti-NK1.1	PBS	*n*	Nr	Ap	Nc	% NK T cells producing IL-4§
1	B6	−	−	+	7	95·3 ± 3	< 5	< 5	< 5
		−	+	−	7	43·7 ± 10**	40·3 ± 3**	9·3 ± 3	27·4 ± 3**
		+	−	−	5	88·7 ± 8	< 5	< 5	< 5
		+	+	−	6	43·7 ± 12**	22·7 ± 3***	25·0 ± 5***	< 5
2	B6	−	−	+	4	89·1 ± 6	5·0 ± 4	5·6 ± 4	< 5
		−	+	−	5	45·4 ± 10**	46·2 ± 12**	8·4 ± 5	32·4 ± 4**
	$\beta 2m^{-/-}$	−	−	+	5	91·2 ± 6	5·3 ± 5	3·6 ± 4	< 5
		−	+	−	5	56·6 ± 12**	16·3 ± 8****	27·1 ± 14****	8·2 ± 2
	$r\beta 2m^{-/-}$	−	+	−	5	57·7 ± 12**	31·5 ± 13**	10·9 ± 4	19·0 ± 3**
	$IL-4^{-/-}$	−	+	−	3	48·3 ± 7**	23·0 ± 10**	31·3 ± 4**	8·2 ± 3

*Mice were inoculated i.v. with anti-CD4 (GK1.5, IgG purified ascites, 1 : 4 dilution, 200 μl/mouse) or control PBS, 200 μl on day −1. On day 0 animals were inoculated i.v. with anti-NK1.1 (PK136, IgG purified ascites; 50 μg/mouse) or PBS.

†Normal (Nr), apoptotic (Ap), and necrotic (Nc) morphologies were assessed by fluorescence microscopy following acridine orange/ethidium bromide staining. The percentage specific events was generated from the formula described in Materials and Methods. Approximately 100 events were counted in five different zones on each of five slides totalling ≈2500 counted events per mouse.

‡All genetically altered mice were bred on the (B6) background. $r\beta_2m^{-/-}$ mice are $\beta_2m^{-/-}$ mice that were reconstituted with NK T cells and antigen presenting cells enriched from thymuses and spleens from wild type ($\beta_2m^{+/+}$) mice. $IL\text{-}4^{-/-}$ mice are interleukin-4 'knockout' mice.

§In separate experiments, NK T cells were analysed for intracellular IL-4 by flow cytometry.

**$P \leq 0.05$; experimental group versus control.

***$P \leq 0.05$; CD4-depleted, anti-NK1.1 treated mice versus CD4-sufficient, anti-NK1.1-treated mice.

****$P \leq 0.05$; anti-NK1.1-treated $\beta_2m^{-/-}$ mice versus anti-NK1.1-treated wild type mice.

(Table 2, experiment 2). It is concluded that $NK1.1^+$ T cells participate in biasing the mechanism of antibody-induced depletion of NK cells toward apoptosis. Potential mechanisms that the $NK1.1^+$ T cell may use to regulate the depletion process include either cell–cell contact, cytokine production, or both.

Role of IL-4 in antibody mediated NK cell depletion

The next postulate we tested was that the IL-4 produced by the mAb-activated NK T cell played a role in the apoptosis induced in NK cells. We reasoned that antibody inoculation might be less efficient in inducing apoptosis in IL-4 deficient mice. Indeed, treatment of IL-4 'knock-out' mice[26] with anti-NK1.1 mAb produced fewer apoptotic cells than seen in mAb-treated wild type mice (Table 2). Thus, IL-4, or a cytokine induced by IL-4 is necessary for the optimal apoptosis of NK cells. Moreover, when β_2m deficient mice were reconstituted with thymus and spleen cells from β_2m-sufficient mice, IL-4 production was restored coincidentally with the apoptotic response to mAb treatment (Table 2) supporting the postulate that the critical cell producing IL-4

in *in vivo* mAb-induced depletion of NK cells was the NK1.1$^+$ T cell.

DISCUSSION

In summary, this study shows that mAb-induced depletion of NK cells *in vivo* occurs via apoptosis; that the NK T cell is resistant to apoptosis but susceptible to stimulation of IL-4 production by NK antigen-specific mAb treatment; and that resistant NK T cells are required for the efficient apoptotic cell death of NK cells induced by mAb treatment *in vivo*.

