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The Marginating-Pulmonary Immune Compartment in rats: Characteristics of continuous inflammation and activated NK cells

Rivka Melamed¹, Ella Rosenne¹, Marganit Benish¹, Yael Goldfarb¹, Ben Levi¹, and Shamgar Ben-Eliyahu^{1,2}

¹ Neuroimmunology Research Unit, Department of Psychology, Tel Aviv University, P.O. Box 39040, 69978 Tel Aviv, Ramit Aviv, Israel

² Outcomes Research(TM) Institute, Louisville, KY, USA

Abstract

A significant role has been indicated for cellular immunity in controlling circulating cancer cells, but most autologous tumor cells seem resistant, in vitro, to NK cell (NKC) and CTL cytotoxicity. Addressing this apparent contradiction, we recently identified a unique leukocyte population, marginating-pulmonary (MP)-leukocytes, which exhibit potent NK cytotoxicity. Here we characterize the MP-compartment in naïve and immunostimulated rats, and assessed its cytotoxicity against “NK-resistant” tumors cells. Animals were treated with poly I-C (3×0.2 mg/kg) or saline, and circulating- and MP-leukocytes were collected and analyzed in terms of cellular composition, cellular activation markers, and NK cytotoxicity of leukocytes and purified NKCs. Compared to circulating-leukocytes, MP-leukocytes showed greater proportion of granulocytes, monocytes, NKCs, and large NKCs; higher expression of activation and adhesion markers (CD25, CD11a, CD11b, and NKR-P1, IFN γ); and elevated NK cytotoxicity of leukocytes and purified NKCs against several syngeneic and xenogeneic NK-resistant target cells (from both F344 and BDX inbred rats). In immunostimulated animals (treated with poly I-C), but not in naïve animals, purified NKCs from the MP-compartment showed markedly superior cytotoxicity, suggesting that poly I-C immunostimulation uniquely affect MP-NKCs, and that in naïve animals other MP-leukocytes support NK cytotoxicity. Overall, the results suggest that the MP-compartment is characterized by a continuous activated inflammatory microenvironment uniquely affected by immunostimulation. If similarly potent MP-NKCs exist in patients, then circulating autologous tumor cells that are considered “NK-resistant” could actually be controlled by MP-NKCs. Innate immunity may assume greater role in controlling malignant spread, especially following immunostimulation.

Keywords

Natural Killer Cells; Cell activation; Tumor Immunity; Lung; Rat

Introduction

Elements of cellular immunity interact with malignant tissue, and can control circulating cancer cells and residual disease even if ineffective against the primary tumor. Specifically, studies in animals and humans have indicated that immune control over circulating tumor cells and micrometastases is carried mainly through cell-mediated immunity (CMI), including CTL,

Address correspondence and reprint requests to: Dr. Shamgar Ben-Eliyahu, Dept of Psychology, Tel Aviv University, Tel Aviv, 69978 Israel, Tel: 972-3-640-7266, Fax: 972-3-640-9520/9547, shamgar@post.tau.ac.il.

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NKT cells, macrophages, DC, and NKC [1,2]. Molecular mechanisms of tumor recognition by NK cells have been recently revealed, and a role for NK cells in controlling leukemia and metastases in humans has received substantial support [3-5].

However, most tumor cells excised from patients and animals are thought to be resistant to NK cells, as circulating leukocytes fail to exhibit significant NK cytotoxicity against these autologous tumor cells [6-8]. Included among such NK-resistant tumors in the inbred Fischer 344 (F344) rat, is the MADB106 tumor line, a non-immunogenic syngeneic mammary adenocarcinoma. MADB106 meets the in vitro criterion of being NK-resistant, as circulating-leukocytes, splenocytes, PBMCs, or leukocytes harvested from the bone marrow [6,9,10] or the lung interstitial and the alveoli compartments (unpublished data from our laboratory), fail to exhibit significant in vitro NK cytotoxicity against MADB106 tumor cells in a standard 4h Cr⁵¹ release assay. However, MADB106 tumor cells are clearly controlled by NK cells in vivo. Earlier work indicated that NK depletion (using the anti-Asialo mAb) elevated MADB106 lung tumor retention and lung metastases, and adoptive transfer of LGL/NK cell restored normal in vivo resistance [6,7]. More recent studies employing selective depletion of NK cells using anti-NKR-P1 mAb (anti-CD161) [11,12], indicated that selective in vivo NK-depletion increased MADB106 lung tumor retention and lung metastases more than 100-fold in both naïve and poly I-C (the immunostimulatory agent polyriboinsinic acid-polyribocytidylic acid) treated rats, and reduced in vivo MADB106 destruction [13-16].

The apparent contradiction between the in vitro resistance of MADB106 tumor cells to NK cytotoxicity, and the marked in vivo susceptibility of these tumor cells to destruction by NK cells prompted us to search for other leukocyte populations that could effectively lyse MADB106 tumor cells in an NK dependent manner. We suspected that a unique population of NK cells or a supporting microenvironment exists in vivo, enabling NK cells to act similarly to in vitro activated lymphocytes (LAK): recognizing what are considered NK-resistant tumor cells, and exhibiting greater cytotoxicity than non-activated NK cells.

Indeed, in our recent studies we reported that a unique leukocyte population – marginating-pulmonary (MP)-leukocytes (leukocytes adhering to the lungs vasculature) – exhibit marked in vitro NK cytotoxicity against the MADB106, and could be responsible for the in vivo destruction of MADB106 tumor cells [17-20]. This MP-leukocytes population also showed an improved capacity to lyse standard xenogeneic YAC-1 target cells [17].

In this study we aimed to (i) test whether the observed enhanced MP-NK cytotoxicity would also occur against other tumor lines and in an additional strain of inbred rats, and (ii) to screen for potential mechanisms through which MP-leukocytes exhibit increased NK cytotoxicity against syngeneic “NK-resistant” tumor cells. To this end we characterized the MP-compartment, comparing it to the circulation, in terms of cellular composition, cellular activation markers, and cytotoxicity of non-purified and purified NK cells. Conducting these studies we used naïve rats and rats repeatedly treated with the acknowledged immunostimulatory/pro-inflammatory agents IL-12 or poly I-C. These agents are used in this study to further understand the impact of inflammation and immune-activation in the MP- and circulating-compartments.

