

Rockefeller University

Digital Commons @ RU

---

Publications

Steinman Laboratory Archive

---

2002

**Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells**

Guido Ferlazzo

Mingli Tsang

Lorenzo Moretta

Follow this and additional works at: <https://digitalcommons.rockefeller.edu/steinman-publications>

---

# Human Dendritic Cells Activate Resting Natural Killer (NK) Cells and Are Recognized via the NKp30 Receptor by Activated NK Cells

Guido Ferlazzo,<sup>1</sup> Ming L. Tsang,<sup>2</sup> Lorenzo Moretta,<sup>3,4</sup>  
Giovanni Melioli,<sup>1</sup> Ralph M. Steinman,<sup>2</sup> and Christian Münz<sup>2</sup>

<sup>1</sup>Laboratorio di Immunoterapia Cellulare, Unità di Immunologia, Istituto Nazionale per la Ricerca sul Cancro, 16132 Genova, Italy

<sup>2</sup>Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, NY 10021

<sup>3</sup>Istituto Giannina Gaslini, 16147 Genova, Italy

<sup>4</sup>Dipartimento di Medicina Sperimentale (DIMES), Università di Genova, 16132 Genova, Italy

## Abstract

During the innate response to many inflammatory and infectious stimuli, dendritic cells (DCs) undergo a differentiation process termed maturation. Mature DCs activate antigen-specific naive T cells. Here we show that both immature and mature DCs activate resting human natural killer (NK) cells. Within 1 wk the NK cells increase two- to fourfold in numbers, start secreting interferon (IFN)- $\gamma$ , and acquire cytolytic activity against the classical NK target LCL721.221. The DC-activated NK cells then kill immature DCs efficiently, even though the latter express substantial levels of major histocompatibility complex (MHC) class I. Similar results are seen with interleukin (IL)-2-activated NK cell lines and clones, i.e., these NK cells kill and secrete IFN- $\gamma$  in response to immature DCs. Mature DCs are protected from activated NK lysis, but lysis takes place if the NK inhibitory signal is blocked by a human histocompatibility leukocyte antigen (HLA)-A,B,C-specific antibody. The NK activating signal mainly involves the NKp30 natural cytotoxicity receptor, and not the NKp46 or NKp44 receptor. However, both immature and mature DCs seem to use a NKp30 independent mechanism to act as potent stimulators for resting NK cells. We suggest that DCs are able to control directly the expansion of NK cells and that the lysis of immature DCs can regulate the afferent limb of innate and adaptive immunity.

Key words: natural killer cells • dendritic cells • NCR • immune regulation • NKp30

## Introduction

Dendritic cells (DCs)\* are sentinels for the immune system. Located in the periphery in an immature form, DCs take up substrates from the surrounding environment such as proteins and dying cells. Upon encounter of microbial antigens or proinflammatory cytokines, the DCs mature, process, and present the internalized antigens on MHC molecules. Mature DCs then prime naive T cells, selected from the recirculating T cell pool in secondary lymphoid organs,

producing for example active antigen-specific MHC class I-restricted CTLs (1–3).

Viruses and tumors often escape the CTL response, by downregulating MHC class I presentation, but a “back up” protective mechanism is provided by NK cells. NK cells share much of the killing machinery with CTLs, except that NK cells are able to recognize targets that have downregulated their MHC class I molecules, the “missing self” mechanism (4, 5). To do so, NK cells use two groups of receptors, inhibitory and activating (6). Inhibitory NK receptors prevent NK activation upon encounter of normal MHC class I levels (7–9). Activating NK receptors recognize diverse ligands. A well-characterized activating system involves the Fc receptor CD16 (10, 11) and killing of antibody-opsonized cells. Other activating receptors are

Address correspondence to Guido Ferlazzo, Laboratorio di Immunoterapia Cellulare, Unità di Immunologia, Istituto Nazionale per la Ricerca sul Cancro, Centro di Biotecnologie Avanzate, Largo Rosanna Benzi, 10, 16132 Genova, Italy. Phone: 39-010-573-7453; Fax: 39-010-573-7462; E-mail: ferlazzo@cba.unige.it

\*Abbreviations used in this paper: DC, dendritic cell; LCL, lymphoblastoid cell line.

termed NKp30, NKp44, and NKp46, but their (non-MHC) ligands have not been identified so far (12–14). MHC class I or MHC class I-related molecules can also act as activating NK ligands, engaging NKG2D or various killer activating receptors (KARs) that are highly homologous in sequence to the corresponding inhibitory NK receptors (15). Finally, there are two molecules 2B4 and NKp80 that seem to act as activating coreceptors, enhancing the function of the above mentioned NK receptors (16). While the ligand for NKp80 is unknown, CD48 appears to be the major ligand for 2B4. Most of these receptor studies involved the use of NK cells activated by IL-2 or antibody-mediated Fc $\gamma$  receptor cross-linking.

However, during the course of infection *in vivo*, NK reactivity peaks before T cell responses (17), the latter being a major source of IL-2 and help for antibody production. Thus, the prior literature on the properties of NK cells primarily reflects NK activation in the wake of adaptive immune responses. The early or innate activation of resting NK cells needs to be addressed.

In this paper we study the potential linkage between two innate responses, the maturation of DCs and the activation of resting NK cells. We will demonstrate that resting NK cells, isolated from human peripheral blood, are activated and expanded directly upon interaction with DCs. The state of NK and DC activation required for these interactions, as well as the underlying NK activating receptor, are characterized in the present work. We will first describe a positive interaction whereby resting NK cells are efficiently expanded and activated by both mature and immature DCs. Then we will outline features of what seems to be a regulatory loop. Activated NK cells, which typically recognize MHC class I-negative targets, selectively kill MHC class I-expressing immature DCs. This recognition relies almost entirely on the NKp30 activating receptors. Mature DCs trigger the same receptor, but escape NK lysis by high MHC class I surface expression. These data demonstrate that the NK response to DCs involves a strong direct response to DC mobilization as well as a potential feedback control limiting the supply of DCs.

## Materials and Methods

**Generation of DCs.** Whole blood from lab donors and leukocyte concentrates served as sources of PBMCs, isolated by density gradient centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech). Positive selection for CD14<sup>+</sup> PBMCs was performed using  $\alpha$ CD14-MicroBeads, MS<sup>+</sup>/RS<sup>+</sup> columns, and MiniMACS separator (Miltenyi Biotec). DCs were generated from CD14<sup>+</sup> PBMCs.  $5 \times 10^5$  CD14<sup>+</sup> PBMCs/ml were plated in 6-well plates with RPMI-1640, plus 1% single donor plasma, glutamine, and gentamicin. rhIL-4 (R&D Systems or Euroclone) and rhGM-CSF (Immunex or Euroclone) were added to a final concentration of 500 and 1,000 U/ml, respectively, at day 0, 2, and 4 in 500  $\mu$ l of fresh medium/well. On day 5 or 6, the floating immature DCs were transferred to new plates at  $3 \times 10^5$  cells/ml and half of the medium was replaced with fresh medium containing IL-1 $\beta$ /IL-6/TNF $\alpha$ /PGE<sub>2</sub> (all from Euroclone or R&D Systems except PGE<sub>2</sub> from Sigma-Aldrich) to mature the DCs for

2 d (18). The maturation cytokines were added to a final concentration of IL-1 $\beta$ , 10 ng/ml, IL-6, 1,000 U/ml, TNF $\alpha$ , 10 ng/ml, and PGE<sub>2</sub>, 1  $\mu$ g/ml.