A previously described subset of T lymphocytes that expresses both the TCR and NK surface molecules is known to promptly produce IL-4 after *in vivo* challenge with anti-CD3.[18–21,47] It is assumed the cells described in this study are similar to the previously described NK T cells that are prompt cytokine producers.[18–21,47] Our studies further characterize the NK1.1$^+$ T cell as expressing several additional NK markers (5E6, AsGM1, LGL-1) and show that these markers respond to *in vivo* ligation by synthesizing IL-4. The mechanism explaining the differential response of NK T cells and NK cells to PK136-induced signalling, cytokine synthesis versus apoptosis, *in vivo* is unknown. Possible explanations for NK T cells surviving *in vivo* mAb treatment include their ability to express the TCR and/or their prompt secretion of IL-4 which may play a pivotal role in the selection of the signalling pathway and the protection of these cells against antibody-induced signalling of apoptosis *in vivo*. Work by Spinozzi *et al.*[48] seems to support the latter explanation since these authors showed that IL-4 protects a population of CD4$^-$ CD8$^-$ αβTCR lymphocytes against CD2-mediated apoptosis *in vitro*. Alternatively, upregulation of the bcl-2 gene in NK T cells might account for the their survival *in vivo*, because work by Carson *et al.*[49] shows that c-kit ligand up-regulates bcl-2 and prevents apoptosis of a subpopulatioin of human CD56^{bright} NK cells.

The significance of the finding that the 5E6 surface receptor is expressed on the NK T cells is intriguing as the 5E6 molecule was shown to be expressed on a subset of NK cells responsible for rejection of H-2^d homozygous bone marrow cell allografts.[37,38] It was further demonstrated that 5E6$^+$ cells augment rather than inhibit growth of H-2^d bone marrow cells (BMC).[37] While it was assumed that 5E6$^+$ cells are NK cells and CD3 negative, it is interesting to speculate that the NK1.1$^+$ 5E6$^+$ T cells, described here, participate in hybrid resistance. Perhaps 5E6$^+$ NK cells reject the graft and 5E6$^+$ T cells produce IL-4 augmenting bone-marrow cell growth, because IL-4 can synergize with granulocyte colony-stimulating factor and IL-11 to stimulate the proliferation of bone marrow cells.[50,51]

Our results raise an interesting question concerning the interpretation of previous *in vivo* antibody depletion studies. It is assumed that all cells expressing the target molecules are deleted and that observed effects are caused by the absence of the deleted cells. However, our study definitively shows that antibody treatment may deplete one subset of cells and activate cytokine production in another 'depletion-resistant' subset. Even if multiple daily inoculations of depleting antibody are given to deplete all subsets expressing the target molecule, 2 hr after the first inoculation one subset may secrete an influential cytokine which may influence, initiate or modify the experimental results in the absence of the target cells. An earlier study by our laboratory showed a role for NK cells in host defence against influenza infection.[52] In light of these new findings, we now suggest that when RAsGM1 antibodies are given *in vivo*, IL-4 secretion by NK1.1$^+$ 5E6$^+$T cells may be initiated, thereby creating a Th2-promoting environment contributing to prevention of the development of appropriate cytotoxic T-cell responses to the virus. This interpretation does not negate the role of NK cells in influenza infection but rather expands the interpretation of the data to include the potential role of NK T cells in down-regulating the subsequent Th1 response needed for protection against virus infection, while up-regulating a non-protective Th2 response.

The correlation of IL-4 production with apoptosis of the NK cells raises the possibility that IL-4 participates in biasing the mechanism of depletion *in vivo* toward apoptosis. Others have reported that apoptosis in mice exposed to ultraviolet irradiation is associated with the increased production of glucocorticoids and IL-4 secretion.[53,54] Moreover, work by Ayroldi *et al.* shows that superantigen induces apoptosis of peripheral T cells and that IL-4 augments the specific deletion of Vβ8$^+$ cells.[55] These authors further speculate that the increased apoptosis observed by superantigen plus IL-4 preferentially deletes Th1 cells, thus perpetuating a Th2-type response. Our findings that *in vivo* treatment of mice with NK marker specific antibodies stimulates apoptosis of NK cells and is associated with the production of IL-4 by NK T cells is yet another example of the relationship of IL-4 with experimentally induced apoptosis. Further support for a role of IL-4 in apoptosis are studies by Adkins and co-workers[56] that show enhanced IL-4 production in thymus of neonatal mice during a crucial time for cellular depletion by apoptosis. Because the process of negative selection in the thymus begins in the postnatal period and is associated with massive cell death, the possibility arises that enhanced IL-4 levels contribute to apoptosis during T-cell differentiation in the neonatal thymus. The exact role for IL-4 in apoptosis *in vivo* needs to be further elucidated, since *in vitro* studies contradict our evidence that IL-4 contributes to apoptosis.[48]