Materials and methods

Animals and counterbalancing

F344 male rats were purchased from Harlan laboratories (Jerusalem, Israel). BDX rats were purchased from Charles River (Sulzfeld, Germany). Both strains were used at the age of 14 to 20 weeks old (age matched within each experiment). Rats were housed 4 per cage on a 12:12 light: dark cycle with free access to food and water, were acclimatized to the vivarium for at

least 3 weeks, and were handled daily during the week before experimentation to reduce potential procedural stress. Body weight, the order of drug administration, blood withdrawal, and lung perfusion, were counterbalanced across groups in each experiment, and control animals were injected with saline. All studies were approved by The Institutional Animal Care and Use Committee of Tel Aviv University.

Poly I-C administration

Poly I-C (Sigma, Israel), a synthetic double stranded RNA that induces a viral-like response through activation of TLR3 [21], was dissolved in PBS. Based on our previous studies with this compound [18], rats were injected intra peritoneal (i.p.) with a low dose of poly I-C (0.2 mg/kg) on days 5, 3, and 1 before being sacrificed for harvesting of circulating and MP-leukocytes.

IL-12 administration

Lyophilized Recombinant murine IL-12 (1×10^7 units/mg, purchased from Cytolab Ltd., Rehovot, Israel) was reconstituted in phosphate-buffered saline (PBS) according to manufacturer's instructions. In order to maintain a relatively constant level of circulating rmIL-12, it was administered subcutaneously ($2 \mu\text{g}/\text{rat}$) in a slowly absorbed emulsion [constituting of four parts of PBS, three parts of mineral oil, and one part of mannide-monooleate – a non specific surface active emulsifier (all purchased from Sigma, Israel) [22]. Based on our experience, using various compounds, this emulsion is absorbed in approximately 36 – 48 h.

Tumor Cell Lines

1. MADB106—Is a selected variant cell line obtained from a pulmonary metastasis of a mammary adenocarcinoma (MADB106) chemically induced in the F344 rat. This syngeneic tumor line is considered NK-resistant in vitro [6,7] and metastasizes only to the lungs following i.v. inoculation [7].

2. YAC-1—The standard target cell line for in vitro assessment of NKC cytotoxicity is a murine T-lymphoma cell line sensitive to NK-cells, originally obtained from a tumor induced by Maloney sarcoma virus in A/Sn mice.

3. P815—A mouse mastocytoma blood NK-resistant cell line. The P815 cell line was kindly provided by Dr. Berke Gideon from weizmann institute Israel.

4. CRNK-16—This syngeneic cell-line in F344 rats is derived from a naturally occurring leukemia that is highly malignant and is the major cause of death in aged F344 rats. Morphologically, these leukemia cells resemble large LGL, and express NKRP-1, CD45 and are negative for CD3. CRNK-16 cell line was kindly provided by Dr. W.H. Chambers, from University of Pittsburgh Cancer Institute.

5. BSp73ASML (ASML)—This syngeneic tumor cell line in BDX rats is derived from an adenocarcinoma of the pancreas. The ASML cells line is highly spontaneously metastasize via the lymphatic vessels [23,24]. ASML cell line was kindly provided by Dr. Zöller, from University of Heidelberg, Germany.

6. BSp73AS (73AS)—A nonmetastasizing pancreatic adenocarcinoma of the BDX vessels [23]. 73AS cell line was kindly provided by Dr. Zöller, from University of Heidelberg, Germany.

7. BSp73AS-14 (AS-14)—BSp73AS cells transfected with CD44v4-v7 cDNA [25]. AS-14 cell line was kindly provided by Dr. Zöller, from University of Heidelberg, Germany.

All cells lines were maintained in 5% CO₂ at 37°C in complete medium (CM) and were used as target cells in the in vitro assessment of NK cell cytotoxicity.

Assessment of leukocyte subsets concentration and molecular phenotypes

A standard procedure for preparing cells for fluorescence activated cell sorter (FACS) analysis was used and described elsewhere [17]. FACS analysis was used to assess the number of different leukocyte subsets in the blood and in the MP-compartment, to assess expression level of different surface molecules on these subpopulations, and to identify and quantify intracellular and membrane ligands. FITC-, PE- or BIOTIN-conjugated + Streptavidin RPE-CY5 were used in a three color FACS analysis. Granulocytes and lymphocytes were identified based on forward and side scatters. The following cell populations were identified based on their CD, using specific mAb: NK cells (CD161^{bright}, also known as NKRP-1^{bright}), T cells (CD5 within lymphocytes), Monocytes (RM1), DC (OX62), CD4 T cells (CD4 within lymphocytes), CD8 T cells (CD8 within lymphocytes). Specific mAb were used to quantify expression levels of CD11a, CD11b, CD11c, CD80, CD25, and MHCII on these leukocyte subsets. All antibodies were purchased from PharMingen or Serotec except RM1 (Bachem). To assess the absolute number of a leukocyte subset per µl blood or MP-perfusate, 600 polystyrene microbeads (20 µm, Duke Scientific, Palo Alto, CA, USA) were added per 1 µl sample. To calculate the number of a specific cell population per µl sample (e.g., NK/µl sample), the number of identified cell (NK) was multiplied by 600 and divided by the number of microbeads. The coefficient of variation for this method is approximately 6% in our laboratory.

Intracellular IFN-γ was quantified using anti-rat IFN-γ, following the Serotec protocol for direct intracellular staining. Briefly: After surface staining and lysing of RBCs, cells were fixed and permeabilized using “LeucopermTM” solution A and B (Serotec) and conjugated antibody recognizing intracellular IFNγ were added to each tube and incubated for 30 minutes. After being washed extensively and fixed in 2% paraformaldehyde, cells were subjected to flow cytometric analysis.

Ex vivo assessment of NK cytotoxicity

NK cytotoxicity was assessed in a 4h Cr⁵¹ release assay against all the above 7 cells lines. The procedures included:

1. Harvesting and preparing of circulating and MP leukocytes—As previously described in details [17], after rats were overdose with halothane and their thoracic cavities opened, blood was drawn from the right cardiac ventricle into a heparinized syringe (30µl per 1ml blood). One ml of blood was washed and used in the assay. MP- leucocytes were collected by injecting heparinized PBS (30 U per 1 ml PBS) into the right cardiac ventricle (15 ml/min) and collecting 30 ml of perfusate from the left ventricle to be concentrated to 1 ml in CM. The first 2 ml of the perfusate was disposed of, as it was contaminated with blood from the lungs and heart.

2. Isolation of PBMC by Ficoll-Hypaque density gradient—Blood (with heparin) was diluted 1:1 with complete medium. Four ml of MP-perfusate or of diluted blood were layered on 3 ml of Ficoll-Hypaque (Sigma, Israel), which contained 5.6% Ficoll and 9.6% diatrizoate with a density of 1.077 ± 0.001 g/ml. Samples were centrifuged for 20 min at 700g at 20°C. The mononuclear cells (MNC) layer (between the plasma and the Ficoll-Hypaque fractions) was carefully collected by pipetation and was washed twice with CM by stepwise

centrifugation for 10 min at 300g and further centrifuged for 10 min at 250g for platelet removal. PBMC were resuspended in 3 ml of CM.

