Interleukin-12 secreted by mature dendritic cells mediates activation of NK cell function

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Received 5 November 2003; revised 15 December 2003; accepted 29 December 2003

First published online 21 January 2004

Edited by Veli-Pekka Lehto

Abstract Dendritic cells (DCs) are known to modulate immune response by activating effector cells of both the innate and the adaptive immune system. In the present study, we demonstrate that co-culture of DCs with paraformaldehyde-fixed tumor cells augments the secretion of interleukin (IL)-12 by DCs and these activated DCs upon co-culture with naive NK cells enhance the cytolytic activity of NK cells against NK-sensitive target YAC-1. Similarly, DCs isolated from tumor-bearing animals also activated NK cells in vitro. For efficient activation of NK cells, the ratio of activated DCs to NK cells is crucial. Addition of anti-IL-12 antibody to the culture system completely abolished activation of NK cells by DCs, suggesting that IL-12 secreted by DCs is an essential factor in NK cell activation. Adoptive transfer of DCs isolated from tumor-bearing animals into normal rats also induced activation of NK cells in normal animals.

Key words: Dendritic cell; Natural killer cell; Cytotoxicity; Activation; Interleukin-12

1. Introduction

Dendritic cells (DCs) are unique antigen-presenting cells [1], as they can both initiate and modulate immune response. Even a small number of DCs and a low level of antigen can elicit a strong immune response by activating naive T cells, B cells [2] and NK cells [3] and also play a critical role in the induction of peripheral immunological tolerance [4]. DCs have been shown to augment the cytotoxic function of NK cells [5,6]. Recent findings that DCs produce interleukin (IL)-2 after microbial challenge suggest this to be the mechanism by which DCs link innate and adaptive immunity [7]. Recently DCs have been shown to produce interferon-γ (IFN-γ) [8], which is essential for both innate and adaptive immunity. Moreover, T-bet (a transcription factor) is required for the optimal production of IFN-γ by both $CD8\alpha^+$ and $CD8\alpha^-$ DCs [9]. Stimuli inducing maturation of DCs include microbial products and interaction with activated T lymphocytes. Mature DCs upregulate co-stimulatory molecules, secrete cytokines and migrate to the secondary lymphoid tissues where they trigger specific effector cell response.

NK cells are the effector cells of innate immunity and mediate spontaneous cytotoxicity against a broad range of targets including major histocompatibility complex (MHC) class I-negative tumor cells [10]. The mechanism of NK cell-mediated oncolysis involves a complex array of events. The cytolytic activity of NK cells is augmented by IL-2 and to a lesser extent by other cytokines including IL-12 and IFN-γ [11]. Recently DCs engineered to express IL-12 have been shown to activate NK cells [12].

AK-5 is a highly immunogenic rat histiocytic tumor, which regresses spontaneously in syngeneic animals when transplanted s.c., whereas it kills 100% of animals when transplanted i.p. [13]. The death of AK-5 cells is achieved through necrosis and apoptosis mediated by NK cells, macrophages and B cells [14,15]. NK cells have been demonstrated to be the major effector cells and require priming/activation before they can participate in the induction of death in AK-5 cells.

In this study, we have studied the role of DCs in AK-5 regression. We observed activation of DCs upon co-culture with fixed tumor cells, which in turn activated NK cells. Similar observations were also made when adoptively transferred mature DCs isolated from 8th day tumor-bearing animals were injected into naive animals, thereby suggesting an important role for mature DCs in the activation of NK cell effector function.

2. Materials and methods

2.1. Animals and tumors

AK-5 tumors were maintained as ascites in 6–8 week old Wistar rats obtained from the inbred colony of this laboratory by injecting 5×10^6 AK-5 cells i.p. Solid AK-5 tumors were obtained by injecting 5×10^6 AK-5 cells s.c. in 6 week old rats. The murine lymphoma cell line YAC-1 was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 50 μg/ml streptomycin.