**Isolation and Culture of NK Cells.** PBMCs were allowed to adhere to plastic, and nonadherent lymphocytes were then extensively washed, treated with  $\alpha$ CD3,  $\alpha$ CD4,  $\alpha$ CD8, and  $\alpha$ CD19 mAbs (Beckman Coulter) for 30 min at 4°C. After two washings, cells were incubated with magnetic immunobeads (Immunotech) coated with anti-mouse IgG. After 15 min, the cells were collected and a highly purified negatively selected population of NK cells was isolated using a Cobalt-Samarium magnet. Alternatively, the NK Cell Isolation Kit (Miltenyi Biotec) was used in which NK cells were negatively selected using  $\alpha$ CD3,  $\alpha$ CD14,  $\alpha$ CD19,  $\alpha$ CD36, and  $\alpha$ IgE depletion. The percentage of NK cells in that population was evaluated using FITC-conjugated  $\alpha$ CD3 and PE-conjugated  $\alpha$ CD56 mAbs (Beckman Coulter) and flow cytometry. Recombinant IL-2 (rIL-2, 100 IU/ml; Proleukin; Chiron Corp.) and PHA (1  $\mu$ g/ml) were added in order to obtain polyclonal NK cell population or, after limiting dilution, NK cell clones. Resting NK/DC cocultures were performed in RPMI-1640 plus 5% human serum in 96 U-bottom well plates.

**Flow Cytometric Analysis.** Analysis of DC surface markers on live cells was performed using the following mAbs in immunofluorescence assays: PE-conjugated anti-CD86 (HA5.2B7, IgG2b), FITC-conjugated anti-CD80 (MAB104, IgG1), PE-conjugated anti-CD1a (BL6, IgG1), FITC-conjugated anti-CD83 (HB15A, IgG2b), FITC-conjugated anti-ICAM-1 (CD54), PE-conjugated anti-CD83 (HB15a, Beckman Coulter), FITC-conjugated anti-HLA-A,B,C (BD PharMingen), FITC-conjugated anti-HLA-DR (BD PharMingen), PE-conjugated anti-CD11c (BD PharMingen), FITC-conjugated anti-CD40, and FITC-conjugated anti-CD25 (both from Immunotech). Anti-CD14 (63D3, IgG1) was provided by D. Vercelli, (Hospital San Raffaele-Dipartimento di Biotecnologie, Milan, Italy). Anti HLA-DR (D1.12,IgG2a) was provided by G. Frumento, Istituto Nazionale Ricerca sul Canero, Genoa, Italy. To analyze the phenotype of NK cells, we used the following FITC-labeled monoclonal antibodies: anti-CD16, anti-CD2, anti-CD7, anti-CD8, anti-CD56, obtained from Beckman Coulter. Anti-NKp30 (A76, IgG1), anti-NKp46 (BAB281, IgG1), anti-NKp44 (Z231, IgG; provided by A. Moretta, University of Genoa, Genoa, Italy) and anti-CD161 (NKR1A; 191B8, IgG2a; provided by A. Poggi and D. Pende, Istituto Nazionale Ricerca sul Canero) mAbs were also analyzed on NK cell surface. Anti-ULBP2 (M311, IgG1; provided by Immunex Corporation, Seattle, WA) and anti-CD48 (TU145, IgM; BD PharMingen) mAbs were employed in order to identify possible NKG2D and 2B4 ligands on DCs. Direct immunofluorescence procedure was performed by diluting fluorochrome-labeled mAb with 1 mg/ml human  $\gamma$ -globulin (human therapy grade from a commercial source), in order to block nonspecific Fc-receptor binding. Cells were then washed and the flow cytometric analysis was performed. Indirect immunofluorescence assays were performed as follows: cell non-specific binding sites were saturated with human  $\gamma$ -globulin and then the relevant mAb was added and incubated for 30 min at 4°C. After extensive washings, FITC-conjugated isotype-specific goat anti-mouse antibodies (GAM; Southern Biotechnology Associates, Inc.) were added and incubated for 30 min at 4°C. Negative controls included directly labeled or unlabeled isotype-matched irrelevant mAbs. Cells were then washed and analyzed by flow cytometry.

**<sup>51</sup>Cr Release Assay.** To evaluate the cytolytic activity of NK cells against DCs, we used autologous or allogeneic DCs at differ-

ent degree of maturation and the LCL 721–221 cell line (19) as target cells as described (20). Briefly,  $10^6$  target cells were incubated with 100  $\mu\text{Ci}$  of  $\text{Na}_2^{51}\text{CrO}_4$  for 60 min at  $37^\circ\text{C}$  and then extensively washed. Supernatants were collected and radioactivity counted on a gamma-counter (Beckman or 1450 MicroBeta TriLux; Wallac). “Specific”  $^{51}\text{Cr}$  release was calculated on the basis of the ratio  $([\text{sample release} - \text{spontaneous release}] / [\text{total release} - \text{spontaneous release}])$ . Assays were performed in triplicate at the indicated effector/target ratios. To analyze the role of NK cell activating receptors and coreceptors in this lysis, anti-p30 (F252, IgM) (12), anti-p44 (KS38, IgM) (13), anti-p46 (KL247, IgM) (14), anti-NKG2D (BAT221, IgG1), anti-2B4 (MA344, IgM), anti-p80 (MA152, IgG1), and anti-HLA class I (A6136, IgM) mAbs (provided by A. Moretta, University of Genoa) were added in saturating amounts in some experiments.