3. NK cells purification—Single-cell suspensions of blood-MNC and MP-MNC fractions were prepared and incubated with FITC-labeled anti-CD161 for FACS sorting (labeling is described above, except that no NaN_3 was added to the staining buffer). Purified blood NK cells or MP-NK cells, as well as large and small NK cells in each compartment, were then obtained. The sorting was conducted on 1.2 envelope length, at a rate of approximately 8000 cells/sec (separating up to two populations). The small NK cell population was contaminated with 2% big NK cells while the big NK cells population was contaminated with 10% small NK cells. Insignificant contaminations occurred between NK and non-NK cells.

4. Preparations and radio-labeling of target cells—Cells lines adhering to culture flasks were removed with trypsin solution (0.25% in PBS), and were washed with CM. For all cell lines, 5×10^6 cells were incubated for 1 h with 100 μCi chromium (Cr^{51}) (Danyl Biotech, Rehovot, Israel) in 100 μl saline, 100 μl fetal calf serum (FCS) and 275 μl CM. Following incubation, cells were washed three times (300g for 10 min) and adjusted to the concentration of $5 \times 10^4/\text{ml}$ in CM.

5. Measurement of NK cytotoxicity—We used a 4 h Cr^{51} release assay to assess NK-mediated lysis of all cell lines, as described elsewhere [17]. Effector cells (i.e., whole blood, MNCs, purified NK cells) were serially diluted and co-incubated with 5000 radiolabeled target cells, yielding different effector to target (E:T) ratios. Our earlier studies indicate that cytotoxicity in this assay, using any of the above effector cell preparation, depends on NK cells, since their selective depletion nullified all specific killing [13].

6. Quantifying NK activity per NK cell—In the first experiment, FACS analysis of the numbers of NK cells in each sample was conducted simultaneously with the assessment of cytotoxicity against the different target cells. Based on the numbers of NK cells/ml samples and cytotoxicity in the different E:T ratios, we used a standard extrapolation approach to calculate NK cytotoxicity based on standard NK:target ratios in all samples. Thus, NK cytotoxicity is presented per the NK:target ratio, rather than per ml sample. In all the other experiments, to achieve standard NK:target ratios, samples were diluted in CM before co-incubation with a constant numbers of target cells. Each sample was diluted to achieve the highest possible designated NK:target ratio (e.g., 32:1, or 16:1) before its serial dilution. In these studies there were at least 6 E:T ratios common to all the samples in each study.

Assessment of lung MADB106 tumor retention, and radio-labeling of tumor cells

To radiolabel MADB106 tumor cells we cultured them in 0.5 $\mu\text{Ci}/\text{ml}$ of ^{125}I -deoxyuridine for 1 day. We then lightly anesthetized rats with halothane, and injected $4 \times 10^5/\text{kg}$ labeled MADB106 cells in 0.5 ml of PBS containing 0.1% bovine serum albumin (BSA) into the tail vein. For more details see [17]. Twenty-one hours following tumor inoculation, we removed the lungs and placed them in a gamma counter for assessment of radioactive content. Lung tumor retention was calculated as the ratio between lung radioactivity and total radioactive content of the injected tumor cells.

Assessment of MADB106 lung metastases

Non-labeled MADB106 tumor cells were injected as above. Three weeks later, lungs were removed and placed for 24 h in Bouin's solution (72% saturated picric acid solution, 23% formaldehyde [37% solution], and 5% acetic acid glacial). Lungs were then washed in ethanol, and two researchers unaware of the lungs' origin independently counted visible surface metastases.

Selective in vivo depletion of LGL/NK cells

Immediately prior to the experiment, approximately 1.5 mg/kg of the anti-NKR-P1 monoclonal antibody (anti-rat NKR-P1A (anti CD161), PharMingen, San Diego, CA, USA) was injected i.v. under light halothane anesthesia. Anti-NKR-P1, originally termed mAb 3.2.3, binds to a surface antigen that is highly expressed on fresh and IL-2-activated NK cells in rats. In vivo treatment of rats with anti-NKR-P1 selectively depletes LGL/NK cells and eliminates NK- and antibody-dependent, non-MHC-restricted cell cytotoxicity. T-cell function and the percentage of T cells, monocytes and polymorphonuclear (PMN) cells are unaffected [12,26]. This antibody renders NK cells ineffective in vivo immediately upon administration, and selectively depletes NK cells within a day [18] [16].

Statistical analysis

Depending on experimental design, we conducted one, two, or three way factorial analysis of variance (ANOVA) with a predetermined significance level of 0.05. When NK cytotoxicity was assessed, two by two repeated measures ANOVA were conducted (repeated E: T ratios). When ANOVA indicated significant group differences, planned contrasts were performed (Fisher's PLSD) based on a priori hypotheses. Data were always assessed to verify normal distribution and group homogeneity of variance, and presented as mean $\pm$ SEM.

Results

A. Higher MP-NK cytotoxicity against various syngeneic cell lines of F344 and BDX rat strains

This study was conducted to clarify whether the enhanced MP-NK cytotoxicity against the syngeneic MADB106 tumor cells observed in our previous studies in F344 rats (compared to circulating-NK cytotoxicity) [17], can be generalized to other target cell lines and an additional strain of inbred rats. To this end we harvested circulating- and MP-leukocytes from naïve and poly I-C immunostimulated F344 and BDX rats, and tested their NK cytotoxicity against various syngeneic and xenogeneic tumor cells. F344 NK cytotoxicity was tested against the syngeneic MADB106 and CRNK-16, and the xenogeneic P815 and YAC-1 tumor cell lines. BDX's NK cytotoxicity was examined against the syngeneic 73AS, AS-14, and ASML, and the allogeneic MADB106 tumor cell lines. Cytotoxicity levels were always presented per equal number of NK cells within the different populations tested for NK activity. A two by two (circulating vs. MP by poly I-C vs. saline) repeated measures (E:T ratios) ANOVA was conducted for each target cell line in each rat strain.

The findings indicate that MP-leukocytes from both rat strains exhibited marked and significant higher NK cytotoxicity against each of the tumor cell lines compared to circulating-leukocytes ($P < 0.005$) (Fig 1A-H). The lysis of all syngeneic tumor lines by circulating leukocytes was very low. Last, poly I-C immunostimulation significantly increased cytotoxicity against all tumor lines, but significantly more profoundly in the MP-compartment and against syngeneic tumor lines. Thus, MP-leukocytes exhibited significant NK cytotoxicity against syngeneic cell lines that would be considered "NK-resistant" in both inbred strains of rats. Consequently, in the following experiments we characterize the MP-compartment in naïve and in immunostimulated F344 rats.

