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Dendritic cells (DC) promote natural killer (NK) cell functions: dynamics of the human DC/NK cell cross talk

Abstract

Dendritic cells (DC) were originally found critical in the setting of cognate immune responses. We first demonstrated that DC can also induce mouse NK cell activation and NK cell dependent-antitumor effects in mice. Here we analyzed the dynamics between DC and NK cells in human in vitro model systems. In the absence of LPS, DC do not trigger resting NK cells. Conversely, in the presence of LPS, resting bulk NK cells interacting with DC acquire CD25 and CD69 surface expression, produce high levels of IFN-gamma and lyse DAUDI

cells. On activated IL-2 dependent NK cell lines, regardless of their differentiation stage, DC maintain or enhance NK cell proliferation and effector functions in the absence of exogenous cytokines. While IL-12, IL-15 and IL-18 are not critical, a direct cell-to-cell contact is mandatory for NK activation by DC and required for optimal proliferation. These data imply that DC also modulate human NK cell innate effector functions.

Full text

INTRODUCTION

NK cells are a subpopulation of lymphoid cells that were originally defined by their ability to lyse infected or transformed target cells without prior immunization. They represent one of the major effector cells of the non-antigen specific innate immunity. The mechanisms involved in target cell recognition and lysis by activated NK cells have been extensively studied, particularly with regard to the role of MHC class I-specific inhibitory receptors [1] and, more recently, to the role of activating receptors [2]. Several infectious disease and tumor models have provided support that early in immune responses, NK cells are recruited and activated locally [3-5]. Some chemokines that have been shown to induce NK cell migration have also been involved in the induction of NK cell effector functions such as macrophage inflammatory protein (MIP)-1alpha, IFN-gamma inducible protein-10 (IP-10) and IL-2 [5-7].

It is still unclear what modulates the NK cell effector functions and proliferation following the initial activation step. Most reports have demonstrated a role for cytokines such as IL-2, IL-12, IL-15, IL-18, IL-21 and type I and II IFN [8-14]. However some studies have suggested that cytokines alone might not be sufficient and shown that the presence of accessory cells such as monocytes/macrophages, K562 or B-EBV cell lines might be required in addition to exogenous cytokines for induction or sustenance of NK cell activation and/or proliferation [15-19].

Dendritic cells (DC) represent a heterogeneous population of antigen-presenting cells (APC) with distinctive stages of development. Tissue-resident immature DC possess the capacity to ingest and process antigens very efficiently. Following different stimuli such as pathogen- or inflammatory-related products, DC are induced to mature and migrate to secondary lymphoid organs. The maturation process is characterized by profound changes in the DC

phenotype. They up-regulate the expression of MHC class I and II, adhesion and costimulatory molecules [20]. If originally DC were described as critical APC capable of stimulating naïve antigen-specific T cells, they rapidly turned out to be cornerstones at the interplay between innate and cognate immune responses. Indeed, the expression of receptors involved in the recognition of pathogen-associated molecular patterns (Toll-like receptor family) [21], their ability to produce cytokines and chemokines following maturation [22, 23] and their capacity of inducing NKT cell activation [24] outline their regulatory role in innate immune responses.

Importantly, we have demonstrated that murine bone marrow-derived DC were capable of activating resting NK cells and that this DC/NK cell cross talk was relevant in antitumor effects *in vivo* [25]. In the present study, we extend these findings in humans. Firstly, we demonstrate, using human monocyte-derived DC (MD-DC) and IL-2 dependent NK cell lines, that MD-DC are capable of enhancing NK cell effector functions and proliferation in the absence of exogenous cytokines. A direct cell-to-cell contact is necessary to promote NK cell functions and required for inducing optimal proliferation. Secondly, in contrast to IL-2 dependent NK cell lines, resting bulk NK cells are only triggered by DC in the presence of LPS or by mature DC. Thus, these data provide evidence that DC act as regulatory cells for NK cells and support the implementation of DC adoptive therapy for immunomodulation of innate immune responses *in vivo*.

MATERIALS AND METHODS

Cell lines

The IL-2-dependent human NK cell lines NKL [26] and NK-92 [27] were maintained in low dosage of IL-2 as previously described. The standard NK cell targets *i.e.* K562, DAUDI, the human B cell line C1R, and its stable HLA-B27 transfectant (C1R-B27) [28] were propagated according to classical protocols already reported.

Generation of MD-DC, NK, monocytes, PBL and B cells

Human PBMC were isolated by Ficoll density centrifugation (Ficoll-Paque, Amersham Pharmacia Biotech AB, Uppsala, Sweden) from cytapheresis buffy coats from normal volunteers according to the institutional guidelines. Half of

isolated cells were frozen in freeze medium (Origène, IGEN International, Gaitherburg, MD). The second half of PBMC were immediately used to generate MD-DC. The percentage of $CD14^+$ cells was determined by flow cytometry and the concentration of cells was adjusted at 2×10^6 $CD14^+$ cells/ml in AIM-V medium (Life Technologies, Cergy-Pontoise, France) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 mug/ml streptomycin (Life Technologies) herein referred to as complete medium. Such $CD14^+$ cell concentration allowed the recovery of $0.5 \pm 0.2 \times 10^6$ MD-DC/well at day 8. Cells were then plated in 24-well plates (1 ml/well) and incubated for 1-2 hours at 37° C. The adherent cells were washed twice and cultured in complete medium supplemented with 1,000 U/ml human GM-CSF (Leucomax, Schering-Plough, Levallois-Perret, France) and 50 U/ml human IL-13 (Sanofi Recherche, Labege, France) or 1,000 IU/ml of rhu IL-4 (Schering Plough, Kenilsworth, NJ). This supplemented medium was changed on day 3 and 5 of culture. For the induction of maturation, cultures of MD-DC were supplemented with 10 mug/ml LPS (Sigma Chemical Co., St Louis, MO) on day 6 of culture for 48 hours before use.

Purified (85-90% $CD56^+/CD3^-$) resting NK cells were obtained from PBL by magnetic cell separation (MidiMacs, Miltenyi Biotech, Malmä, Sweden) using a two step protocol: PBMC were first incubated with purified anti-CD3, anti-CD14, anti-CD19 mAbs (Becton Dickinson) for 30 min followed by negative selection with goat anti-mouse microbeads (Miltenyi Biotech).