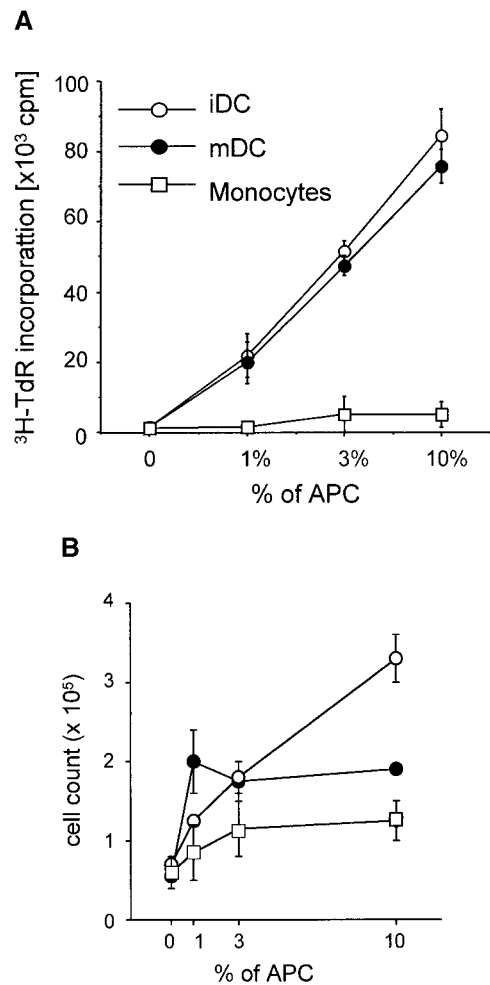
**Cytokine Assays.** To detect the production of  $\text{IFN-}\gamma$  by NK/DC cultures, cells were cultured in RPMI 1640 plus 5% human serum in 96-well round bottom microtiter plates. The supernatants of the cultures were collected after 48 h or 7 d and assayed in commercial ELISA (Biosource International or Endogen). Similarly to cytolytic assay, the involvement of natural cytotoxicity receptor (NCR) was investigated by mAb-mediated masking of anti-p30, anti-p44, and anti-p46.

**Proliferation Assay.**  $10^5$  NK cells were incubated with DCs at the indicated ratios for 5 d in RPMI-1640 plus 5% human serum in 96-well round bottom microtiter plates. 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine was added per well overnight and harvested with a Harvester Mach IIIM (Tomtec) and counted in a 1450 MicroBeta TriLux (Wallac). Counts represent mean values of duplicates or triplicates.

## Results

**DCs Can Stimulate Proliferation of Resting NK Cells.** Several reports recently described interactions between “in vitro” cytokine-activated NK cells and DCs. To evaluate the ability of DCs to stimulate resting NK cells, the latter were purified from peripheral blood by negative selection. When autologous monocytes, immature DCs or mature DCs were compared as stimulators of NK cells, the responses were different (Fig. 1). Monocytes were unable to induce DNA synthesis in NK cells (Fig. 1 A) or expand NK cell numbers (Fig. 1 B). Mature and immature DC/NK cocultures on the other hand induced active DNA synthesis and NK cell expansion. Just 1–10% autologous DCs could stimulate high levels of DNA synthesis (Fig. 1 A) and two- to fourfold increases in NK cell numbers by day 7 (Fig. 1 B). Cultures without the addition of NK cells showed no proliferation above background (data not shown).

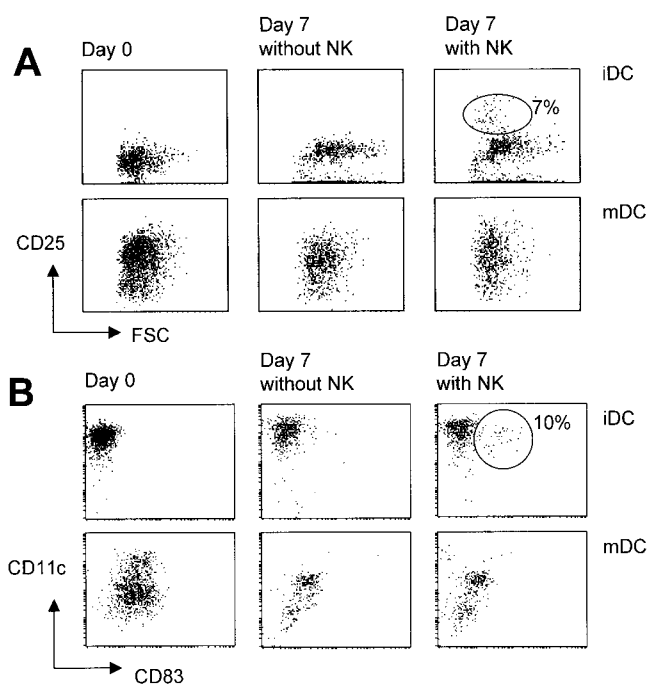
The number and the surface phenotype of NK cells and DCs recovered from 7 d cultures were verified by FACS<sup>®</sup>. The NK cells retained the  $\text{NKp30}^+$ ,  $\text{NKp46}^+$ ,  $\text{NKG2D}^+$ ,  $\text{CD161}^+$ ,  $\text{CD56}^+\text{CD3}^-$  phenotype. Slight increases of expression of the inducible molecules  $\text{NKp44}$ ,  $\text{CD25}$ , and  $\text{HLA-DR}$  were detected after coculture with either immature or mature DCs (data not shown). The numbers of mature DCs did not substantially change upon culture, and their surface phenotype was retained, as assessed by surface expression of  $\text{CD83}$  and  $\text{CD25}$ . In contrast, 70% of the im-



**Figure 1.** DCs stimulate proliferation and expansion of NK cells. (A) Small numbers of mature (mDC) and immature DCs (iDC) stimulate proliferation of negatively selected, peripheral blood NK cells, whereas monocytes had little or no stimulating activity. (B) Immature and mature DCs expand NK cell numbers by two- to fourfold, whereas monocytes sustain the initial number of added NK cells ( $10^5$ ). Count of viable cells was performed by trypan blue exclusion. Similar results were obtained in five independent experiments.

mature DCs were lost during culture, and among the surviving cells, only  $\sim 10\%$  had acquired the  $\text{CD83}$  and  $\text{CD25}$  markers, typical of mature DCs (Fig. 2). The small fraction of mature DCs by itself probably does not account for the proliferative response of NK cells detected in NK/immature DC coculture, as mature DCs were not 10 times more efficient than immature DCs in the induction of NK proliferation (Fig. 1). Instead, the proliferative responses of NK cells to mature or immature DCs were comparable (Fig. 1). DC cultures in the absence of NK cells showed no change in phenotype (Fig. 2). We conclude that DCs are able to induce NK proliferation and that this stimulatory capacity of DCs is maturation independent.