B. Different Leukocyte subset composition of the MP-compartment vs. the circulation

In this study we examined the leukocyte subset composition of the MP- and circulation-compartments of F344 rats. Data is based on four replications, each containing 6-8 naïve rats or rats treated with poly I-C. Cells subtypes were identified by FACS analysis as described above. All the results reported below were statistically significant at ($P < 0.05$) and many were also at ($P < 0.001$). The findings indicate that the MP-compartment exhibit a markedly different

cellular composition than the circulation. Specifically, the “innate immune division”: granulocytes, monocytes, and NK cells constituted 52% ($\pm 1.52\%$) of the overall MP-leukocytes population, compare to 27.9% ($\pm 0.66\%$) in the circulation ($P < 0.05$) (Fig 2). Following poly I-C administration (known to induces an anti-viral immune activatory responses), the proportion of the “inflammatory innate division” in the MP-compartment further increased, reaching 61.1% ($\pm 2.56\%$) ($P < 0.05$), while in the circulation these cells composition increased only to 30.6% ($\pm 1.48\%$) ($P < 0.05$).

On the other hand, the percentage of $CD4^+$ T cells, $CD4^+CD8^+$ T cells, and $CD4^+CD25^+$ T cells, which are aspects of adaptive immunity and regulatory immune cells, were each higher in the circulation than in the MP-compartment, as was the percentage of DCs (Fig.2) ($p < 0.05$ for each population). Poly I-C further decreased the percentage of MP- $CD4^+CD8^+$ T cells and MP- $CD4^+CD25^+$ T cells.

C. Phenotypes of an active inflammatory state in the MP-leukocytes population

We further characterized the activation state of the MP-leukocyte subpopulations by comparing their surface molecular determinants to those in the circulating. We found that MP-granulocytes, MP-monocytes, and MP-NK cells (see below), have phenotypic signatures of inflammation (Fig.3). Specifically, these leukocytes subpopulations demonstrated significantly higher expression levels of the adherence receptor CD11b (Fig.3A-B) (an acknowledged marker for inflammation [27,28] ($p < 0.05$), and poly I-C further increased the expression of this determinant ($p < 0.05$). In addition, MP-monocytes exhibited significant increase in surface expression of the activation marker NKR-P1(CD161) (Fig.3C) ($p < 0.05$), another indication for inflammation [29,30]. Moreover, a mature characteristic of DC was more prominent in the MP-compartment, as indicated by a significantly higher percentage of $CD80^+MHCII^+$ DC (Fig.3D) ($p < 0.05$), and poly I-C further increased the prevalence of this characteristics in both compartments ($p < 0.05$).

Last, the ratio of $CD4^+$ T cells to $CD8^+$ T cells, which is a common index of an inflammatory immune status [31], was significantly lower in the MP- compartment ($p < 0.05$), and poly I-C further decreased it in both compartments ($p < 0.05$) (Fig.3E). Thus, the above findings point at several phenotype characteristics that are all associated with an active inflammatory status of the MP-compartment.

D. The NK subpopulation

D.1 NK cells' number, percentage, and size, is greater in the MP-compartment

—The following results were evident in numerous studies conducted in our laboratory in the past 5 years, and the following is a representative study. Eleven rats were pretreated with poly I-C and 11 with saline, and leukocytes were harvested from the circulation- and MP-compartments for FACS analysis. NK cells percent was significantly higher in the MP-compartment than in the circulation (7.5 ± 0.69 vs. 4.8 ± 0.33), and poly I-C increased %NK cells in both compartments, but markedly and significantly more in the MP-compartment (18 ± 3.3 vs. 7.1 ± 1.1 , respectively) (Fig.2). The total number of NK cells per total MP-leukocytes and per ml blood increased following poly I-C treatment ($p < 0.05$ for both), but significantly more in the MP-compartment (4.2 ± 2 fold vs. 1.5 ± 1.0) ($p < 0.05$) (Fig.4B). With respect to the size of NK cells – in the rats, NK cells can be categorized into two distinct subpopulations: large NK cells (larger than $18 \mu m$) and small NK cells (Fig 4A). Importantly, and as is in our previous studies [17], MP-NK cells contained a three-fold higher percentage of large NK cells than circulating NK cells (30% vs. 10%, respectively) ($p < 0.05$) (Fig 4C). Interestingly, poly I-C that significantly elevated the percentage and the total number of NK cells in the two immune compartments did not affect the above ratios of large NK cells in either compartment.

D.2 Different expression levels of activation and adhesion surface markers on small and large NK cells in the two immune compartments—We also characterized the status of activation and adhesion surface markers on small and large NK cells in the MP and circulating, using aliquots from the above study. The most profound differences were evident between large and small NK cells. Compared to small NK cells, large NK cells were characterized by significantly higher expression levels of the adhesion molecules CD11b (Mac-1) and CD11a (LFA-1), CD25 (IL-2 receptors), and CD161 (NKR-P1), (Fig.5A-D) ($p < 0.05$ for all). These differences were evident in both compartments, and in both poly I-C and saline treated rats. In addition, the percentage of NK cells that were positive for expressing the IL-2 receptor and the CD11a and cd11b adhesion molecules was significantly higher in large NK cells than in small NK.

The second most profound difference was the expression levels of the above surface determinants between the two immune compartments. MP-NK cells exhibited significantly higher expression levels than circulating NK cells with respect to CD11b (two-fold difference), and CD11a (Fig.5A-B). CD 25 showed the same difference, but only with respect to small NK cells (Fig.5C), and CD161 only with respect to large NK cells in rats not treated with poly I-C (Fig.5D), ($p < 0.05$ for all).

By and large, poly I-C was the factor that least profoundly affected the above measures. It either increased or decreased their expression depending on the immune compartment and the size of the NK population (Fig.5A-D).

D.3 Higher intracellular interferon gamma in MP-NK cells—IFN- γ , a prominent Th1 cytokine, promotes the differentiation of naïve T cells to committed Th1 cells, which accelerate inflammation. Given the small percentage of NK cells that were positive to intracellular IFN- γ (3-5%), and given the technical procedure for intracellular staining, it was impossible to reliably distinguish small vs. large NK cells. The results are therefore presented for the entire NK cell population in each compartment. Intracellular IFN- γ was expressed by a significantly higher percentage of MP-NK cells, compared to circulating NK cells ($p < 0.05$), and poly I-C further increased this percentage in both compartments ($p < 0.05$) (Fig.6). Thus, intracellular IFN- γ is another index pointing at the pro-inflammatory status of MP-NK cells.