Monocytes and PBL were obtained from the frozen batch of autologous PBMC the day of co-culture assays. The cells were thawed in medium supplemented with 625 U/ml DNase (Sigma Chemical Co.), washed and adjusted at a concentration of 1.5×10^6 $CD14^+$ cells/ml. 0.5 ml was then plated in 24-well plates and incubated for 1-2 hours at 37° C. The adherent monocytes were washed twice and 0.5 ml of medium was added in wells. In some experiments, monocytes were collected using a cell scraper, and the purity of the generated $CD14^+$ population was found to be 85-95% as determined by flow cytometry. The absolute number of monocytes/well was $0.5 \pm 0.1 \times 10^6$/well. The nonadherent PBL were collected, resuspended at a concentration of 1×10^6/ml, and plated in 24-well plates (0.5 ml).

B cells were isolated from PBL by magnetic cell separation using a B cell isolation kit (Miltenyi Biotech) according to the manufacturer's instructions. 0.5×10^6 B

cells were then plated in 24-well plates in 0.5 ml. The purity of the isolated $CD45^+/CD19^+$ cells was found to be 90-95% as determined by flow cytometry.

Co-culture assays

For functional assay, NK cell lines were deprived from IL-2 for 48 hours, then collected, washed and resuspended in IL-2 free NK cell medium at a concentration of 1×10^6/ml. Alternatively, purified resting bulk NK cells from healthy volunteers were used in the co-culture with DC without exogenous cytokines. 0.5 ml of the NK cell suspension was plated in 24-well plates containing 0.5 ml of medium, MD-DC, monocytes, PBL or B cells. The optimal ratio of stimulating DC to effector NK cell for co-culture was found to be 0.5:1 to 1:1. In some experiments, neutralizing anti-IL-12 (goat IgG), anti-IL-15 (mouse IgG1) and anti-IL-18 (mouse IgG2a) (R&D Systems Inc., Minneapolis, MN) or isotype matched mAbs were added to co-cultures or single cultures at 10 mug/ml each, a concentration which represented 15 to 200 times (depending on the Ab) the neutralizing $dose_{50}$ determined by the manufacturer. For transwell experiments, NK cells were cultured in 24-well plates equipped with a transwell insert (Costar, Cambridge, MA). MD-DC were in the lower chamber in 0.6 ml culture medium. NK cells were added at a concentration of 5×10^6/ml in 0.1 ml either together with MD-DC in the lower chamber or by themselves in the upper chamber of the transwell plates.

For NK cell immunophenotyping studies, single NK cell or DC cultures and DC/NK cell co-cultures with or without LPS were analyzed according to size and granulosity and after 3 color staining using anti-CD69-FITC, anti-CD25-PE mAb and anti-CD56 PerCP (Becton Dickinson) in a FACSCalibur (Becton Dickinson, San Jose, CA).

For proliferation studies, similar protocols were followed with the exception that NK cell lines were not deprived from IL-2 before co-culture assays. Half of the co-culture was renewed daily for 6 days using IL-2 free NK cell medium. NK cells were labeled with 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) as previously described [29]. Briefly, NK cells were resuspended at 5×10^6/ml in RPMI-1640, without serum, and incubated with CFSE (5 muM) for 10 min at 37° C. Unincorporated dye was immediately quenched by adding an equal volume of FCS. Cells were then extensively washed before performing co-culture assays.

Proliferation and survival assays

Six day co-cultures (DC/NK or NK alone) were collected and viable trypan blue-excluded cells counted. The CFSE content was monitored on viable cells, selected by propidium iodide, using a FACSCalibur (Becton Dickinson). For the cell viability, the percentage of NK cells was evaluated by flow cytometry as the percentage of CFSE positive cells. For experiments using non CFSE labeled cells, the percentage of $CD2^+/CD3^-$ viable NK cells was determined after staining with a PE-conjugated anti-CD2, and a FITC-conjugated anti-CD3 mAbs (Becton Dickinson). The absolute number of viable NK cells was calculated based on the total number of viable cells and the determined percentage of NK cells.

NK cell functional assays

For cytotoxicity assays, cells were collected after 18 hours of co-culture. Viable trypan blue-excluded NK cells were counted and used as effector cells. Cytotoxicity of NK cells was measured in a standard 4 hours ^{51}Cr-release assay using $Na_2{}^{51}CrO_4$-labeled K562, C1R, C1R-B27 cells, monocytes or MD-DC as targets. Experiments were conducted in triplicate at various E:T ratios. For human IFN-gamma ELISA, the supernatants from co-cultures or single cultures were collected after 18 hours and assayed in commercial ELISA for human IFN-gamma (PharMingen, San Diego, California, USA). The detection limit of this assay was 4.7 pg/ml.

Statistical analyses

Statistical analyses of experimental results were performed using the Student's two paired t test.

RESULTS

MD-DC induce enhancement of NK cell line functions

We first investigated whether DC could maintain or enhance IL-2 deprived-NK cell line cytolytic activity against K562 or C1R targets. DC generated from monocytes in GM-CSF/IL-13 (or GM-CSF/IL-4) for 8 days exhibited the classical phenotypic features of immature DC *i.e.* expression of CD1a, HLA-DR dim, low levels of CD40, CD80, CD86 and no lineage specific markers (CD3, CD14, CD20,

CD56). Following an incubation with LPS for 48 hours, flow cytometry analysis revealed an enhanced expression of HLA-DR, CD40, CD80 and CD86 (data not shown). When the two IL-2 dependent-NK cell lines NKL or NK-92 cells were co-cultured for 18 hours at a ratio of 1:1 with immature MD-DC in the absence of IL-2, a significant increase of NK cell cytolytic activity was detected against the NK sensitive target K562 (Figure 1A). Similarly, co-culture of NK cells with LPS-stimulated DC at the same ratio promoted potent NK cell activation (Figure 1B). The MD-DC population cultured alone at a similar concentration did not yield substantial K562 lysis. To address the question whether MD-DC-mediated NK cell activation would overcome an inhibitory signal, we tested the lytic activity of the LIR-1/ILT-2 expressing NKL cells alone or following co-culture with MD-DC against C1R or C1R-B27 cells. C1R-B27 is a target cell expressing the MHC class I molecule HLA-B27 known to be the ligand for the inhibitory receptor LIR-1/ILT-2 [30]. While co-culture of NKL cells with DC promoted an increased lysis of C1R targets, it did not allow NKL cells to circumvent the HLA-B27 mediated-inhibitory signal since no significant increase in their cytolytic activity against the C1R-B27 cells was achieved (Figure 1C). Interestingly and in line with our previous mouse data [25], the co-culture of IL-2 deprived-NK cells with DC trigger the IFN-gamma production from NK cells *in vitro* (data not shown).