ACKNOWLEDGMENTS

The authors thank Dr E. Podack, University of Miami School of Medicine, Miami, FL and Drs J. W. Streilein and A. Taylor, Schepens Eye Research Institute, Harvard Medical School, Boston, MA for their critical reviews of the manuscript; Drs J. R. Ortaldo, NCI, Frederick, MD for the anti-LGL-1 (4D11) mAb; Drs V. Kumar and M. Bennett, University of Texas Health Science Center, Dallas, TX for hybridomas 5E6 and 2B4; and Dr T. Mercolino, OrthoDiagnostics, Rariton, NJ for the PermeaFix. We thank Dr R. Levy, University of Miami School of Medicine, Miami, FL, for supplying backup reagents for many pilot experiments. We thank Vania Fernandez, University of Miami, for expert technical assistance; Magda Guzman, University of Miami, and Anita Pearson, Schepens Eye Research Institute, for preparation of the manuscript. This work was supported in part by NIH, HL33709 and a Gift Giving Award from Johnson and Johnson.

REFERENCES

1. Smith C.A., Williams G.T., Kingston R., Jenkinson E.J. & Owen J.J. (1989) Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature* **337**, 181.

2. Shi Y.F., Sahai B.M. & Green D.R. (1989) Cyclosporin A inhibits activation-induced cell death in T-cell hybridomas and thymocytes. *Nature* **339,** 625.
3. Shi Y.F., Bissonnette R.P., Parfrey N., Szalay M., Kubo R.T. & Green D.R. (1991) *In vivo* administration of monoclonal antibodies to the CD3 T cell receptor complex induces cell death (apoptosis) in immature thymocytes. *J Immunol* **146,** 3340.
4. Brunner T., Mogil R.J., Laface D. *et al.* (1995) Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature* **373,** 441.
5. Van Parijs L., Ibraghimov A. & Abbas A.K. (1996) The roles of costimulation and Fas in T cell apoptosis and peripheral tolerance. *Immunity* **4,** 321.
6. Cohen J.J., Duke R.C., Fadok V.A. & Sellins K.S. (1992) Apoptosis and programmed cell death in immunity. [Review]. *Annu Rev Immunol* **10,** 267.
7. Trinchieri G. (1989) Biology of natural killer cells. [Review]. *Adv Immunol* **47,** 187.
8. Yu Y.Y., Kumar V. & Bennett M. (1992) Murine natural killer cells and marrow graft rejection. [Review]. *Annu Rev Immunol* **10,** 189.
9. Höglund P., Sundback J., Olsson-Alheim M.Y. *et al.* (1997) Host MHC class I gene control of NK-cell specificity in the mouse. [Review]. *Immunol Rev* **155,** 11.
10. Raulet D.H., Held W., Correa I., Dorfman J.R., Wu M.F. & Corral L. (1997) Specificity, tolerance and developmental regulation of natural killer cells defined by expression of class I-specific Ly49 receptors. [Review]. *Immunol Rev* **155,** 41.
11. Scharton T.M. & Scott P. (1993) Natural killer cells are a source of interferon gamma that drives differentiation of $CD4^+$ T cell subsets and induces early resistance to *Leishmania major* in mice. *J Exp Med* **178,** 567.
12. Trinchieri G. (1995) The two faces of interleukin 12: a pro-inflammatory cytokine and a key immunoregulatory molecule produced by antigen-presenting cells. [Review]. *Ciba Found Symp* **195,** 203.
13. Koo G.C. & Peppard J.R. (1984) Establishment of monoclonal anti-NK-1.1 antibody. *Hybridoma* **3,** 301.
14. Brooks C.G., Burton R.C., Pollack S.B. & Henney C.S. (1983) The presence of NK alloantigens on cloned cytotoxic T lymphocytes. *J Immunol* **131,** 1391.
15. Karlhofer F.M. & Yokoyama W.M. (1991) Stimulation of murine natural killer (NK) cells by a monoclonal antibody specific for the NK1.1 antigen. IL-2-activated NK cells possess additional specific stimulation pathways. *J Immunol* **146,** 3662.
16. Abo T., Watanabe H., Sato K. *et al.* (1995) Extrathymic T cells stand at an intermediate phylogenetic position between natural killer cells and thymus-derived T cells. [Review]. *Nat Immun* **14,** 173.
17. Watanabe H., Miyaji C., Kawachi Y. *et al.* (1995) Relationships between intermediate TCR cells and $NK1.1^+$ T cells in various immune organs. $NK1.1^+$ T cells are present within a population of intermediate TCR cells. *J Immunol* **155,** 2972.
18. Arase H., Arase-Fukushi N., Good R.A. & Onoe K. (1993) Lymphokine-activated killer cell activity of $CD4^-CD8^-$ TCR alpha $beta^+$ thymocytes. *J Immunol* **151,** 546.