2.2. Antibodies

Monoclonal antibody (mAb) OX-6 (MHC-II) was from Pharmingen, USA. Hybridoma OX-62 (αε-integrin) was kindly provided by Dr. M.J. Puklavec and B7.2 (CD86) by Dr. Vijay Kuchroo. Anti-mouse IL-12 (c17.15, p40 subunit) and anti-mouse IFN-γ (XMG1.2) were kindly provided by Dr. G. Trinchieri and Dr. R.L. Coffman respectively. Anti-mouse Ig Alexa 568 was from Molecular Probes and anti-mouse Ig fluorescein isothiocyanate (FITC) was purchased from Amersham, UK.

2.3. Isolation of DCs and NK cells from spleen

Spleens from normal or different day (s.c.) tumor-bearing rats were removed aseptically and teased in RPMI 1640 medium. Mononuclear cells were obtained after fractionation on Ficoll-Hypaque density gradient. DCs and NK cells were isolated using magnetic beads (Dynal, Chantilly, VA, USA) coated with mAb OX-62 and anti-CD161 mAb respectively. For further DC purification, cells were plated in RPMI 1640 containing 10% FCS overnight at 37°C in a CO_2 incubator and

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0014-5793/04/$30.00
doi:10.1016/S0014-5793(04)00026-2

the non-adherent cells were removed. DCs isolated by this procedure were >90% pure.

2.4. Co-culture of DCs with tumor cells

AK-5 tumor cells were washed with phosphate-buffered saline (PBS) and fixed in 3% paraformaldehyde for 30 min at room temperature. DCs (5×10^5/ml) were co-cultured with fixed AK-5 cells at different DC to AK-5 cell ratios in RPMI 1640 medium supplemented with 10% heat-inactivated FCS for 18 h.

2.5. Co-culture of DCs with NK cells

DCs isolated from 8th day tumor-bearing rats or previously co-cultured for 18 h with fixed tumor cells (50:1) or from normal rats were further co-cultured with naive NK cells at different ratios (3:1 to 0.5:1). After 18 h incubation, NK cells were separated on magnetic beads and tested for their cytolytic activity against YAC-1 cells.

2.6. RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using Trizol reagent (Gibco BRL). Single-stranded cDNA was prepared in a 20 μl reaction volume using 500 ng of oligo(dT)12–18 primer, 15 units of AMV reverse transcriptase (Promega) and 2 μg of RNA. 50 μl PCR reactions containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl pH 9.2, 200 mM of each dNTP, 0.1% Triton X-100, 200 nM of each primer, 1.25 U Taq polymerase and 2 μl of cDNA solution were set up. Primers specific for cytokines IL-2, IL-12 and IFN-γ were used along with GAPDH primers. The PCR products were confirmed after hybridization with IL-2-, IL-12- or IFN-γ-specific cDNA probes.

2.7. MTT assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay used in measuring antitumor cell-mediated cytotoxicity was modified for adherent effector cells [16]. Briefly, experimental wells contained both DCs and tumor cells (E:T = 50:1) and control wells included DCs or tumor cells alone. Cells were cultured for 18 h, followed by incubation with MTT for 2 h at 37°C. The generated formazan was read at 590 nm and the percentage cytotoxicity was calculated.

2.8. Immunofluorescence and flow cytometry

DCs isolated from normal or tumor-bearing animals were fixed in 3% paraformaldehyde at room temperature for 30 min and treated with primary antibodies against OX-62, OX-41, OX-6 and B7-2 for 1 h at room temperature. The secondary antibodies used were FITC or Alexa-568-conjugated anti-mouse Ig. The cells were analyzed by flow cytometry.