**Resting NK Cells Expanded by DCs Secrete  $\text{IFN-}\gamma$  and Acquire Cytolytic Activity.** Apart from proliferation and expansion, the NK cells also secreted  $\text{IFN-}\gamma$  and acquired cy-

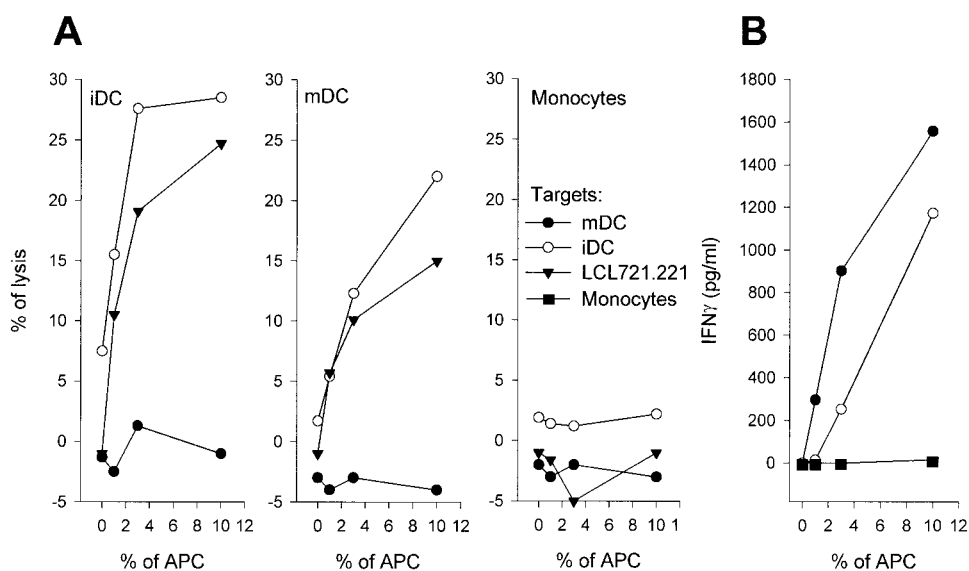


**Figure 2.** The phenotype of DCs after coculture with autologous freshly isolated NK cells. NK cells and DCs were cultured under the same conditions described in Materials and Methods for proliferation assay. The NK/DC ratio in the coculture was 10:1. Surface expression of two DC maturation markers, CD25 (A) and CD83 (B), is shown for DCs gated either as large (FSC, A) or CD11c<sup>+</sup> (B) cells. Left column: phenotype of the immature (iDC) and mature DCs (mDC) added to the NK/DC cocultures. Mature DCs were all positive for CD11c and CD83, and 70–80% were CD25<sup>high</sup>. Immature DCs had low expression of CD83 and CD25. Middle column: immature and mature DCs after 7 d without NK cells. Right column: immature and mature DCs after 7 d with NK cells. Around 10% of immature DCs expressed the mature DC phenotype upon NK coculture. These data are representative of two experiments.

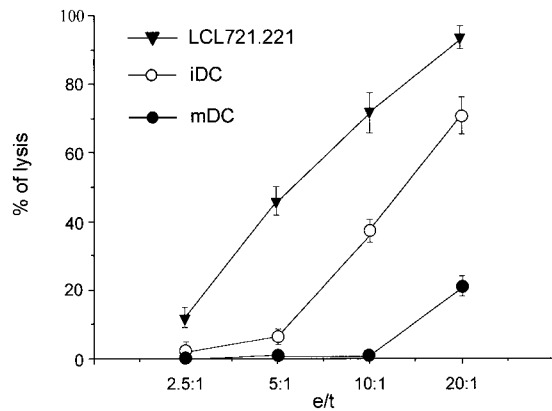
cytolytic activity, lysing the MHC class I-negative target, LCL721.221 (Fig. 3 A). Immature DCs were lysed comparably to the LCL (Fig. 3 A), even though the immature DCs had high levels of MHC class I as reported (21), but

mature DCs were protected from this cytolytic activity. Monocyte cocultured NK cells developed no cytolytic activity against either target. Although not shown, neither mature nor immature DCs could directly kill against LCL721.221, thus ruling out any possible effect of these cells on the observed cytolytic activity. NK acquisition of cytolytic capacity was mirrored in the amount of IFN- $\gamma$  secreted into the medium. NK cells secreted IFN- $\gamma$  in response to both immature and mature DCs, but not monocytes (Fig. 3 B). These data suggest that in addition to expansion, NK cells also acquire effector (cytokine plus cytolytic) functions after coculture with DCs.

**NK Cells Activated with IL-2 Also Kill Immature DCs.** In a second set of experiments we analyzed the interaction occurring between IL-2-activated NK cells and mature or immature DCs. Purified polyclonal NK cell, generated in the presence of IL-2 for 10–30 d, were tested for their ability to lyse autologous or allogeneic DCs. Both immature (i.e., cultured 7 or 8 d in GM-CSF plus IL-4) and mature (cultured for 5 or 6 d in GM-CSF plus IL-4 and 2 additional days in IL-1 $\beta$ , TNF $\alpha$ , IL-6, and PGE<sub>2</sub>) DCs were analyzed. A substantial difference was again evident in the susceptibility to NK lysis of mature versus immature DCs, in that only immature DCs were lysed with high efficiency (Fig. 4). NK-mediated lysis of DCs was comparable to the lysis of MHC class I-negative lymphoblastoid cell line LCL721.221, known to be a highly susceptible target for NK cells. Similar data have been obtained in six additional experiments using different donors. Autologous and allogeneic immature DCs were both lysed by NK cell populations. Likewise, the analysis of four NK cell clones confirmed that maximal NK-mediated cytotoxicity was exerted on immature DCs, while mature DCs were relatively resistant (not shown). Thus, our data confirm the ability of NK cells to kill DCs and clearly indicate that immature DCs are more susceptible to NK-mediated attack than their mature counterparts.



**Figure 3.** DCs induce cytolytic function and IFN- $\gamma$  secretion by DC-activated NK cells isolated from blood. (A) NK cells were cocultured with immature (iDC) or mature DCs (mDC) for 7 d (the two panels to the left) and then tested for cytolytic activity against DCs and the standard NK target, LCL721.221. The latter and immature DCs were lysed comparably. In contrast, NK cells cocultured with monocytes did not develop any cytolytic activity. (B) NK cells secreted IFN- $\gamma$  in response to immature and mature DCs, but not monocytes. Similar results after 7 d coculture were obtained in three experiments. The E/T ratio in these experiments was 30:1.