D.4 Can higher MP-NK cytotoxicity be ascribed to potent individual NK cells or to cellular composition of the MP-compartment?—This study was conducted to test whether the enhanced MP-NK cytotoxicity observed in our previous studies (compared to circulating-NK cytotoxicity) is ascribed to potent individual NK cells, or to the cellular composition of the MP-compartment. To this end we studied NK cytotoxicity against MADB106 and YAC-1 tumor cells following increasing levels of NK cell purification, in animals that were or were not treated with poly I-C (at least 8 animals per group in each purification level). Cytotoxicity levels are always presented per equal number of NK cells within the different populations tested for NK activity. A two by two (circulating vs. MP by poly I-C vs. saline) repeated measures (E: T ratios) ANOVA was conducted for each purification level and each target cell.

D.4.a NK Cytotoxicity by the entire leukocyte population: MP-leukocytes exhibited marked and significant NK cytotoxicity compared to circulating-leukocytes against MADB106 (7A) and YAC-1 target cells (7D) ($P < 0.05$ for each). Poly I-C had no significant effects.

D.4.b NK Cytotoxicity by MNCs: NK cells were enriched by separating the MNC fraction from each compartment. MP-MNCs exhibited markedly and significantly higher NK cytotoxicity against the MADB106 (Fig.7B) and YAC-1 (7E) target cells ($P < 0.05$ for both).

Poly I-C increased NK cytotoxicity, but significantly so only in MP-MNC cytotoxicity against the MADB106. No other comparison reached statistical significance.

D.4.c NK Cytotoxicity by Purified NK cells: No differences were evident between MP- and circulating- purified NK cells from naïve rats. However, poly I-C increased NK cytotoxicity only in the MP-compartment, in a marked and significant manner (Fig 7C and 7 F) ($P < 0.05$), as indicated by a significant interaction between the compartment and the effects of poly I-C ($P < 0.05$).

The “no NK fraction” showed no cytotoxicity against either tumor lines (data not shown.)

D.4.d No differences in NK cytotoxicity between purified small and large NK cells:

Because leukocytes from the MP compartment have a greater percentage of large NK cells, we sought to determine whether this higher percentage contributes to the higher cytotoxicity exhibited by the MP population. To this end, we directly compared cytotoxicity of large to small NK cells that originated from the circulation or the MP compartment. No appreciable differences in NK cytotoxicity were observed between purified small and purified large NK cells in the circulation or in the MP compartment against the MADB106 or the YAC-1 tumor cells (data not shown).

As in the previous study (D.4.c), poly I-C significantly increased purified MP-NK cell activity, but not circulating NK activity, against MADB106 tumor cells. This increase was very similar in small and large NK cells. Similar findings were observed with respect NK cytotoxicity against YAC-1 cells (data not shown).

D.4.e. Comparison between the above three levels of MP-NK cell's purification with respect to MADB106 cytotoxicity: To compare cytotoxicity levels between the above three levels of MP-NK cell's purification, we compared cytotoxicity against MADB106 tumor cells in the same E:T ratio (16:1). Circulating NK cells of each purification level exhibited 5-12% cytotoxicity; lower than MP-NK cell cytotoxicity evident in each of the three purification levels. With respect to MP-NK cell, as seen in Fig. 7G, the more we enriched NK cells (from all leukocytes, to MNC, to purified NK cells) the more the cytotoxicity decreased and the smaller the differences between the cytotoxicity of the two compartments became. However, pre-exposure to poly I-C maintained the levels of MP-NK cytotoxicity and thus the difference between the two compartments (Fig.7G). Therefore, the enhanced MP-NK cytotoxicity observed in untreated rats (no poly I-C) compared to the circulation may be ascribed to interaction of MP-NK cells with activated MP-leukocytes (which are absent in purified NK cells). On the other hand, in poly I-C treated rats purified MP-NK cells are independently more potent than circulating NK cells.

E. NK cells are required in vivo to control MADB106 metastases in naïve and poly I-C treated rats

To understand the in vivo role of NK cells in controlling MADB106 metastasis we selectively depleted NK cells in naïve and in poly I-C treated rats immediately prior to inoculating rats with MADB106 tumor cells. Twenty-four hours after inoculation with radiolabeled MADB106 tumor cells, rats were sacrificed and lung tumor retention was assessed ($n = 40$ in these four groups). In the second set of experiments, three weeks after inoculation with MADB106 tumor cells, rats were sacrificed and surface lung metastases were counted ($n = 40$ in these four groups). In both sets of experiments, selective depletion of NK cells markedly and significantly reduced resistance to MADB106 metastasis as reflected by increased lung tumor retention (Fig. 8A, $p < 0.001$) or increased number of metastases (Fig.8B, $p < 0.001$). The magnitude of the effects of depletion on lung tumor retention were similar in control and poly I-C treated rats

(376 and 321-fold, respectively). Similar effects were evident when metastases were counted (Fig.8B).

These findings indicate that NK cells play a critical role in controlling MADB106 lung tumor retention and metastases in both naïve rats and in poly I-C treated rats.

F. NK cells are required *in vitro* for lysing MADB106 tumor cells in naïve and in poly I-C or IL-12 stimulated rats

To assess the *in vitro* capacity of NK cells in lysing MADB106 tumor cells, we conducted a selective *in vivo* depletion of NK cells in naïve and immuno-stimulated rats, and harvested MP-leukocytes for *in vitro* assessment of NK cytotoxicity. Specifically, rats were subcutaneously injected every other day for 4 injections with 2 µg IL-12, with 0.2 mg/kg poly I-C (one vehicle, then 3 poly I-C injections) or with vehicle. On the day of the last injection, rats from each group were subdivided and injected with either vehicle or with 1.5 mg/kg anti-NKR-P1 to deplete NK cells ($n = 6-7$ for group). A day later, we collected MP-leukocytes and assessed NK cytotoxicity. Selective depletion of NK cells significantly reduced *ex vivo* NK cytotoxicity in control, poly I-C, and IL-12 treated rats (Fig.8C, $P < 0.001$).