Flow cytometry analyses of NKL and NK-92 cells following a co-culture for 18 hours with MD-DC did not highlight the modulation of expression of activating or inhibitory receptors such as CD161 (NKRP1-A), CD85 (LIR-1/ILT-2), NKG2-A, CD94, 2B4 and NKp46 (data not shown).

In these experiments, the absolute number of NK cells following co-culture with MD-DC for 18 hours and up to 6 days was significantly higher compared with NK cells cultured alone (Figure 2A). As for the DC-mediated increase of NK cell cytotoxicity, the induction of DC maturation did not modify the NK cell recovery following co-culture of NK cells with DC (Figure 2A). Similar results were obtained with the NKL cell line (data not shown). On average, NK cell recovery at day 6 was 30% *versus* 1% of the initial cell input, in the presence or absence of DC respectively. To further evaluate whether this enhanced NK cell recovery involved cell division or prolonged cell survival, NK cells were labeled with the fluorescent dye CFSE. The co-culture of NK-92 cells for 6 days with MD-DC stimulated or not with LPS resulted in NK-92 cell proliferation (Figure 2B). In contrast, NK cells did not divide when cultured separately at the same concentration. Similar results were obtain-ed with the NKL cell line (data not

shown). NK cell proliferation in the presence of MD-DC for 6 days was associated with an increased cytolytic activity (data not shown).

Therefore, MD-DC are able not only to enhance the effector functions of IL-2 deprived-NK cell lines but also to induce their proliferation in the absence of exogenous cytokines.

Direct cell contact requirements

To determine the involvement of soluble factors in the MD-DC-mediated NK cell triggering, we compared the cytolytic activity of NK cells recovered following co-culture with or separated from MD-DC by a porous membrane (transwells). As shown in Figure 3A, the enhanced cytolytic activity of NK cells was detected only when NK cells were co-cultured in close contact with immature MD-DC but not when the cells were separated by a porous membrane, indicating that the DC-mediated NK cell activation required a direct cell-to-cell contact. Similar results were obtained with LPS-stimulated MD-DC (data not shown). However, such settings do not preclude a role of NK cell stimulating factors such as IL-12, IL-15 and IL-18. In order to formally test the involvement of these cytokines in the DC-mediated NK cell activation, NK cells were co-cultured with immature MD-DC in the presence of different combinations of neutralizing anti-IL-12, anti-IL-15 and anti-IL-18 or isotype matched mAbs. These cytokines did not play a major role since concomitant neutralization of all of them did not inhibit increase of NK cell cytolytic activity following co-culture with MD-DC (Figure 3A).

While the separation of DC from NK cells in a transwell completely prevented the DC-mediated NK cell cytolytic activity, it only partially abrogated the NK cell proliferation induced by DC. These data suggest that both, a cell to cell contact and soluble factors are directly involved in the DC triggering of NK cell proliferation. Indeed, the absolute number of viable NK-92 cells recovered following co-culture with DC through a porous membrane was higher than that achieved without DC but lower than the number of NK cells recovered following co-culture in close contact with MD-DC, stimulated or not with LPS (Figure 3B). This decreased absolute number of NK-92 cells was associated with a reduced cell proliferation as determined by the monitoring of the CFSE content (data not shown).

Thus, the activation of NK cell cytolytic activity by MD-DC was dependent on a direct cell-to-cell contact. By contrast, this direct intercellular interaction was not a strict requirement for DC-mediated NK cell proliferation but was required for an optimal cell division.

Myelomonocytic cells trigger NK cell activation

To investigate the specificity of the MD-DC regulatory effects on NK cell lines, we evaluated the effects of other hematopoietic cells on NK cell cytolytic activity and proliferation. Results indicated that monocytes were as potent as MD-DC at similar co-culture ratios in increasing NK cell cytolytic activity against C1R (Figures 4A, B), and in inducing their proliferation (Figure 4C). However, these effects were specific of the myelomonocytic lineage since neither B cells nor whole peripheral blood lymphocytes (PBL) could stimulate NK cell lytic activity (Figure 4B) or proliferation (Figure 4C). Myeloid APC can therefore enhance NK cell functions *in vitro*.

Since co-culturing NK cells with NK-sensitive targets might trigger their effector functions [4], it was important to determine whether MD-DC are lysed during the co-culture. As shown in Figure 4A (inset), MD-DC and monocytes, in contrast to C1R, did not represent targets for NK cells in our system. Moreover, the co-culture of NK cell lines with an NK-sensitive target such as K562 did not induce NK cell line lytic activity (Figure 4B). Thus, the DC-mediated NK cell activation could not be accounted for by the recognition of target cells during the co-culture.

Resting bulk NK cells are only triggered by activated or mature DC

Since it was shown in a mouse model system that DC but not macrophages selectively trigger NK cell functions [25], we postulated that the requirements for NK cell activation in NK cell lines might differ from those of bulk resting NK cells. Therefore, we next investigated the DC/NK cell cross talk using purified bulk NK cells from human healthy volunteers. Following purification from peripheral blood, bulk NK cells displayed a resting phenotype *i.e.* did not express CD25 nor CD69, did not spontaneously produce IFN-gamma, did not lyse DAUDI cells. Interestingly, follo- wing a co-culture of 24 hours with allogeneic MD-DC, NK cells became capable of lysing DAUDI cells (Figure 5A) and producing high levels of IFN-gamma (Figure 5B) but only when such allogeneic MD-DC were