19. Arase H., Arase N., Nakagawa K., Good R.A. & Onoe K. (1993) $NK1.1^+CD4^+$ $CD8^-$ thymocytes with specific lymphokine secretion. *Eur J Immunol* **23,** 307.
20. Yoshimoto T. & Paul W.E. (1994) CD4pos, NK1.1pos T cells promptly produce interleukin-4 in response to *in vivo* challenge with anti-CD3. *J Exp Med* **179,** 1285.
21. Yoshimoto T., Bendelac A., Watson C., Hu-Li J. & Paul W.E. (1995) Role of $NK1.1^+$ T cells in a TH2 response and in immunoglobulin E production. *Science* **270,** 1845.
22. Seder R.A. & Paul W.E. (1994) Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells. [Review]. *Annu Rev Immunol* **12,** 635.
23. Bendelac A., Lantz O., Quimby M.E., Yewdell J.W., Bennink J.R. & Brutkiewicz R.R. (1995) CD1 recognition by mouse $NK1^+$ T lymphocytes. *Science* **268,** 863.
24. Howie S.E., Harrison D.J. & Wyllie A.H. (1994) Lymphocyte apoptosis: mechanisms and implications in disease. [Review]. *Immunol Rev* **142,** 141.
25. Köller B.H., Marrack P., Kappler J.W. & Smithies O. (1990) Normal development of mice deficient in beta 2 M, MHC class I proteins, and $CD8^+$ T cells. *Science* **248,** 1227.
26. Kühn R., Rajewsky K. & Muller W. (1991) Generation and analysis of interleukin-4 deficient mice. *Science* **254,** 707.
27. Duke R. & Cohen J.J. (1995) Morphological biochemical and flow cytometric assays of apoptosis. In: *Current Protocols in Immunology* (eds J. E. Coligen, A. M. Kruisbeek, E. M. Shevach & W. Strober), Vol. 2, 2nd edn, p. 3.17.1. John Wiley & Sons, New York.
28. Asea A. (1995) Role of histamine in the regulation of natural killer cells. thesis, University of Goteborg, Sweden, p.1.
29. Hansson M., Asea A., Hermodsson S. & Hellstrand K. (1996) Histaminergic regulation of NK-cells: protection against monocyte-induced apoptosis. *Scand J Immunol* **44,** 193.
30. Gorczyca W., Gong J. & Darzynkiewicz Z. (1993) Detection of DNA situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res* **53** 1945.
31. Berguer R. & Ferrick D.A. (1995) Differential production of intracellular gamma interferon in alpha beta and gamma delta T-cell subpopulations in response to peritonitis. *Infect Immun* **63,** 4957.
32. Openshaw P., Murphy E.E., Hosken N.A. *et al.* (1995) Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J Exp Med* **182,** 1357.
33. Yokoyama W.M. (1995) Natural killer cell receptors. [Review]. *Curr Opin Immunol* **7,** 110.
34. Hackett J., JR., Bennett M., Koo G.C. & Kumar V. (1986) Origin and differentiation of natural killer cells. III. Relationship between the precursors and effectors of natural killer and natural cytotoxic activity. *Immunol Res* **5,** 16.
35. Koo G.C., Dumont F.J., Tutt M., Hackett J., JR & Kumar V. (1986) The NK-1.1(-) mouse: a model to study differentiation of murine NK cells. *J Immunol* **137,** 3742.
36. Brennan J., Mager D., Jefferies W. & Takei F. (1994) Expression of different members of the Ly-49 gene family defines distinct natural killer cell subsets and cell adhesion properties. *J Exp Med* **180,** 2287.
37. Murphy W.J., Raziuddin A., Mason L., Kumar V., Bennett M. & Longo D.L. (1995) NK cell subsets in the regulation of murine hematopoiesis. I. $5E6^+$ NK cells promote hematopoietic growth in $H\text{-}2^d$ strain mice. *J Immunol* **155,** 2911.
38. Yu Y.Y., George T., Dorfman J.R., Roland J., Kumar V. & Bennett M. (1996) The role of Ly49 A and 5E6 (Ly49C) molecules in hybrid resistance mediated by murine natural killer cells against normal T cell blasts. *Immunity* **4,** 67.
39. Moingeon P., Chang H.C., Sayre P.H. *et al.* (1989) The structural biology of CD2. [Review]. *Immunol Rev* **111,** 111.
40. Garni-Wagner B.A., Purohit A., Mathew P.A., Bennett M. & Kumar V. (1993) A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells. *J Immunol* **151,** 60.
41. Seki S., Abo T., Ohteki T., Sugiura K. & Kumagai K. (1991) Unusual alpha beta-T cells expanded in autoimmune lpr mice are probably a counterpart of normal T cells in the liver. *J Immunol* **147,** 1214.
42. Emoto M., Emoto Y. & Kaufmann S.H. (1995) IL-4 producing