Discussion

Our experiments indicate that the pulmonary vasculature contains a unique leukocyte population, markedly different from the circulation, which exhibits characteristics of active inflammation, and may define a unique vasculature microenvironment. MP-NK cells themselves resemble *in vitro* IL-2 activated NK cells, but exist endogenously. As will be elaborate below, MP-NK cells contain greater proportion of large NK cells, and exhibit higher levels of activation markers and intracellular IFN γ . Importantly, compared to circulating NK cells, equal number of MP-NK cells in the context of MP-leukocytes, exhibit higher NK cytotoxicity against several syngeneic and xenogeneic target cells, in both the F344 and BDX inbred rat strains. In animals challenged with poly I-C, purified MP-NK cells show markedly greater cytotoxicity than purified circulating NK cells.

Inflammation is commonly a local protective response against foreign invaders, or altered or deteriorating self. The first cells responding to inflammation-induced chemotactic signals are constituents of innate immunity, of these granulocytes, monocytes, and NK cells are prominent [32,33]. Suggesting the inflammatory status of the MP-compartment is its composition of leukocyte subtypes, which contains 52% of these innate immune cells, compare to 28% in the circulation. Following poly I-C administration into the intraperitoneal cavity, which induces an anti-viral response, the proportion of “inflammatory cells” in the MP-compartment further increased, reaching 61%, while in the circulation these “inflammatory cells” increased only to 31%. Further, the total number of MP-NK cells increased by 4-fold following poly I-C administration, while the number of circulating NK cells increased only by 1.5-fold. These effects of poly I-C suggest that following an immune inflammatory challenge in remote locations (i.e., the intraperitoneal cavity, where the poly I-C was injected), the MP-compartment is further triggered into a pro-inflammatory status.

Inflammation is also associated with cell activation and leukocyte adhesion to vascular endothelium at the site of inflammation. ICAM-1, which is constitutively expressed by pulmonary endothelium [34,35], can be augmented by a variety of inflammatory mediators, thus further increasing leukocyte adhesion. The adhesion of circulating leukocytes to ICAM I-III endothelial ligands is mediated through integrin molecules, including LFA-1 (CD11a-CD18) and Mac-1 (CD11b-CD18) [36]. Surface CD11b can be up-regulated several folds by chemotactic factors (by transporting intracellular granular CD11b content to cell surface). Thus, the approximately 2-fold higher expression levels of CD11b in NK, monocytes, and

granulocytes (“innate division”) of the MP-leukocytes evident in the current study could reflect a local chemotactic proinflammatory signal, or the recruitment of activated cells to this region [27,28]. CD11a is constitutively expressed on the cell membrane and is characterized by firm adhesion capacity to endothelial ICAM-1 ligands. Thus, CD11a higher expression levels of the “innate division” of MP-leukocytes evident herein (data shown regarding NK cells only) may be indicative of continuous presence of these cell populations in the MP-compartment.

With respect to cell activation, the co-stimulatory NKR-P1 receptor and the IL-2 receptor (CD25), are acknowledged markers of activated NK cells that exhibit higher natural cytolytic function against certain, but not all, target cells [37]. In the current study MP-NK cells (small and large) exhibit higher expression levels of the surface receptor for IL-2 (CD25). In addition, both CD25 and NKR-P1 receptors are over-expressed on large NK cells relative to small NK-cells. Large NK constitute 30% of the MP-NK population relative to only 10% in the circulation, further contributing to higher expression and prevalence of these markers within the MP-leukocyte population. Last, Steiniger [29] reported that elevated expression of NKR-P1 receptor on surface monocytes is an indication of inflammation, as was indeed seen within MP-monocytes in our study.

Our findings regarding dendritic cells (DCs) support our hypothesis that the MP-compartment is in a continuous or prolonged inflammatory status. Pathogen-associated compounds, such as those recognized by Toll-like receptors (TLRs), activate immature DCs, which then undergo dramatic phenotypic changes that end in the acquisition of a mature phenotype. The mature DC phenotype is characterized by a reduced capacity to capture antigens, high expression of MHC-II at the cell surface, and high expression of T-cell costimulatory molecules such as CD40, CD80, and CD86, which enable mature DCs to stimulate naive T cells. In the current study we found that MP-DCs contain higher percentage of mature DCs compared to circulating DCs, as indicated by higher expression levels of the CD80 and MHC-II receptors on DCs. Thus it seems that the MP-compartment is in a continuous, rather than an early inflammatory status.

The ratio of CD4⁺ T to CD8⁺ T cells is a common index of immune inflammatory status. In humans, a ratio of approximately 1.5-2 characterizes healthy individuals, while below 1 is associated to severe infection [31]. In the current study, we found the CD4⁺ T/CD8⁺ T ratio to be significantly reduced in the MP-compartment compared to the circulation (and below 1), and poly I-C further decreased it in both compartments.

Lastly, large cell size is another phenotype of activated cells [38,39]. Biron [38] verified that endogenous NK cells isolated from non-infected mouse spleen cell preparations contain small to medium-size cells, but NK cells activated during infection are capable of blastogenesis in vivo and have a broad size distribution including large-size population. In this study, MP-NK cells exhibited a significantly greater percentage of large NK cells than circulating NK cells (approximately 30% vs. 10% respectively), further supporting a local activated microenvironment.

Overall, the above findings indicate several changes in cellular phenotype and composition that are all indicative of active pro-inflammatory status of the MP-compartment.

Another indicator of NK cells activation status is their functional capacity. Compared to circulating NK cells, equal number of MP-NK cells within the MP-leukocytes milieu of both rat strains studied exhibited significantly higher NKC cytotoxicity against various syngeneic and xenogeneic target cells. Thus, higher NKC cytotoxicity is not restricted to F344 rat's MP-NK cells against the specific syngeneic tumor line (MADB106), but rather is a more generalized phenomenon.

The potential supporting role of non-NK leukocytes in NKC cytotoxicity within the circulation and the MP compartments was studied herein using the MADB106 and the YAC-1 target cell lines in the F344 rat. The more we enriched NK cells (from all leukocytes, to MNC, to purified NK cells), the lower MP-NK cytotoxicity levels became, and the smaller the differences between cytotoxicity per NK cell from the two immune compartments became (Fig. 7G). However, pre-exposing rats to poly I-C maintained the difference and the absolute levels of MP-NKC cytotoxicity (Fig. 7G & 7A-F).

This decrease in cytotoxicity along NK purification could result from a lack of NK cell interactions with supporting leukocytes or their secretions. This support seems less critical for purified MP-NK cells taken from poly I-C treated rats [40]. Indeed, NK cells reciprocally interact with other leukocyte subpopulations, including NKT cells, monocytes [41], and DCs [42-44]. They might be activated by ligands expressed on other cells and by soluble signaling molecules (e.g. IFN- α /b, IL-12) [45-49]. The unique impact of poly I-C on MP-NKC activity might be mediated by direct stimulatory effects on primed MP-NK cells, and by indirect effects through other cells. Alternatively, poly I-C might have protected MP-NKCs against the stress and the delay induced by the in vitro procedures of purification (including density gradient and FACS sorting). Indeed, in a recent study we reported that the same in vivo poly I-C regimen protects MP-NK cells, but not circulating NK cells, from in vitro suppression by PGE₂ and corticosterone [19].