2.9. Cytotoxicity assay

Cytotoxicity assay was performed by a 4 h ^{51}Cr release assay. Activated NK cells were isolated from the DC-NK co-culture experiment or NK cells from mature DC adoptively transferred animals and were incubated with ^{51}Cr-labelled YAC-1 cells (E:T = 25:1) for 4 h. ^{51}Cr released in the medium was counted in a Packard Gamma Counter and the percentage cytotoxicity was calculated.

2.10. Enzyme-linked immunofiltration assay (ELIFA)

The cytokine levels in the co-culture supernatants were quantified by ELIFA (Pierce, USA). The culture supernatant was filtered through a nitrocellulose membrane, allowing the protein to bind to the membrane. The membrane was blocked with PBS-bovine serum albumin solution followed by treatment with primary antibody for IL-12 or IFN-γ and after washing it was treated with horseradish peroxidase-conjugated anti-rat Ig antibody. The membrane was washed and the signal bound to the membrane was developed for peroxidase.

2.11. Adoptive transfer experiments

For adoptive transfer of mature DCs (from 8th day s.c. tumor-bearing rat spleens), two groups of normal Wistar rats were injected i.p. twice each week for 3 weeks (2×10^6 DCs/dose/animal). The control group received immature DCs obtained from naive Wistar rats. One group of rats was killed after the scheduled dose of i.p. injections and spleens were removed to isolate NK cells which were checked further for cytotoxicity against YAC-1 cells. In the other group of animals, AK-5 tumor was injected i.p. (1×10^6/rat) and the animals were monitored for tumor growth (ascites).

2.12. Statistical analysis

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

3. Results

3.1. Activation of DCs by fixed tumor cells

DCs isolated following the procedure described above were >90% pure as assessed by dendritic morphology and phenotypic markers such as αε-integrin (OX-62) (Fig. 1A) and up-regulation of MHC class II (OX-6) and CD86 (B7-2) upon stimulation leading to maturation. To investigate whether fixed tumor cells could activate DCs upon co-culture, several parameters of DC activation were studied. We tested the ability of fixed AK-5 cells to upregulate DC maturation markers, viz., secretion of IL-12 and IFN-γ and upregulation of the surface expression of MHC-II and CD86.

DCs were incubated with fixed AK-5 cells at different ratios (DC:AK-5, 100:1–1:1) for 18 h before analyzing for the secretion of IL-12 and IFN-γ (Fig. 1B) or for phenotype evaluation of MHC-II and CD86 (Fig. 1C). Differences in IL-12 and IFN-γ secretion between DCs incubated with or without fixed AK-5 cells were significant. In addition, fixed AK-5 tumor cells induced DC activation in a dose-dependent manner (Fig. 1B). The augmentation in the activation of DCs following exposure to fixed AK-5 cells was not due to the binding of mAb OX-62, because the control DCs were also isolated using the same procedure. In addition fixed AK-5 cells did not produce either IL-12 or IFN-γ (data not shown).

DCs isolated from different day AK-5 tumor-bearing rats showed expression of several markers as analyzed by RT-PCR for cytokines IL-2, IL-12 and IFN-γ (Fig. 2). DCs from 8th day tumor-bearing rats were mature as analyzed by the mRNA transcript levels of IL-12 (whereas IL-2 transcripts were present at very low levels). IFN-γ transcripts were highly expressed in all the samples of DCs collected from different day tumor-bearing rats. They also showed upregulation of MHC-II and CD86 expression (Fig. 1C). For subsequent experiments, like NK cell activation assays and adoptive transfer experiments, DCs isolated from 8th day tumor-bearing rats were used.

3.2. Immature but not mature DCs induced death in tumor cells

To rule out the possibility that mature DCs directly induce death signal in tumor cells, AK-5 tumor cells were co-cultured with immature DCs or mature DCs isolated from different day (s.c.) tumor-bearing rat spleens (E:T = 50:1). After 18 h incubation, cell viability was checked by the MTT assay. We observed significantly higher apoptotic cell death in tumor cells co-cultured with immature DCs, whereas the apoptotic potential of DCs decreased gradually with maturation after tumor transplantation (Fig. 3).