$CD4^+$ TCR $\alpha\beta^{int}$ liver lymphocytes: influence of thymus, beta 2-microglobulin and NK1.1 expression. *Int Immunol* **7,** 1729.

43. Mason L., Giardina S.L., Hecht T., Ortaldo J. & Mathieson B.J. (1988) LGL-1: a non-polymorphic antigen expressed on a major population of mouse natural killer cells. *J Immunol* **140,** 4403.
44. Kasai M., Iwamori M., Nagai Y., Okumura K. & Tada T. (1980) A glycolipid on the surface of mouse natural killer cells. *Eur J Immunol* **10,** 175.
45. Mathieson P.W., Qasim F.J., Thiru S., Oldroyd R.G. & Oliveira D.B. (1994) Effects of decomplementation with cobra venom factor on experimental vasculitis. *Clin Exp Immunol* **97,** 474.
46. Hercend T. & Schmidt R.E. (1988) Characteristics and uses of natural killer cells. *Immunol Today* **9,** 291.
47. Bendelac A., Killeen N., Littman D.R. & Schwartz R.H. (1994) A subset of $CD4^+$ thymocytes selected by MHC class I molecules. *Science* **263,** 1774.
48. Spinozzi F., Nicoletti I., Agea E. *et al.* (1995) IL-4 is able to reverse the CD2-mediated negative apoptotic signal to $CD4^-$ $CD8^-$ alpha beta and/or gamma delta T lymphocytes. *Immunology* **86,** 379.
49. Carlson W.E., Haldar S., Baiocchi R.A., Croce C.M. & Caligiuri M.A. (1994) The c-kit ligand suppresses apoptosis of human natural killer cells through the upregulation of bcl-2. *Proc Natl Acad Sci, USA* **91,** 7553.
50. Araneo B.A., Dowell T., Moon H.B. & Daynes R.A. (1989) Regulation of murine lymphokine production *in vivo*. Ultraviolet radiation exposure depresses IL-2 and enhances IL-4 production by T cells through an IL-1-dependent mechanism. *J Immunol* **143,** 1737.
51. Jacobsen F.W., Keller J.R., Ruscetti F.W., Veiby O.P. & Jacobsen S.E. (1995) Direct synergistic effects of IL-4 and IL-11 on proliferation of primitive hematopoietic progenitor cells. *Exp Hematol* **23,** 990.
52. Stein-Streilein J. & Guffee J. (1986) *In vivo* treatment of mice and hamsters with antibodies to asialo GM1 increases morbidity and mortality to pulmonary influenza infection. *J Immunol* **136,** 1435.
53. Wyllie A.H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284,** 555.
54. Sonoda Y. (1994) Interleukin-4: a dual regulatory factor in hematopoiesis. [Review]. *Leukemia Lymphoma* **14,** 231.
55. Ayroldi E., Cannarile L., d'Adamio F. & Riccardi C. (1995) Superantigen-induced peripheral T-cell deletion: the effects of chemical modification of antigen-presenting cells, interleukin-4 and glucocorticoid hormones. *Immunology* **84,** 528.
56. Adkins B., Ghanei A. & Hamilton K. (1993) Developmental regulation of IL-4, IL-2, and IFN-gamma production by murine peripheral T lymphocytes. *J Immunol* **151,** 6617.