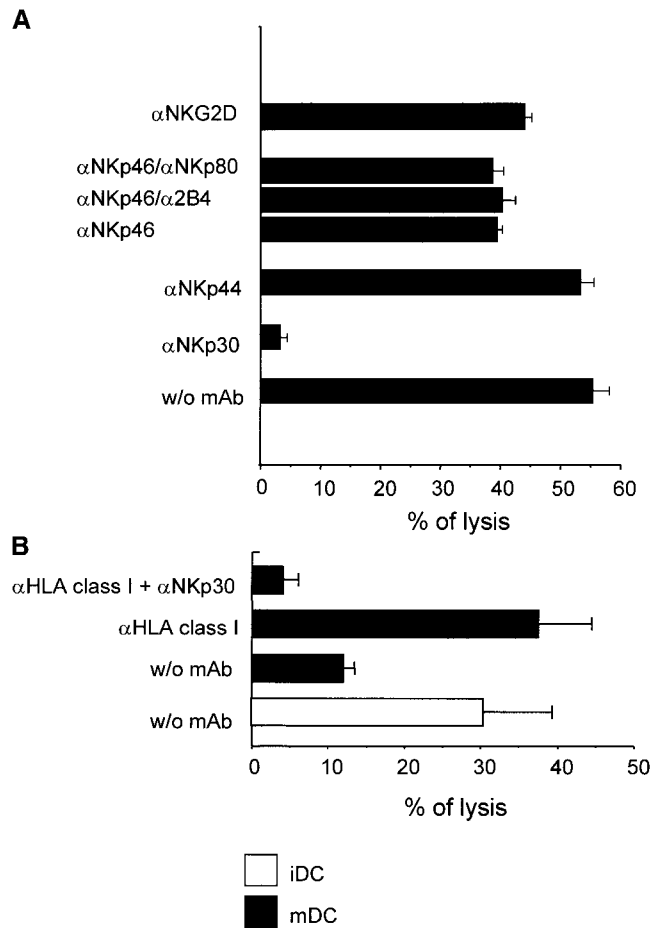


**Figure 4.** NK-mediated lysis of mature vs. immature DC. IL-2-activated autologous NK cells were tested in standard  $^{51}\text{Cr}$  release assay at various E/T ratios. Both mature (mDC) and immature DC (iDC) were analyzed. MHC class I<sup>-</sup> LCL721.221 cells were used as an NK-sensitive control. The data shown were obtained with a polyclonal NK population cultured for 20 d in the presence of IL-2 (mean of triplicates).

*The NKp30 Receptor Primarily Mediates the Activating Signal in DC Recognition by IL-2-activated NK Cells.* As the NK-mediated killing of tumor as well as normal cells can utilize a number of triggering receptors and coreceptors, we analyzed whether mAb-mediated masking of these receptors could affect the NK-mediated cytolysis of immature DCs. As shown in Fig. 5 A, the addition of anti-NKp44 mAb had no effect on cytolysis, while some degree of inhibition could be observed with anti-NKp46 and anti-NKG2D mAbs. In contrast, a marked inhibition was detected upon addition of anti-NKp30 mAb. Indeed, in the presence of this mAb the NK-mediated cytolysis was virtually abrogated. 2B4 and NKp80 have been shown to function as coreceptors, rather than as true receptors, for NK-mediated lysis. This coreceptor activity has been primarily documented in association with NKp46. Although immature DCs used as targets did not appear to express CD48, i.e., the major ligand for 2B4, we could not exclude the presence of still undefined ligand(s). Thus, we further investigated whether the simultaneous addition of mAbs to 2B4 or NKp80 to anti-NKp46 mAb could lead to a more marked inhibitory effect. Fig. 5 A shows that these coreceptors did not play any functional role in lysis of immature DCs.

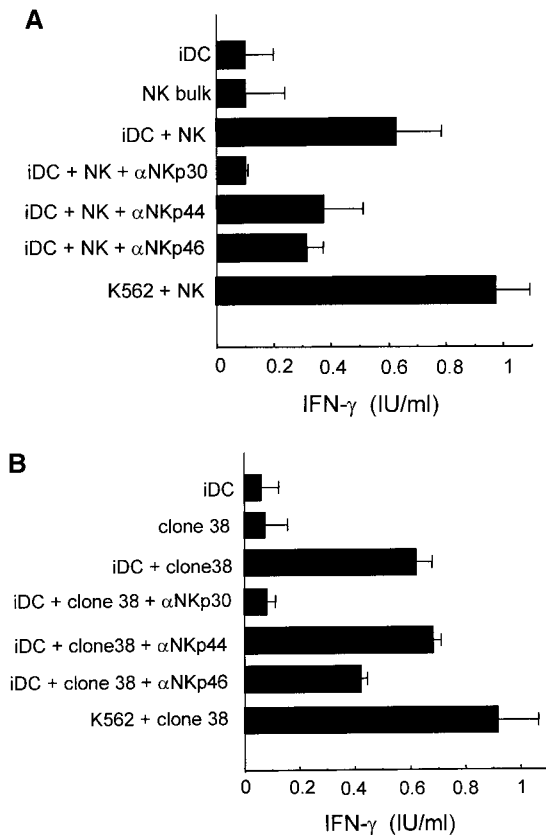
We also demonstrated that mature DCs after blocking of MHC class I molecules with a HLA-A,B,C specific mAb, were lysed by NK cells as efficiently as immature DCs (Fig. 5 B). Again the activation of IL-2-activated NK cells was mediated by NKp30 and could be blocked by anti-NKp30 mAb (Fig. 5 B).

Then we tested if anti-NKp30 could block the expansion of resting NK cells by DCs. Saturating amounts of antibody were added to cultures at day 0, 2, and 4. Proliferation was assessed at day 4. Although this antibody reliably ablated the lysis of DCs by activated NK cells (Fig. 5), it did not block DC-induced expansion of resting NK cells (not shown).



**Figure 5.** Role of activating NK receptors and coreceptors in the lysis of DCs. (A) IL-2-activated NK cells were analyzed for their cytolytic activity against autologous immature DCs in the absence or in the presence of the indicated mAb. The E/T ratio was 20:1. The experiment shown is representative of six independent experiments and data are mean of triplicates. (B) Black bars represent lysis of mature DCs; the white bar refers to the lysis of immature DCs. Mature DCs display a higher susceptibility to NK-mediated lysis after coating with anti-HLA class I mAb (A6136, IgM) and NKp30 plays a major role in this lysis. The E/T ratio was 20:1. Data are representative of two independent experiments performed in triplicates.