3.3. Mature DCs activate resting NK cells

Mature DCs obtained either from 8th day (s.c.) tumor-bearing rat spleens or after co-culture with fixed AK-5 cells were able to activate resting NK cells. Activation of NK cells was checked after prior co-culture with mature DCs at different ratios (3:1 to 0.5:1) for 18 h or naive NK cells for induction of cytotoxicity against YAC-1 cells. NK cell cytotox-

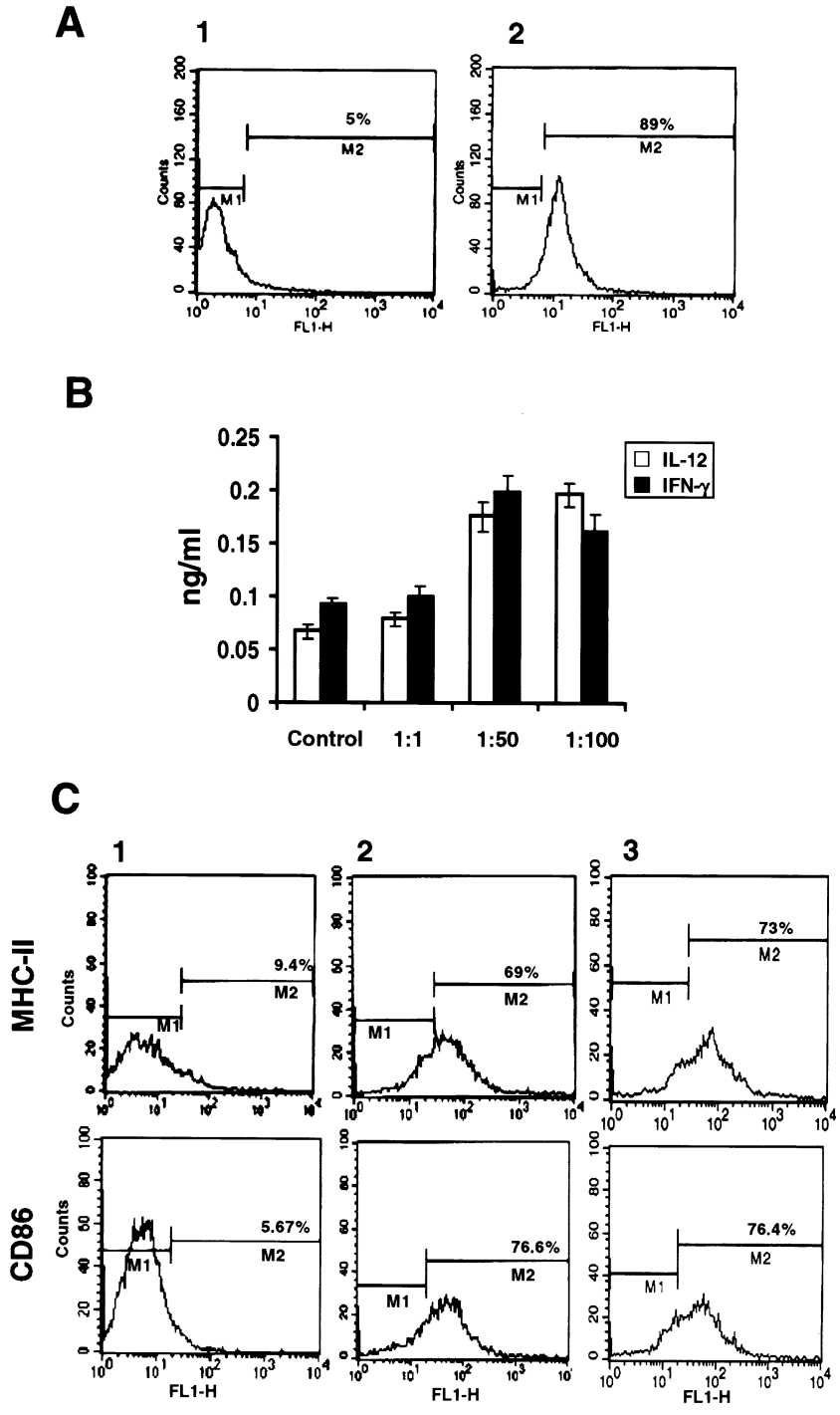

Fig. 1. A: DCs were isolated from rat spleens by positive selection using mAb OX-62 conjugated to magnetic beads. Panel 1, secondary antibody control; panel 2, OX-62-positive cells. B: Cytokine secretion by DCs after co-culture with fixed AK-5 cells. DCs (2×10^5) were incubated with fixed AK-5 cells at different ratios for 18 h. IL-12 and IFN-γ levels in the supernatants were assayed by ELIFA. The levels shown are mean ± S.D. from three different experiments. C: Upregulation of MHC-II (OX-6) and CD86 (B7.2) in activated DCs after co-culture with fixed AK-5 cells for 18 h or DCs isolated from 8th day (s.c.) tumor-bearing rat spleens. Panel 1 shows control DCs, panel 2 shows DCs co-cultured with fixed AK-5 cells and panel 3 shows DCs from tumor-bearing animals.

icity was highly augmented after an 18 h co-culture with mature DCs (Fig. 4).

3.4. Role of IL-12 in NK cell activation