Last, the intracellular level of IFN- γ is another index of potential innate resistance to intracellular pathogens and NK-dependent resistance to tumor metastasis. IFN- γ secretion causes activation of committed Th1 cells, which then promote cytotoxic T cell and macrophage activation, and cause further NK cell activation [50]. Thus, the 2-fold higher percentage of positive intracellular IFN- γ in MP-NK cells, evident in our study, is another indication for the potency of MP-NK cells and their pro-inflammatory immune status. This index further increased in both immune compartments following poly I-C administration.

The in vivo and the biological significance of MP-NK cells are suggested by the NK-depletions studies, assessing in vitro and in vivo resistance to MADB106 cells. While circulating NK cells showed no significant in vitro NK cytotoxicity against MADB106 cells, MP-NK cells showed marked cytotoxicity against MADB106 cells. Selective in vivo NK-depletion significantly and markedly reduced MP-NK cytotoxicity against MADB106 in vitro, and dramatically elevated in vivo levels of MADB106 lung tumor retention and number of lungs metastases. Conversely, poly I-C elevated MP-NK cytotoxicity against MADB106 in vitro, and reduced in vivo levels of MADB106 lung tumor retention and number of lungs metastases. These findings clearly suggest the significant role of MP-NK cells in controlling MADB106 tumor cells in vivo, and thus MP-NK cells' biological significance. This does not exclude a role for other mechanisms in controlling MADB106 tumor in vivo, or in mediating the beneficial effects of immunostimulation [51,52].

Based on the above, it is our hypothesis that the MP-leukocyte population is in a state of continuous activation: the lungs are continuously in contact with inhaled particles, many of which are able to act as antigens (e.g., LPS). Consequently, the MP-compartment is constantly exposed to several defused nonspecific activating factors, such as the proinflammatory cytokines IL-2 or IFN- γ . Bryceson [53] showed that resting NK cells require ligation of at least two receptors to trigger lysis, but IL-2-primed NK cells required only one triggering ligand. Maintenance of a pool of primed MP-leukocyte including MP-NK cells during normal environmental exposure could be advantageous and of adaptive value for the organism. MP-leukocytes are strategically located to physically interact with all circulating aberrant cells, as the small diameter of the lungs' capillaries enforce circulating cells to contact the vascular endothelium and MP-leukocytes before they can pass through. MP-leukocytes can thus be

critical for preventing pathogens that escaped the bronco-alveolar space and the lung interstitium barriers from invading the circulation and causing systemic infections, as well as for resisting autologous circulating infected or malignant cells. In the case of circulating cancer cells, MP-leukocytes may have a single opportunity to lyse tumor cells before the latter seed and form metastases in their preferred tissue organ.

It would be interesting to determine whether the MP-compartment is a location that manifests continuous inflammatory characteristics because it involves ongoing interactions with airborne infectious agents, as suggested above. Alternatively, activated leukocytes with a proinflammatory profile, which originated at other body locations, could migrate and adhere to the lungs' capillaries. This study is insufficient to quantify the relative contribution of each of these non-exclusive mechanisms. The latter mechanism is clearly indicated by our findings that intraperitoneal poly I-C immunostimulation potentiated the activated/inflammatory characteristics of the MP-compartment markedly more than it impacted the circulating compartment.

Potent leukocyte populations, similar to MP-population, may be found at other local vasculature systems in the interface with "pathogen-rich environments". Noteworthy, leukocyte recruitment to capillaries has been observed in hepatic sinusoids[54], pulmonary capillaries[55], and in the glomerulus[56]. Such populations seem continuously in contact with pathogens or endogenous antibodies and could consequently become activated leukocytes and play a key role in host defense against systemic virally infected cells and tumor cells. In fact, there is similarity between MP-NK cells and Pit cells (hepatic NK cells). Pit cells are located in the liver sinusoids where they adhere to endothelial cells[57,58]. They were suggested to resemble *in vitro* IL-2 activated NK cells because they exhibit greater cytotoxicity than circulating NK cells, are able to recognize a larger spectrum of tumor cells (the NK-resistant P810 tumor cells), and have enhanced expression of the LFA-1 adhering molecule. Similarly to lungs, the liver is continuously exposed to a large variety of antigens derived from the gastrointestinal tract, including dietary antigens, pathogens, and toxins.

Thus primed or activated vasculature leukocytes, specifically MP-NK cells, might have a significant physiological role, albeit they constitute only a small portion of the entire host cell population. Given the ability of MP-leukocyte to efficiently exhibit NK cytotoxicity against syngeneic "NK-resistant" target cells, such as the MADB106, it could be suggested that if a similar compartment exists in humans, then innate immunity may have a greater role in controlling circulating malignant cells than is currently believed. Innate immunity may also hold greater therapeutic potential, especially following immunostimulation.

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Fig.1. Higher MP-NK cytotoxicity against syngeneic, allogeneic, and xenogeneic cell lines of the F344 and BDX rat strains

MP-leukocytes from F344 (Fig. 1A-D) and BDX's (Fig. 1E-H) rats exhibited marked and significant higher NK cytotoxicity against various syngeneic, allogeneic, and xenogeneic tumor cells compare to leukocytes from the circulation ($P < 0.005$, for each tumor line). NK cytotoxicity in F344 rats was tested against the syngeneic MADB106 (Fig. 1A) and CRNK-16 (Fig. 1B), and the xenogeneic P815 (Fig. 1C) and YAC-1 (Fig. 1D) tumor cell lines. NK cytotoxicity in BDX rats was examined against the syngeneic 73AS (Fig. 1E), AS-14 (Fig. 1F), and ASML (Fig. 1G), and the allogeneic MADB106 (Fig. 1H) tumor lines. Cytotoxicity levels are presented in all figures using the same NK:target cell ratio. The lysis of all syngeneic tumor lines by circulating leukocytes was very low (Fig. 1A, B, E, F, and G). Poly I-C immunostimulation significantly increased MP-NK, but not circulating-NK cytotoxicity, against all tumor lines (excluding the xenogeneic YAC-1, in which cytotoxicity increased in both compartments). Data are expressed as mean $\pm$ SEM.