*Activated NK/DC Interaction Leads to the Production of IFN- $\gamma$ .* As important biological effects of NK cell triggering may also be related to cytokine production, we further investigated whether IL-2-cultured NK cells also secrete IFN- $\gamma$  upon interaction with DCs. To this end, we analyzed both polyclonal NK cell populations and NK cell clones. As shown in Fig. 6 A, activated NK cells exposed to immature DCs released amounts of IFN- $\gamma$  comparable to those induced by NK cell interaction with K562, i.e., a target cell highly susceptible to NK-mediated lysis. Note that neither NK cells nor DCs alone released substantial amounts of IFN- $\gamma$ . Again, addition of anti-NKp30 mAb virtually abrogated IFN- $\gamma$  production. On the other hand, addition of anti-NKp46 mAb had only a marginal inhibitory effect, while anti-NKp44 mAb had no effect. Fig. 6 B shows data obtained with a representative autologous NK cell clone. Allogeneic NK clones that detect MHC class I levels



**Figure 6.** Activated NK cells produce IFN- $\gamma$  upon interaction with autologous immature DCs. Polyclonal (A) or clonal (B) NK cell populations were cocultured with DCs in the absence or in the presence of the indicated mAbs. The E/T ratio was 20:1. The K562 cell line was used as positive control. IFN- $\gamma$  in the supernatants was detected by ELISA after 48 h. iDC: immature DCs; NK bulk: polyclonal NK population; clone 38: a representative NK cell clone; iDC + NK: NK cells cocultured with DCs alone or in the presence of the indicated mAbs. Data shown are the mean of triplicates. Similar results were obtained in five independent experiments.

through the CD94/NKG2A receptor and therefore different HLA haplotypes with similar efficiency behaved the same way (data not shown). Again a sharp inhibitory effect could be documented only by the addition of anti-NKp30 mAb. Although not shown, mature DCs failed to induce IFN $\gamma$  production by activated NK cell lines and clones.

## Discussion

In the mouse, there is recent evidence that DCs are able to activate NK cells which in turn can retard the growth of experimental tumors (22). We now find that human DCs also have a major effect on resting NK cells from human blood. DCs were able to induce proliferation and cytolytic function for MHC class I low targets. Activated NK cells also could kill MHC class I-positive DCs, but only immature DCs. This NK cell recognition was primarily through the activatory NK receptor NKp30. In contrast to the interaction of activated NK cells with DCs, NKp30 does not seem to be involved in activation of resting NK cells by

mature or immature DCs. The costimulatory molecule B7-1/CD80 (23–26) and several cytokines (IL-12 [27–29]; IL-15 [30–33]; and IFN $\alpha$ / $\beta$  [34–38]) have been implicated in NK activation, and early during infection, DCs can act as a major source of these stimuli (39, 40). We consider cell contact important in NK activation by DCs, as separation of DC and NK populations in transwells inhibited the interaction (data not shown). This is consistent with findings in the mouse (22, 41).

Where does this NK activation take place *in vivo*? DCs typically migrate from peripheral tissue to secondary lymphoid organs (1), whereas resting NK cells have been found in peripheral blood, spleen and bone marrow (17, 42). We suggest that resting NK cells may be activated by DCs in these compartments, rather than in the usual sites for T cell activation, in lymph nodes. An increase of DCs in peripheral blood would therefore signal inflammation or pathogen encounter in the periphery and alarm NK cells. In contrast, monocytes which are found in peripheral blood in high numbers do not activate resting NK cells (Figs. 1 and 3). After expansion and activation, the NK cells can home to sites of infection in peripheral tissues. Indeed, NK cells have been detected early in inflamed tissues and are known to migrate in response to a number of chemokines (17).

Activated NK cells can destroy virus-infected cells and tumor cells that have downregulated MHC class I products in order to escape T cell recognition (17, 22, 43–45). Especially in human Herpesvirus infections, low NK reactivity has been associated with increased susceptibility to infection (46–48). The Herpesviruses, especially HSV and CMV, encode gene products that interfere with MHC class I antigen presentation escaping T cell surveillance (49). Therefore, NK cells seem to be crucial in the immune control of these viruses and MHC class I<sup>low</sup> tumor cells. Our findings suggest that mature DCs are able to activate NK cells *in vivo*, although their site of action (or route of administration in the case of DC-based immunotherapy) needs to be worked out, as mentioned above.

Several groups have reported that activated NK cells can kill immature, but to a lesser extent, mature DCs (50–53). We could confirm these observations (Figs. 4 and 5) and demonstrate that activated NK cells also secrete IFN- $\gamma$  in response to immature DCs (Fig. 6). Recognition of immature DCs seems to be mainly mediated through the NK activating receptor NKp30 and to a lesser extent by NKp46, NKp44, and NKG2D (Figs. 4 and 5). In contrast the three activating NK receptors NKp30, NKp44, and NKp46 synergize in tumor cell recognition (12) and the recognition of mouse target cells by human NK cells is mainly mediated by NKp46 (12, 54). Recently, NK recognition of mature DCs has been reported to be mediated by NKp30 and NKp46 (55). However, as mature DCs are protected from NK lysis by high MHC class I surface expression (53; Fig. 5 B) and high MHC class I surface expression is actually a characteristic of mature DCs (1, 56), we attribute the differences between the study of Spaggiari and colleagues and ours mainly to the different DC culturing conditions,

achieving under the influence of only TNF $\alpha$  (55) a lower degree of maturation.

Our data imply that DCs express one or more ligands for NKp30. Lysis of immature DCs by IL-2 activated NK cells is nearly completely abrogated by antibody blocking of NKp30, while NKp44, NKp46, and NKG2D have only little effect. These findings are also consistent with the presence of low levels of ligands for NKp46 and NKG2D. While the NKp46 ligand is still unknown, we could analyze the expression of MICA and ULBP2, two of the known ligands of NKG2D receptor. We found that immature DCs are MICA- and ULBP2-negative (unpublished data). However, they could express low levels of other known NKG2D ligands like MICB or ULBP1. Moreover, immature DCs did not express CD48, the major 2B4 ligand (unpublished data). In this respect, DCs differ from the other hematopoietic mononuclear cell populations. As immature DCs have substantial MHC class I surface expression levels, the density of NKp30 ligand has to be sufficiently high to overcome KIR-mediated inhibition of IL-2-activated NK cells by immature DCs. We also demonstrated that NK-mediated lysis of mature DCs is comparable to the lysis of immature DCs after antibody blocking of MHC class I molecules, disrupting the KIR/MHC class I interaction. As in the case of immature DCs, lysis of mature DCs was NKp30 dependent. Taken together, these data suggest that both immature and mature DCs express high levels of NKp30 ligand and should be exploited for the identification of this molecule.

Activation of resting NK cells by immature and mature DCs could serve several purposes. Steady-state migration of immature DCs could be involved in NK homeostasis, while increased migration from inflammation sites of mainly mature DCs would lead to fast NK cell expansion and influx of activated NK cells. Killing of MHC class I expressing immature DCs by activated NK cells could have several implications for the continuing immune response. One possible function is to limit the generation of mature DCs by removal of their precursors, the immature DCs. This could limit the supply of DCs terminating the immune responses after clearance of the infection. Another possible outcome relates to recent information that immature DCs may induce IL-10-producing regulatory T cells (57, 58). These T cells can suppress effector T cell functions and could be counterproductive to clearance of the pathogen or tumor. Removal of immature DCs by activated NK cells might be a way to prevent induction of suppressor T cells specific for antigen at the site of inflammation. This study suggests a regulatory circuit between NK cells and DCs, in which resting NK cells require DCs for activation, and then limit DC function selectively at the level of immature DCs.