Mature DCs secrete various cytokines that are involved in activating NK cells. IL-12 and IFN-γ are NK cell stimulatory cytokines, secreted during tumor development. We investigated the role of IL-12 in DC-mediated NK cell activation. Addition of neutralizing anti-IL-12 mAb to NK-DC co-culture experiments did block activation of NK cell cytotoxicity (Fig. 5A). Inhibition of NK cell activity by anti-IL-12 antibody was significant ($P < 0.001$) as compared to activated NK

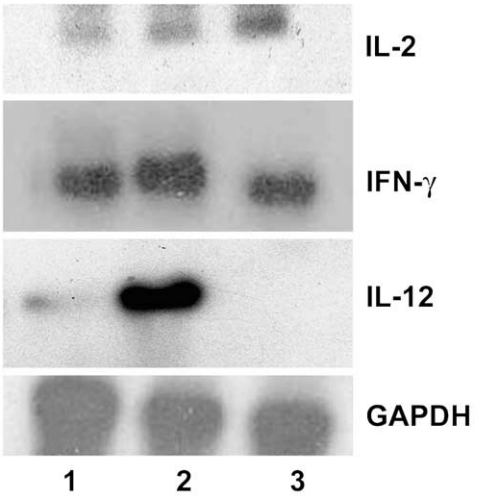

Fig. 2. Expression of transcripts for IL-2, IL-12, IFN-γ cDNAs were prepared from DCs obtained from (1) naive rats, (2) 8th day tumor-bearing rats, and (3) 16th day tumor-bearing rats. GAPDH is shown as loading control. The results shown are representative of three similar experiments.

cells by mature DCs. However, culture supernatants from mature DC alone did not activate NK cells (data not shown), suggesting that in addition to the cytokines secreted by DCs, cell-to-cell contact may also be essential for activation.

3.5. NK cell activation by mature DCs in vivo

In order to study activation of NK cells by DCs in vivo, we adoptively transferred mature or immature DCs obtained from 8th day tumor-bearing or naive rats respectively. NK cells obtained from DC-injected animals showed augmented cytotoxicity against YAC-1 cells as compared to NK cells obtained from control groups (Fig. 5B). Another set of animals that had received DC injections were challenged with AK-5 cells i.p., monitored for tumor growth and mortality. Although a considerable delay in tumor growth was observed, none of the DC-injected animals survived (data not shown).