Fig.2. Different leukocyte subsets composition in the MP-compartment vs. the circulation
The MP-compartment exhibited a markedly different cellular composition than the circulation: the "innate immune division", including granulocytes, monocytes, and NK cells, constituted 52% ($\pm 1.52\%$) of the MP-leukocytes population, compared to 27.9% ($\pm 0.66\%$) in the circulation ($p < 0.05$). Contrary, the T cell subpopulations, including CD4⁺, CD4⁺CD8⁺ T cells, and regulatory (CD4⁺ CD25⁺) T cells, as well as DC, exhibited lower percentage in the MP compartment compared to the circulation ($P \leq 0.005$). Following poly I-C administration, the proportion of the "innate immune division" in the MP-compartment further increased, reaching 61.1% ($\pm 2.56\%$) ($P < 0.05$), and also increased in the circulation to only 21% ($\pm 1.48\%$) ($P < 0.05$).

Fig.3. Phenotypes of an active inflammatory state in the MP-leukocytes population

Compared to the circulation, MP-granulocytes exhibited higher expression levels of CD11b (Fig.3A) ($P < 0.05$), and MP-monocytes expressed higher levels of CD11b and NKR-P1 (Fig. 3B-C). MP-DCs contained a higher percentage of mature DCs compared to circulating DCs, as indicated by higher expression levels of the CD80⁺MHC-II⁺ cells (Fig.3D), and poly I-C further increased it in both compartments. The CD4⁺T/CD8⁺T ratio was significantly reduced in the MP-compartment compared to the circulation, and poly I-C further decreased it in both compartments (Fig.3E). # indicate significant difference between the MP compartment and the blood compartment, and * indicates significant difference within a compartment, between poly I-C and naive animals. Data are expressed as mean $\pm$ SEM.

Fig.4. NK cells' number, percentage, and size, is greater in the MP-compartment

Leukocytes were labeled by two fluorescent markers to identify CD161 (FL-1, for NK cells) and CD5 (FL-2, for T cells). Together with FSC and SSC, small NK cells and large NK were identified, and are circled in panel A (T cells, granulocytes, and plastic beads are not marked in the figure). In the 4 scattergrams of panel A, FSC by FL-1 are presented for circulating and MP-leukocytes, treated or not with poly I-C. See that NK cells can be clearly categorized into two distinct subpopulations: large NK cells (larger than 18 μ m) and small NK cells (Fig 4A). The total number of NK cells per MP-leukocytes and per ml blood increased following poly I-C treatment ($p < 0.05$ for both), but significantly more in the MP-compartment (4.2 ± 2 fold vs. 1.5 ± 1.0) ($p < 0.05$) (Fig.4B). MP-NK cells contained a three-fold higher percentage of large NK cells than circulating NK cells (30% vs. 10%, respectively) ($p < 0.05$) (Fig 4C), and poly I-C did not affect this ratio in neither compartments. # indicate significant difference between the MP compartment and the blood compartment, and * indicates significant difference within a compartment, between poly I-C and naive animals. Data are expressed as mean $\pm$ SEM.

Fig.5. Different expression levels of activation and adhesion surface markers on small and large NK cells in the two immune compartments

Compared to small NK cells, large NK cells were characterized by significantly higher expression levels of the adhesion molecules CD11b (Mac-1) (Fig.5A) and CD11a (LFA-1) (Fig.5B), and of CD25 (IL-2 receptor) (Fig.5C) and CD161 (NKR-P1) (Fig.5D), ($p < 0.05$ for all). These differences were evident in both compartments, and in both poly I-C and saline treated rats. In addition, MP-NK cells also exhibit significantly and markedly higher expression levels of CD11b and CD11a compared to circulating-NK cells (Fig 5A-B). # indicate significant difference between the MP compartment and the blood compartment, and * indicates significant difference within a compartment, between poly I-C and naive animals. Data are expressed as mean $\pm$ SEM

Fig.6. Higher intracellular interferon gamma in MP-NK cells

Intracellular IFN- γ was expressed by a significantly higher percentage of MP-NK cells, compared to circulating NK cells (#) ($p < 0.05$), and poly I-C further increased this percentage in both compartments ($P < 0.05$ across compartments) (Fig.6). Data are expressed as mean $\pm$ SEM.

Fig.7. NK cytotoxicity against MADB106 and YAC-1 target cells along increasing levels of NK cell purification

A & D. Circulating and MP-leukocytes: MP-leukocytes exhibited marked and significant NK cytotoxicity compared to circulating-leukocytes against MADB106 (7A) and YAC-1 target cells (7D) ($P < 0.05$ for each). Poly I-C had no significant effects.

B & E. Circulating and MP-MNC: MP-MNC exhibited marked and significant higher NK cytotoxicity compared to circulating-MNC against the MADB106 (Fig.7B) and YAC-1 (7E) target cells ($P < 0.05$ for both). Poly I-C increased NK cytotoxicity, but significantly so only in MP-MNC cytotoxicity against the MADB106.

C & F. Purified circulating and MP-NK cells: No differences were evident between MP and circulating purified NK cells from naïve rats. However, poly I-C increased NK cytotoxicity only in the MP compartment, in a marked and significant manner (Fig 7C and 7 F) ($P < 0.05$), as indicated by a significant interaction between the compartment and the effects of poly I-C ($P < 0.05$). In all panels, data are expressed as mean $\pm$ SEM.

G. Comparison of MADB106 cytotoxicity by MP-NK cells between the three purification levels at a common E:T ratio (16:1) (Taken from A-C). Cytotoxicity levels of circulating NK cells of all purification levels are below the dashed line (5-12%). The more we enriched the part of NKCs (from all leukocytes, to MNC, to purified NK cells) the more MP-NK cell

cytotoxicity decreased, and the smaller the differences between the cytotoxicity of the two immune compartments became. However, pre-exposure to poly I-C maintained the levels of MP-NK cytotoxicity and thus the difference between the two compartments.

Fig.8. The effect of in vivo NK depletion on controlling MADB106 metastases and on in vitro MADB106 lysis

Selective elimination of NK cells markedly increased lung tumor retention (A) and lung tumor metastases (B) in both poly I-C treated and untreated rats ($n = 40$, $P < 0.001$). (Notice the logarithmic scale). In vitro MP-NK activity against MADB106 target cells was significantly greater in control and in IL-12 or poly I-C treated rats compared to NK-depleted rats. Depletion of NK cells markedly decreased NK activity to a similar level in IL-12, poly I-C, and vehicle treated groups (C) ($n = 53$, $P < 0.001$). This, together with the inability of circulating and other populations of NK cells to lyse MADB106, suggest the role of MP-NK cells in controlling MADB106 cells in vivo. Data are expressed as mean $\pm$ SEM.