We thank Alessandro Moretta for the kind gift of antibodies.

This work was supported by grants awarded by Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.) and Istituto Superiore di Sanita' (I.S.S.). R.M. Steinman was supported by a grant from the Cancer Research Institute and the National Institutes of

Health, AI40045 and AI40874. C. Münz was supported by the Leukemia Research Foundation of America and the Leukemia & Lymphoma Society.

Submitted: 02 July 2001

Revised: 26 November 2001

Accepted: 10 December 2001

## References

1. Banchereau, J., and R.M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature*. 392:245–252.
2. Thery, C., and S. Amigorena. 2001. The cell biology of antigen presentation in dendritic cells. *Curr. Opin. Immunol.* 13: 45–51.
3. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y.J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18:767–811.
4. Ljunggren, H.G., and K. Karre. 1985. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. *J. Exp. Med.* 162:1745–1759.
5. Ljunggren, H.G., and K. Karre. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today*. 11:237–244.
6. Yokoyama, W.M., and W.E. Seaman. 1993. The Ly-49 and NKR-P1 gene families encoding lectin-like receptors on natural killer cells: the NK gene complex. *Annu. Rev. Immunol.* 11:613–635.
7. Lanier, L.L. 1998. NK cell receptors. *Annu. Rev. Immunol.* 16:359–393.
8. Moretta, A., and L. Moretta. 1997. HLA class I specific inhibitory receptors. *Curr. Opin. Immunol.* 9:694–701.
9. Colonna, M. 1996. Natural killer cell receptors specific for MHC class I molecules. *Curr. Opin. Immunol.* 8:101–107.
10. Perussia, B., V. Fanning, and G. Trinchieri. 1983. A human NK and K cell subset shares with cytotoxic T cells expression of the antigen recognized by antibody OKT8. *J. Immunol.* 131:223–231.
11. Lanier, L.L., J.J. Ruitenberg, and J.H. Phillips. 1988. Functional and biochemical analysis of CD16 antigen on natural killer cells and granulocytes. *J. Immunol.* 141:3478–3485.
12. Pende, D., S. Parolini, A. Pessino, S. Sivori, R. Augugliaro, L. Morelli, E. Marcenaro, L. Accame, A. Malaspina, R. Biassoni, et al. 1999. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *J. Exp. Med.* 190:1505–1516.
13. Vitale, M., C. Bottino, S. Sivori, L. Sanseverino, R. Castriconi, E. Marcenaro, R. Augugliaro, L. Moretta, and A. Moretta. 1998. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *J. Exp. Med.* 187:2065–2072.
14. Sivori, S., M. Vitale, L. Morelli, L. Sanseverino, R. Augugliaro, C. Bottino, L. Moretta, and A. Moretta. 1997. p46, a novel natural killer cell-specific surface molecule that mediates cell activation. *J. Exp. Med.* 186:1129–1136.
15. Lanier, L.L. 2001. On guard-activating NK cell receptors. *Nat. Immunol.* 2:23–27.
16. Moretta, A., C. Bottino, M. Vitale, D. Pende, C. Cantoni, M.C. Mingari, R. Biassoni, and L. Moretta. 2001. Activating receptors and coreceptors involved in human natural killer cell-mediated cytotoxicity. *Annu. Rev. Immunol.* 19:197–223.