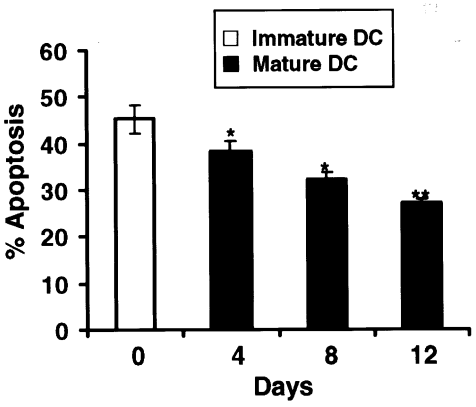

Fig. 3. Induction of apoptosis in AK-5 cells by immature and mature DCs isolated from different day (s.c.) tumor-bearing rat spleens. The results shown are representative of three similar experiments. $*P < 0.01$, $**P < 0.001$ with respect to immature DC control.

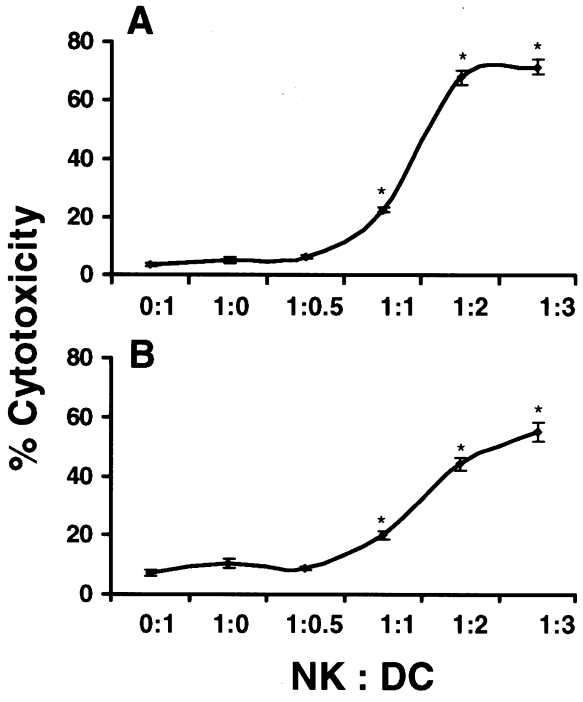

Fig. 4. A: Activation of naive NK cells by mature DCs isolated from 8th day (s.c.) tumor-bearing rat spleens. NK cells and DCs were co-cultured for 18 h at different ratios. NK cell cytotoxicity was measured against YAC-1 target cells. The results shown are representative of three similar experiments. $*P < 0.001$ with respect to naive NK cell control. B: Activation of naive NK cells by preactivated DCs (fixed AK-5 cells co-cultured with DCs; DC:AK-5 = 50:1). NK cells and DCs were co-cultured for 18 h at different ratios. Activation of NK cells was assayed by measuring cytotoxicity against YAC-1 cells. $*P < 0.001$.

4. Discussion

This study demonstrates the activation of DCs after their interaction with fixed tumor cells and in turn induction of lytic function by NK cells and the requirement for cell-secreted accessory signals for the augmentation of the lytic function. It also demonstrates that adoptively transferred mature DCs are able to activate NK cells for their cytotoxic potential against the tumor. NK cells have been shown to be the effector cells during AK-5 tumor regression [17].

A variety of factors can induce maturation/activation following antigen uptake and processing within DCs, including whole bacteria or bacterial-derived antigens (e.g. lipopolysaccharide (LPS)), inflammatory cytokines (tumor necrosis factor-α, IL-1β, IL-6 and prostaglandin E_2), ligation of select cell surface receptors (e.g. CD40), viral products (e.g. double-stranded RNA), tumor products (heat shock proteins, tumor-associated antigens) etc. [17]. DC maturation is a process where MHC and co-stimulatory molecules are upregulated (e.g. CD80, CD86, CD40, OX-40-L and LBB1L). Simultaneously, they become primed to synthesize cytokines (IL-12, IL-15 and IL-18) essential for the development of the innate and the adaptive immune system [18].