phenotypically mature following a stimulation of 24-48 hours in 10 mug/ml of LPS. Mature MD-DC expressed CD83 and displayed higher levels of MHC class II and CD86 molecules associated with stronger allostimulatory capacities (data not shown) compared to their immature counterparts. Indeed, immature MD-DC could not trigger resting NK cell functions (Figure 5). At the onset of an inflammatory stimulation, both DC and NK cells might be recruited concomitantly and interact to initiate innate immune responses. Therefore, we investigated the DC/NK cell cross talk in the presence of LPS. LPS was added during the 24 h-co-culture of immature MD-DC with resting bulk NK cells. Indeed, most NK cells turned into $CD25^+/CD69^+$ double positive cells in the presence of LPS while remaining in the resting phenotype in the absence of LPS (Figure 6A). In accordance with this activated phenotype, resting NK cells in the presence of MD-DC+LPS exhibited potent cytolytic activity (Figure 6B) against DAUDI and produced high amounts of IFN-gamma (Figure 6C). In the absence of MD-DC, LPS did not induced NK cell activation (data not shown). These interactions required a cell-to-cell contact since NK cells remained in a resting state when physically separated from MD-DC stimulated with LPS (transwell experiments not shown). Following activation by DC in the concomitant presence of LPS, NK cells, in turn enhanced LPS-induced DC activation. Indeed, control MD-DC maintained for 24 hours in LPS slightly up-regulated CD86 cell surface expression that could be further enhanced in the presence of NK cells [31]. Moreover, activated DC-mediated NK cell triggering was found not only in allogeneic but also in autologous settings, in normal volunteers and neuroblastoma bearing patients [31].

Altogether, following a stress of inflammation, DC acquire the capacity to promote NK cell effector functions.

DISCUSSION

At ports of entry, dendritic cells and NK cells are cornerstones modulating innate immune responses *in vivo*. The present study investigated the dynamics between these two players in human *in vitro* model systems. Firstly, in the absence of an ongoing inflammatory stimulation, immature DC do not trigger resting NK cell effector functions. In contrast, in the presence of LPS or in the presence of mature DC, resting NK cells up-regulate the CD25 and CD69 molecules, produce IFN-gamma and lyse DAUDI cells. Interestingly both immature or mature DC could maintain or enhance NK cell effector functions and

proliferation of IL-2 activated NK cell lines. We showed that a direct cell-to-cell contact was strictly required for triggering NK cell cytolytic activity and necessary for their optimal expansion. Importantly, these data have been extended by experiments using autologous cells derived from children with metastatic neuroblastoma. In this study, immature MD-DC were able to maintain, in the absence of exogenous cytokines, the long term survival of autologous lymphokine activated-killer cells ($CD56^+/CD3^-$) and their high cytolytic activity against both classical NK sensitive targets and neuroblastoma cell lines for up to 6 days [31].

Several cytokines such as type I IFN [32], IL-12 [33], IL-15 [10], IL-18 [13] and IL-21 [12] are potent inducers of NK cell activation, and some of them are known to be secreted by human DC following maturation stimuli [8, 22]. In a recent report, it has been suggested that the increased NK cell cytotoxicity following co-culture of NK cells with DC was partially dependent on soluble factors but not IL-12 or IL-18 [34]. A very recent study has suggested that NK activation by DC is not dependent on type I IFN [35] and our results showed that the incubation of DC/NK co-culture with anti-IL-12, anti-IL-15 and anti-IL-18 mAbs did not reduce the NK cell lytic activity (Figure 3A). In accordance with very recent reports [35-37], our results support the idea that a direct cell-to-cell contact is necessary for DC-mediated NK cell activation. In this respect, we found that the co-culture of NK cells and DC separated by a porous membrane completely abolished the NK activation (Figure 3A). Thus, although it remains possible that DC-derived soluble factors might be necessary to drive full NK cell activation, a direct interaction between the two cell types is a mandatory pathway for NK cell triggering by DC.

Interestingly, we were also able to demonstrate that MD-DC can induce the proliferation of two NK cell lines in the absence of exogenous cytokines (Figure 2B and data not shown). The partial, but significant inhibition of MD-DC-mediated NK proliferation in transwell experiments (Figure 3B) indicates that direct cellular interactions, although not mandatory, are required for an optimal NK cell proliferation. Among the soluble factors that could be involved in the DC-mediated NK cell proliferation, IL-21 and IL-15 represent likely candidates. The NK-92 cell line express the IL-21 receptor [12]. When deprived of IL-2, the NKL cell line express high levels of the alpha and beta components of IL-2 receptor [26], both utilized by IL-15 [10] and, in contrast to many others cytokines, IL-15 alone does support the growth of NK cells [38]. Thus, DC could substitute for the classical

EBV cell line based-feeder to support growth and differentiation of NK cell lines *in vitro*.

In order to assess whether the modulation of NK cell functions was selectively induced by DC or could be attributed to a non specific cell-to-cell interaction, we tested other hematopoietic cells. The modulation of NK cell functions was clearly specific of myeloid APC since B cells (Figure 4B) or unfractionated PBL (Figure 4C) were unable to up-regulate NK cell functions. However, our results indicate that not only DC but also monocytes were capable of triggering NK cell cytolytic activity (Figure 4A, B) as well as their proliferation (Figure 4C). In contrast with this result, a very recent study showed that monocytes did not activate NK cell functions [36]. This apparent discrepancy might be explained by the use of IL-2 deprived NK cell lines in our study whereas resting NK cells were used in the other study. Indeed, in the absence of a concomittant inflammatory stimuli, the DC/NK cell cross-talk did not lead to the enhanced cytolytic activity nor IFN-gamma production of freshly isolated NK cells (Figures 5 and 6 and [37]). In contrast, in the study of Ferlazzo et al. [36], the enhanced lytic activity of NK cells cultured with immature DC was observed even in the absence of LPS. This difference might be accounted for by the DC/NK cell ratio (1/10 in Ferlazzo's work) and the time lag of the coculture (7 *versus* 2 days).