17. Biron, C.A., K.B. Nguyen, G.C. Pien, L.P. Cousens, and T.P. Salazar-Mather. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu. Rev. Immunol.* 17:189–220.
18. Jonuleit, H., U. Kuhn, G. Muller, K. Steinbrink, L. Paragnik, E. Schmitt, J. Knop, and A.H. Enk. 1997. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur. J. Immunol.* 27:3135–3142.
19. Shimizu, Y., and R. DeMars. 1989. Production of human cells expressing individual transferred HLA-A,-B,-C genes using an HLA-A,-B,-C null human cell line. *J. Immunol.* 142:3320–3328.
20. Semino, C., G. Pietra, A. Semino, M. Falco, M. Varaldo, and G. Melioli. 1998. Normal epithelial cells modulating HLA class I surface molecules are susceptible to lysis mediated by CD3<sup>+</sup> and CD3<sup>-</sup> “nonspecific” killer cells. *Cell. Immunol.* 190:183–190.
21. Albert, M.L., S.F. Pearce, L.M. Francisco, B. Sauter, P. Roy, R.L. Silverstein, and N. Bhardwaj. 1998. Immature dendritic cells phagocytose apoptotic cells via  $\alpha\text{v}\beta 5$  and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J. Exp. Med.* 188:1359–1368.
22. Fernandez, N.C., A. Lozier, C. Flament, P. Ricciardi-Castagnoli, D. Bellet, M. Suter, M. Perricaudet, T. Tursz, E. Maraskovsky, and L. Zitvogel. 1999. Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. *Nat. Med.* 5:405–411.
23. Geldhof, A.B., G. Raes, M. Bakkus, S. Devos, K. Thielemans, and P. De Baetselier. 1995. Expression of B7-1 by highly metastatic mouse T lymphomas induces optimal natural killer cell-mediated cytotoxicity. *Cancer Res.* 55:2730–2733.
24. Yeh, K.Y., B.A. Pulaski, M.L. Woods, A.J. McAdam, A.A. Gaspari, J.G. Frelinger, and E.M. Lord. 1995. B7-1 enhances natural killer cell-mediated cytotoxicity and inhibits tumor growth of a poorly immunogenic murine carcinoma. *Cell. Immunol.* 165:217–224.
25. Wu, T.C., A.Y. Huang, E.M. Jaffee, H.I. Levitsky, and D.M. Pardoll. 1995. A reassessment of the role of B7-1 expression in tumor rejection. *J. Exp. Med.* 182:1415–1421.
26. Chambers, B.J., M. Salcedo, and H.G. Ljunggren. 1996. Triggering of natural killer cells by the costimulatory molecule CD80 (B7-1). *Immunity.* 5:311–317.
27. Kobayashi, M., L. Fitz, M. Ryan, R.M. Hewick, S.C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* 170:827–845.
28. Naume, B., M. Gately, and T. Espevik. 1992. A comparative study of IL-12 (cytotoxic lymphocyte maturation factor)-, IL-2-, and IL-7-induced effects on immunomagnetically purified CD56<sup>+</sup> NK cells. *J. Immunol.* 148:2429–2436.
29. Robertson, M.J., R.J. Soiffer, S.F. Wolf, T.J. Manley, C. Donahue, D. Young, S.H. Herrmann, and J. Ritz. 1992. Response of human natural killer (NK) cells to NK cell stimulatory factor (NKSF): cytolytic activity and proliferation of NK cells are differentially regulated by NKSF. *J. Exp. Med.* 175:779–788.
30. Fehniger, T.A., and M.A. Caligiuri. 2001. Interleukin 15: biology and relevance to human disease. *Blood.* 97:14–32.
31. Carson, W.E., J.G. Giri, M.J. Lindemann, M.L. Linett, M. Ahdieh, R. Paxton, D. Anderson, J. Eisenmann, K. Grabstein, and M.A. Caligiuri. 1994. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J. Exp. Med.* 180:1395–1403.
32. Flamand, L., I. Stefanescu, and J. Menezes. 1996. Human herpesvirus-6 enhances natural killer cell cytotoxicity via IL-15. *J. Clin. Invest.* 97:1373–1381.
33. Atedzoe, B.N., A. Ahmad, and J. Menezes. 1997. Enhancement of natural killer cell cytotoxicity by the human herpesvirus-7 via IL-15 induction. *J. Immunol.* 159:4966–4972.
34. Trinchieri, G., D. Santoli, and H. Koprowski. 1978. Spontaneous cell-mediated cytotoxicity in humans: role of interferon and immunoglobulins. *J. Immunol.* 120:1849–1855.
35. Santoli, D., G. Trinchieri, and H. Koprowski. 1978. Cell-mediated cytotoxicity against virus-infected target cells in humans. II. Interferon induction and activation of natural killer cells. *J. Immunol.* 121:532–538.
36. Gidlund, M., A. Orn, H. Wigzell, A. Senik, and I. Gresser. 1978. Enhanced NK cell activity in mice injected with interferon and interferon inducers. *Nature.* 273:759–761.
37. Welsh, R.M., Jr., and R.M. Zinkernagel. 1977. Heterospecific cytotoxic cell activity induced during the first three days of acute lymphocytic choriomeningitis virus infection in mice. *Nature.* 268:646–648.
38. Welsh, R.M., Jr. 1978. Cytotoxic cells induced during lymphocytic choriomeningitis virus infection of mice. I. Characterization of natural killer cell induction. *J. Exp. Med.* 148:163–181.
39. Reis e Sousa, C., S. Hieny, T. Scharton-Kersten, D. Jankovic, H. Charest, R.N. Germain, and A. Sher. 1997. In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. *J. Exp. Med.* 186:1819–1829.
40. Biron, C.A. 2001. Interferons  $\alpha$  and  $\beta$  as immune regulators – a new look. *Immunity.* 14:661–664.
41. Shaw, S.G., A.A. Maung, R.J. Steptoe, A.W. Thomson, and N.L. Vujanovic. 1998. Expansion of functional NK cells in multiple tissue compartments of mice treated with Flt3-ligand: implications for anti-cancer and anti-viral therapy. *J. Immunol.* 161:2817–2824.
42. Salazar-Mather, T.P., R. Ishikawa, and C.A. Biron. 1996. NK cell trafficking and cytokine expression in splenic compartments after IFN induction and viral infection. *J. Immunol.* 157:3054–3064.
43. Herberman, R.B., M.E. Nunn, H.T. Holden, and D.H. Lavrin. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *Int. J. Cancer.* 16:230–239.
44. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol.* 47:187–376.
45. Moretta, A., R. Biassoni, C. Bottino, M.C. Mingari, and L. Moretta. 2000. Natural cytotoxicity receptors that trigger human NK-cell-mediated cytotoxicity. *Immunol. Today.* 21:228–234.
46. Ching, C., and C. Lopez. 1979. Natural killing of herpes simplex virus type 1-infected target cells: normal human responses and influence of antiviral antibody. *Infect. Immun.* 26:49–56.
47. Sullivan, J.L., K.S. Byron, F.E. Brewster, and D.T. Purtilo. 1980. Deficient natural killer cell activity in x-linked lymphoproliferative syndrome. *Science.* 210:543–545.
48. Quinnan, G.V., Jr., N. Kirmani, A.H. Rook, J.F. Manischewitz, L. Jackson, G. Moreschi, G.W. Santos, R. Saral,

- and W.H. Burns. 1982. Cytotoxic T cells in cytomegalovirus infection: HLA-restricted T-lymphocyte and non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone-marrow-transplant recipients. *N. Engl. J. Med.* 307:7–13.
49. Tortorella, D., B.E. Gewurz, M.H. Furman, D.J. Schust, and H.L. Ploegh. 2000. Viral subversion of the immune system. *Annu. Rev. Immunol.* 18:861–926.
  50. Carbone, E., G. Terrazzano, G. Ruggiero, D. Zanzi, A. Ottaiano, C. Manzo, K. Karre, and S. Zappacosta. 1999. Recognition of autologous dendritic cells by human NK cells. *Eur. J. Immunol.* 29:4022–4029.
  51. Wilson, J.L., L.C. Heffler, J. Charo, A. Scheynius, M.T. Bejarano, and H.G. Ljunggren. 1999. Targeting of human dendritic cells by autologous NK cells. *J. Immunol.* 163:6365–6370.
  52. Geldhof, A.B., M. Moser, L. Lespagnard, K. Thielemans, and P. De Baetselier. 1998. Interleukin-12-activated natural killer cells recognize B7 costimulatory molecules on tumor cells and autologous dendritic cells. *Blood.* 91:196–206.
  53. Ferlazzo, G., C. Semino, and G. Melioli. 2001. HLA class I molecule expression is up-regulated during maturation of dendritic cells, protecting them from natural killer cell-mediated lysis. *Immunol. Lett.* 76:37–41.
  54. Biassoni, R., A. Pessino, C. Bottino, D. Pende, L. Moretta, and A. Moretta. 1999. The murine homologue of the human NKp46, a triggering receptor involved in the induction of natural cytotoxicity. *Eur. J. Immunol.* 29:1014–1020.
  55. Spaggiari, G.M., R. Carosio, D. Pende, S. Marcenaro, P. Rivera, M.R. Zocchi, L. Moretta, and A. Poggi. 2001. NK cell-mediated lysis of autologous antigen-presenting cells is triggered by the engagement of the phosphatidylinositol 3-kinase upon ligation of the natural cytotoxicity receptors NKp30 and NKp46. *Eur. J. Immunol.* 31:1656–1665.
  56. Hashimoto, S.I., T. Suzuki, S. Nagai, T. Yamashita, N. Toyoda, and K. Matsushima. 2000. Identification of genes specifically expressed in human activated and mature dendritic cells through serial analysis of gene expression. *Blood.* 96:2206–2214.
  57. Jonuleit, H., E. Schmitt, G. Schuler, J. Knop, and A.H. Enk. 2000. Induction of interleukin 10-producing, nonproliferating CD4<sup>+</sup> T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J. Exp. Med.* 192:1213–1222.
  58. Dhodapkar, M.V., R.M. Steinman, J. Krasovsky, C. Münz, and N. Bhardwaj. 2001. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J. Exp. Med.* 193:233–238.