In this study we have shown activation of immature DCs by paraformaldehyde-fixed tumor cells. Recently we have observed NKR-P2/NKG2D expression on DCs (unpublished observations), which may be interacting with its ligand on fixed AK-5 tumor cells thereby inducing activation of DCs.

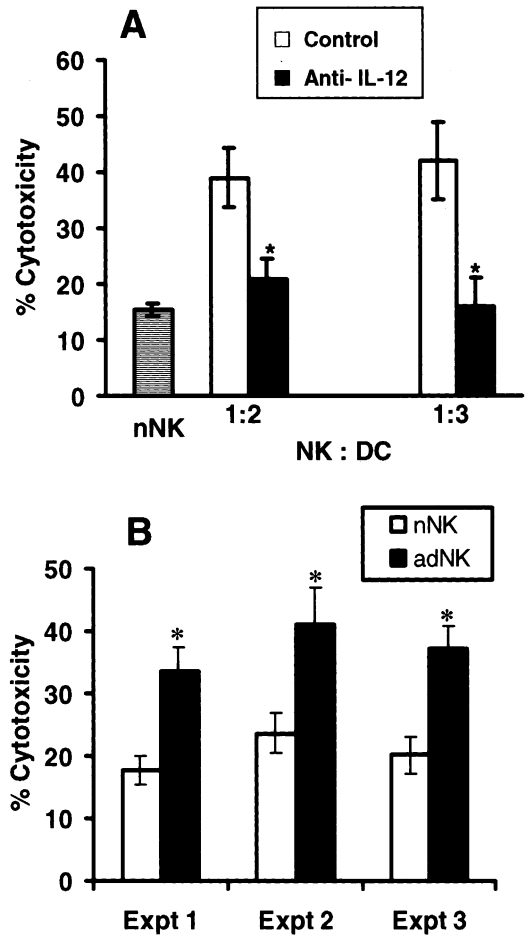

Fig. 5. A: Role of IL-12 secreted by DCs on NK cell activation. IL-12 secreted by DCs was neutralized by anti-IL-12 antibody and its effect on NK cell cytotoxicity against YAC-1 was studied. In the presence of anti-IL-12 mAb (10 μg/ml), NK cell cytotoxicity was inhibited significantly. Controls were treated with isotype-matched antibody. B: Adoptive transfer of mature DCs (from 8th day tumor-bearing rats) to normal rats and its effect on NK cell activation. NK cells from animals that had received activated DCs showed significantly higher cytotoxic activity against YAC-1 cells as compared to naive NK cells (nNK). adNK denotes NK cells obtained from animals that were injected with activated DCs. $^{*}P < 0.001$ with respect to control.

The activated DCs secreted increased amounts of IL-12 and expressed MHC class II and CD86 co-stimulatory molecules (Fig. 1), which are the hallmark of DC maturation. DCs isolated from tumor-bearing rats also showed high transcripts for genes encoding IL-12 and IFN-γ (Fig. 2), in addition to the expression of maturation markers like MHC-II and CD86 (Fig. 1C). Microorganisms or microbial products only seem to induce the transcription of the gene encoding IL-2 in DCs [7]. Recent studies have established DCs as an important source of IFN-γ. After a 72 h stimulation with IL-12 and IL-18, DCs secreted substantial amounts of IFN-γ. T-bet influenced the generation of type I immunity not only by controlling Th1 lineage commitment in the adaptive immune system but also by directly influencing the transcription of IFN-γ gene in DCs [9].