The molecular mechanisms underlying the DC/NK cell interactions remain to be elucidated. A simple explanation could be that the NK activation is due to the recognition of MD-DC as target cell. However, we were able to exclude this possibility in that 1) DC were not targets for IL-2 deprived NK cell lines (Figure 4A, inset) and 2) NK cell targets such as K562 cells could not trigger NK cell cytolytic activity (Figure 4B). MD-DC can enhance IL-2 dependent NK cell line as well as autologous lymphokine activated-NK cell effector functions [31] indicating that the effect of MD-DC was not due to MHC mismatching between DC and NK cell lines *i.e.* lack of appropriate MHC class I specificity for the NK cell inhibitory receptors. It has been shown that the expansion and proliferation of NK cells following LPS stimulation [39] or co-culture with mature DC [40] required CD80/86 costimulation although the role of these molecules in NK cell activity remains controversial [41]. Moreover the role of these molecules in our system is unlikely since the NKL cell line does not express CD28 [26]. We found that the B7/CD28 interaction was not critical in the mouse DC/NK cell cross talk. Among the potential molecules that could participate in the DC/NK cell interaction, the CD40/CD40L interaction has been shown to be partially involved in the mature

DC-induced NK cell proliferation [40]. Other candidates could be the adhesion receptors as a very recent study has shown that the NK cell lysis of DC is mediated by the LFA-1-dependent activation of the calmodulin kinase II [42]. However a very recent study failed to demonstrate a role for the CD40L and the LFA-1 molecules in the DC-mediated NK cell activation [35]. It is possible that different receptors may be used depending upon the activation state of the NK cell and the availability of the relevant ligands at the surface of the stimulating cells [43]. It is conceivable that the DC/NK cell interactions allow the down-modulation of inhibitory receptors or up-regulation of activating receptors and/or costimulatory molecules. However our preliminary phenotypic analyses of NK cells did not reveal any significant down- or up-regulation of such receptors following co-culture with DC. It is noteworthy that the DC-mediated NK cell activation did not allow NKL cells to overcome the C1R-B27-mediated MHC class I inhibitory effects (Figure 1C).

It is conceivable that following a stress in the peripheral tissues, DC and NK cells could be both recruited and interact together at sites of injury. Indeed, the DC/NK cell dynamics leading to NK cell activation was optimal in the presence of LPS or mature DC (Figure 6). Similarly, mouse bone marrow derived-DC interact with liver tissue resident NKT cells and keep them in check at an immature stage through inhibitory receptor/H-2 molecule interaction. Following inflammation, DC polarize NKT cells towards Th1 secretion pattern in a B7/CD28 dependent manner [44]. In the presence of a concommitant inflammatory stimulus (LPS, type I IFN, mycobacterium tuberculosis) the DC/NK cell cross talk leads to not only NK cell activation but also DC maturation [35, 37]. Indeed, DC acquire enhanced expression levels of costimulatory molecules and membrane MHC class II localization but also the CC chemokine receptor 7 and produce large amounts of TNF-alpha. Interestingly, the DC/NK cell cross-talk, when dealing with pre-activated NK cells, leads to either DC activation or DC death, depending on the ratio between DC and NK cells [35]. The *in vivo* relevance of these findings remains to be determined, as discussed by L. Zitvogel [45].

In contrast to tissue resident NKT cells, NK cells need to be recruited from blood. DC and NK cells respond to similar chemokines such as macrophage inflammatory protein (MIP)-1alpha, monocyte chemotactic protein (MCP)-1 and macrophage-derived chemokine (MDC) [5, 46-49]. In viral models, APC and NK were found in close contact in spleens of virally infected mice [50]. At the onset of a tumor, activated NK cells were found infiltrating the deposits at early stages

of development [3], as well as immature DC [51]. Moreover, we reported in a mesothelioma model that the interaction between NK cells and DC resulted in NK cell activation [25]. Once NK cells are activated, two scenarios could take place, i) a negative feed back with the down-modulation of NK effectors or ii) the maintenance and/or amplification of NK cell functions. The down-modulation of NK cell functions could occur following interactions with their targets expressing ligands for inhibitory receptors [1] associated with anergy or apoptosis [52]. However, NK cells, as important links between innate and cognate immunity, should be able to trigger and sustain local immune responses. This prolonged efficiency could imply a proliferation phase following the activation step. Our data support that the sentinel DC play a regulatory role in inducing NK cell proliferation and maintaining their effector functions.

CONCLUSION

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Figures

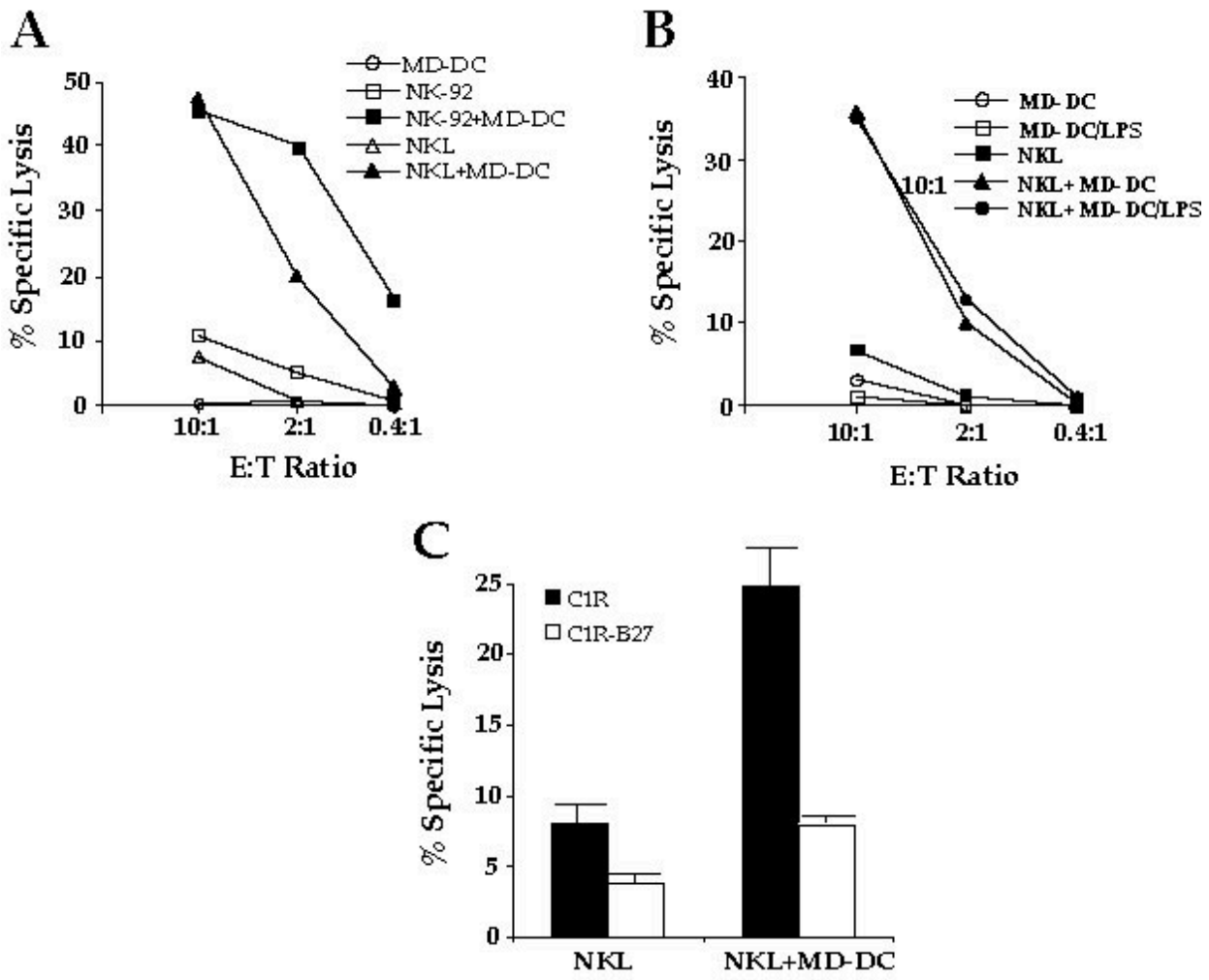


Figure 1

Activation of NK cell effector functions by MD-DC stimulated or not with LPS. Forty-eight hours IL-2 deprived-NK-92 and NKL cells were cultured for 18 hours either alone without IL-2 or with immature MD-DC (MD-DC) or with LPS-stimulated MD-DC (MD-DC/LPS) at a ratio of 1:1. The cytolytic activity of NK cells was tested against K562 cells **(A** and **B)**, C1R and C1R-B27 cells **(C)**. Results are expressed at various E:T ratios **(A** and **B)** or at a ratio of 2:1 **(C)** and represent the means of triplicate wells. s.e.m. were consistently less than 10% of means. The results correspond to one representative experiment out of three to five.

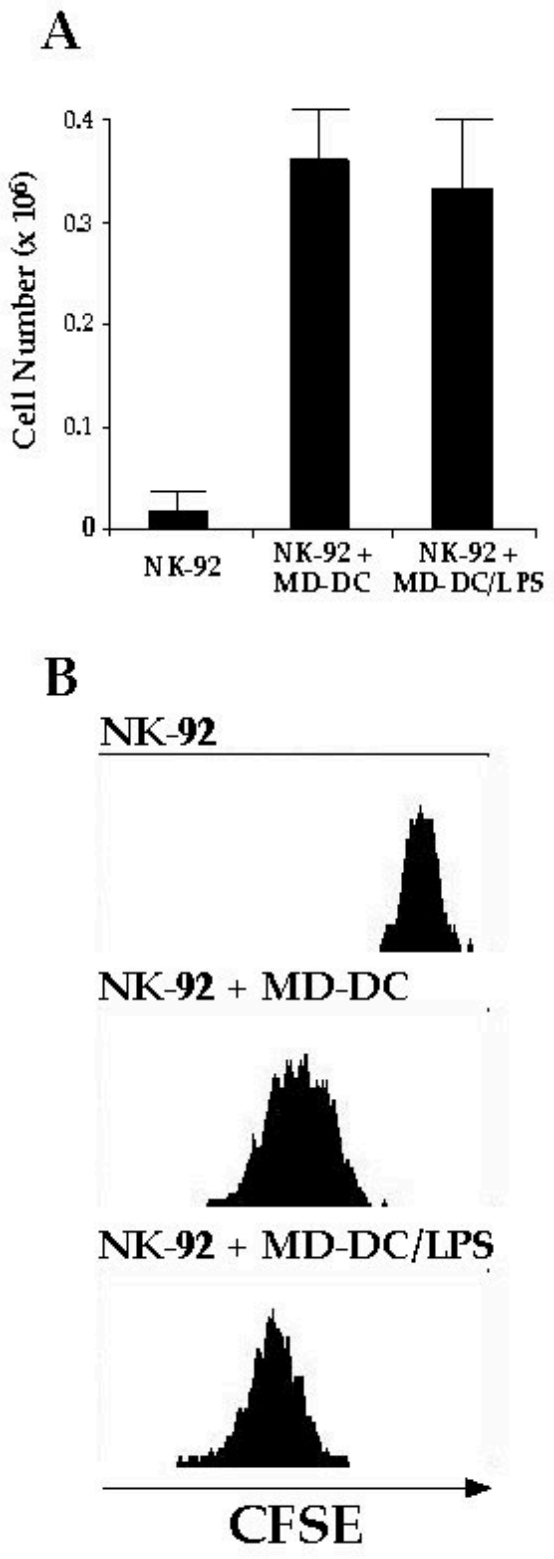


Figure 2

Induction of NK cell proliferation by immature or mature MD-DC. NK-92 cells were labeled with CFSE and co-cultured with immature MD-DC (MD-DC) or mature MD-DC (MD-DC/LPS). After 6 days, the overall number of viable cells was determined and the CFSE content was

analyzed by flow cytometry. **A:** NK-92 absolute cell number was determined as described in Methods. **B:** Histograms are gated on viable NK cells and show the CFSE intensity. Similar results were obtained in three independent experiments.