Immature DCs have been shown to be more effective in mediating tumor cell apoptosis than mature DCs, inducing mitochondrial damage initially followed by DNA fragmentation [16]. It is known that immature, but not mature DCs readily ingest apoptotic bodies and carry out antigen presentation. We tested the oncolytic activity of mature DCs isolated from different day tumor-bearing rats and compared it with that of immature DCs in MTT and 18 h ^{51}Cr release assays. Only in the MTT assay could we see a significant difference in the ability to induce death signals in tumor cells (Fig. 3) by immature DCs; however, in the ^{51}Cr release assay, we were unable to detect cell death (data not shown) suggesting that DCs may first induce mitochondrial damage and may require a longer period of acquaintance with tumor cells for DNA damage to occur. Maturation of DCs is known to be accompanied by their multiple phenotypic and functional changes including that of antigen-presenting activities and a significant decrease of their tumoricidal function.

Previous reports in murine and human models demonstrated that DCs were able to activate NK cells which in turn retarded the growth of experimental tumors [3,19,20]. In the presence of a maturation stimulus DCs activated NK cells that in turn strongly enhanced DC maturation. However, the requirement for direct NK-DC contact, presence of soluble factors and DC maturation varied in the different studies. Fresh NK cells were activated as indicated by the induced expression of the CD69 antigen, and their cytolytic activity was strongly augmented by contact with LPS-treated mature DCs or with immature DCs in the presence of the maturation stimulus LPS [5].

We observed that DCs from tumor-bearing rats as well as DCs previously activated with fixed AK-5 cells were able to activate NK cells and render them cytolytic against NK-sensitive target YAC-1 cells, whereas immature DCs were unable to activate NK cells. DC maturation stimulus is very important in activating NK cells. Moreover, when anti-IL-12 antibody was introduced in the DC-NK co-culture system, NK cells significantly lost their cytolytic activity. Spent culture medium from mature DCs alone was unable to activate NK cells, suggesting that in addition to IL-12, cell–cell contact and NK to DC ratios were crucial in achieving significant activation in NK cells.

So far, several methods using DCs to induce antitumor immunity have been investigated: DCs pulsed with proteins or peptides extracted from tumor cells [21–23], DCs transfected with genes encoding tumor-associated antigens [24], DCs cultured with tumor cells [25] and DCs fused with tumor cells [26–29]. Evidence of clinical improvement such as regression of metastasis and enhanced T cell immunity, antigen-specific proliferative responses and delayed-type hypersensitivity reactions was observed in some cases, even in late stages of the disease.

We adoptively transferred mature DCs isolated from 8th day tumor-bearing rats into normal Wistar rats. These rats also achieved activation of NK cells in vivo, and induced significant cytotoxicity against YAC-1 cells in a 4 h ^{51}Cr release assay (Fig. 5B). Adoptively transferred rats were challenged by transplanting a low dose of AK-5 tumor cells i.p. (1×10^{6} AK-5 cells/rat) and monitored for ascites development and delay in mortality. Compared to normal rats, adoptively transferred rats showed slow progression in ascites formation and delay in mortality. But we could not achieve complete protection against AK-5 tumor challenge (data not shown). This could be attributed to the rapid growth of tumor cells in the peritoneum thereby not giving enough time for the

host immune system to mount an effective anti-tumor response. Alternatively, overexpression of CD95-L by day 3 and day 4 ascitic tumor cells may be involved in depleting the peritoneum of $CD95^+$-activated lymphocytes [30].

Acknowledgements: The authors are grateful to Drs. Puklavec and Vijay Kuchroo for providing hybridomas OX-62 and B7.2, Dr. R.L. Coffman for anti-IFN-γ antibody and Dr. G. Trinchieri for anti-IL-12 antibody. Technical help provided by Mrs. Ch. Varalakshmi, Mrs. A. Leela Kumari and A. Mubarak Ali is acknowledged. Mr. Dwarakanath helped us in tumor maintenance and animal handling. Mrs. T. Hemalatha typed the manuscript. Financial support was provided by the Department of Science and Technology, Government of India.

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