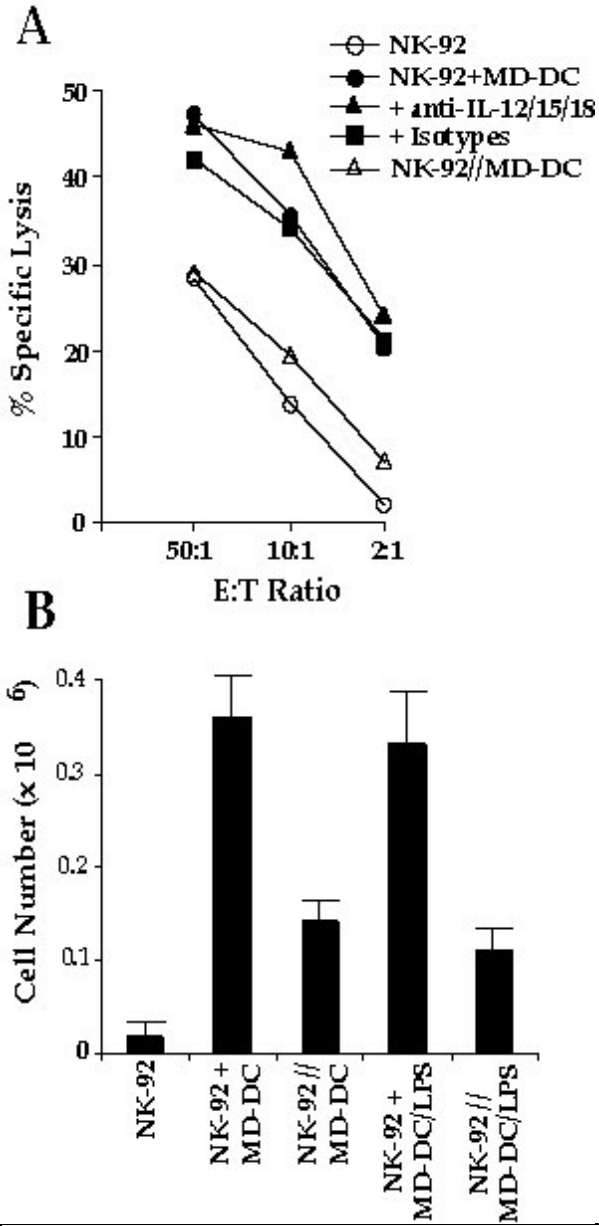


Figure 3

Cell contact requirement of the DC-mediated NK cell activation. A: 48 hours IL-2 deprived-NK-92 cells and immature MD-DC were cultured either together (NK-92 + MD-DC) in the same wells in absence or in presence of neutralizing anti-IL-12, anti-IL-15 and anti-IL-18 or isotype

matched mAbs (10 mug/ml each) or separated by a porous membrane (NK-92//MD-DC) at a ratio of 1:1. After 18 hours, viable NK cells were tested against C1R cells in a cytotoxicity assay. Results are shown at various E:T ratios and represent the means of triplicate wells. s.e.m. were consistently less than 10% of means. Results correspond to one experiment repeated once with similar data. **B:** NK-92 cells and immature MD-DC (MD-DC) or LPS-stimulated MD-DC (MD-DC/LPS) were cultured either together (NK-92 + MD-DC+/- LPS) in the same wells or separated by a porous membrane (NK-92//MD-DC+/-LPS) at a ratio of 1:1. After 6 days, the overall number of viable cells was determined, cells were stained with a PE-conjugated anti-CD2 and a FITC-conjugated anti-CD3 mAbs and analyzed by flow cytometry. The absolute number of viable NK-92 cells was determined as described in Methods. This figure is representative of three independent experiments.

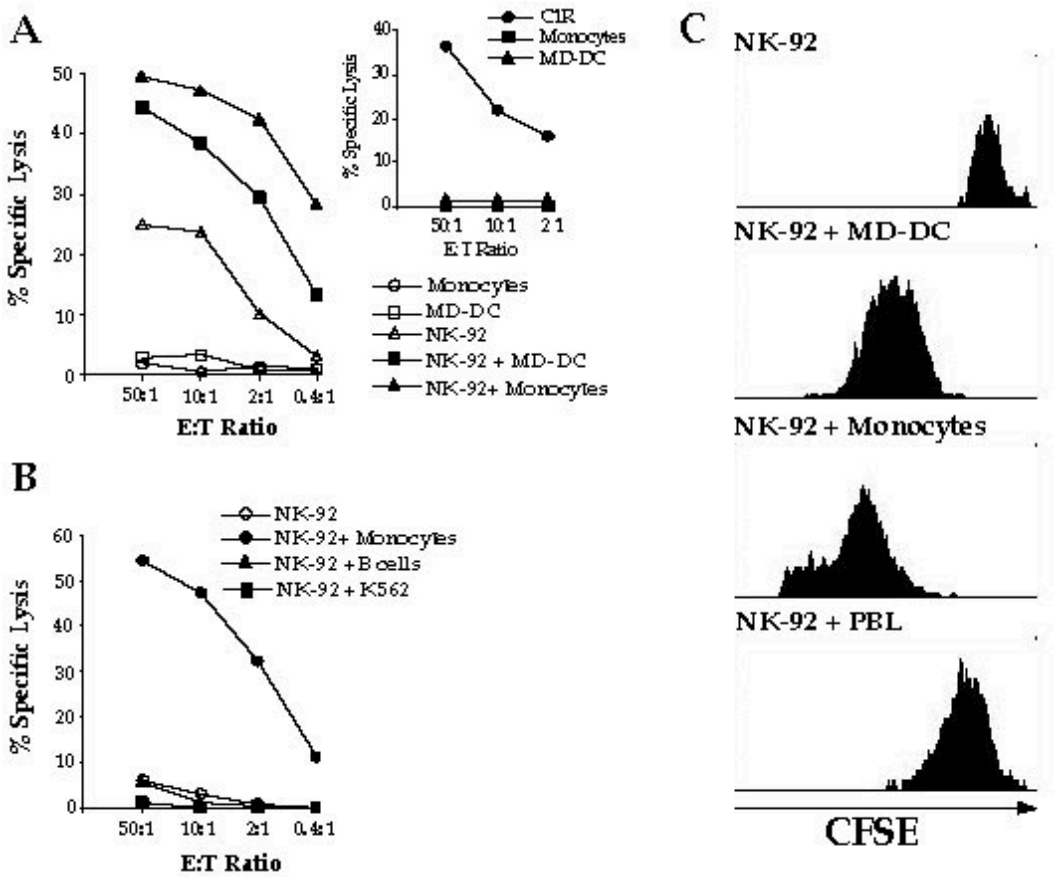


Figure 4

Specificity of the DC/NK cell interaction. Forty-eight hours IL-2 deprived-NK-92 cells were co-cultured with either immature MD-DC **(A)**, monocytes **(A** and **B)**, B cells **(B)** or K562 cells **(B)**. The lytic activity of NK cells against C1R cells was tested 18 hours later by a chromium release assay. **Inset:** the lytic activity of NK-92 cells was tested before co-culture against the immature MD-DC and monocytes used for the co-culture assay and C1R cells. Results are expressed as a percentage of specific lysis at various E:T ratios and represent the means of triplicate wells. s.e.m. were consistently less than 10% of means. Data represent one out of two independent experiments achieving similar results. **C:** NK-92 cells were labeled with CFSE and co-cultured with immature MD-DC, monocytes or PBL at a ratio of 1:1. After 6 days, the overall viable cell number was determined for each culture condition, and the CFSE content was analyzed by flow cytometry. Histograms are gated on viable NK cells and show the CFSE intensity. Similar results were obtained in two independent experiments.

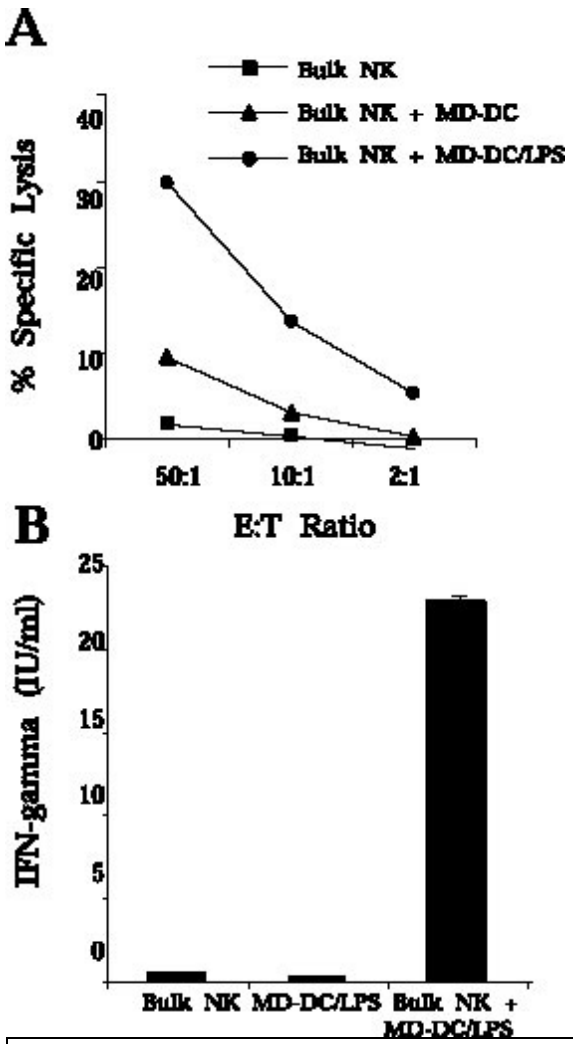


Figure 5

Immature and mature DC differentially activate resting bulk NK cells. Immature day 7 MD-DC, or MD-DC induced to mature in LPS from day 5 to day 7, were co-cultured for 24 hours in the presence of allogeneic resting purified bulk $CD3^-/CD56^+$ NK cells in 96 well plates. **A:** The lytic activity of the co-culture against DAUDI cells was assayed at various E:T ratio. **B:** The IFN-gamma levels of the co-culture supernatants were tested in ELISA.

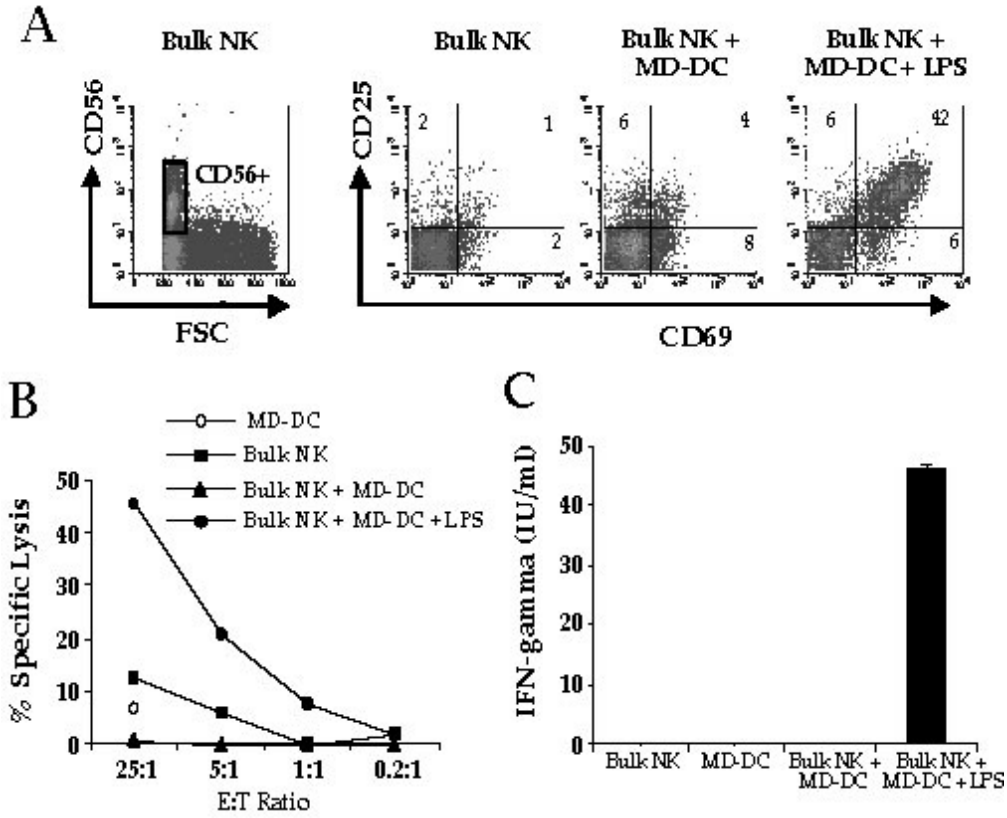


Figure 6

Dynamics of the DC/NK cell interaction during ongoing LPS stimulation. Immature MD-DC and resting bulk $CD3^-/CD56^+$ NK cells were co-cultured in the presence or absence of LPS for 24 hours. **A:** Phenotypic analyses of gated NK cells in the co-culture revealed a strong up-regulation of CD25 and CD69 in the presence of MD-DC + LPS. **B:** The lytic activity of the co-culture in the presence or absence of LPS against DAUDI cells was assayed at various E:T ratio. **C:** The IFN-gamma levels of the co-culture supernatants were tested in ELISA.

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all-trans-retinoic acid, 13 Cis retinoic acid, interferon-alpha, cervical squamous cell carcinoma.

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CSF-1, tumour progression, metastasis, de-differentiation.

The effect of interleukin-1beta (IL-1beta) and tumor necrosis factor-alpha (TNF-alpha) on granulocyte

macrophage-colony stimulating factor (GM-CSF) production by neuronal precursor cells

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IL-1beta, TNF-alpha, GM-CSF, neuron, mRNA, protein.

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