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The marginating-pulmonary immune compartment in mice exhibits increased NK cytotoxicity and unique cellular characteristics

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Abstract

To test whether marginating-pulmonary (MP) leukocytes in mice have a unique potential to identify and destroy aberrant circulating cells, we compared MP to circulating leukocytes with respect to natural killer (NK) cytotoxicity, proinflammatory characteristics, molecular determinants of activation, and response to IL-12 immunostimulation. Cytotoxicity was assessed employing the YAC-1, B16F10, and 3LL target lines. C57BL/6 mice were injected with either saline or murine IL-12 (0.1 or 0.5 µg/mouse), either once or three times 48-h apart. Twenty-four hours after last injection, cardiac blood was withdrawn and MP leukocytes were collected by forced lung perfusion. NK cytotoxicity, cellular composition, and surface molecular markers were studied. MP leukocytes exhibited greater NK cytotoxicity than circulating leukocytes against the syngeneic B16F10 and 3LL tumor lines, but not against the allogeneic YAC-1 line. NKG2D and IL-12 receptor expression predicted NK cytotoxicity in circulating leukocytes, but not in MP leukocytes. IFN γ -receptor, IL-12-receptor, CD69, CD11a, and CD11b showed different patterns of expression in the two leukocyte populations, suggesting pro-inflammatory characteristics of the MP compartment. IL-12 stimulation caused differential effects on these markers and also elevated cytotoxicity in both compartments, but in different effector: target ratio-dependent patterns. MP leukocytes may play a critical role in eliminating aberrant circulating cells due to their enhanced NK cytotoxicity and given their strategic location in the lungs vasculature, which forces physical interactions with all circulating aberrant cells. MP-NK cells are unique in their cytotoxic mechanisms against syngeneic targets and in their activation profile and response to immunostimulatory agents.

Keywords

Marginating-pulmonary; Cell surface markers; NK cytotoxicity; Inflammation

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Conflict of interest

There are no financial and commercial conflicts of interest.

Introduction

Unique characteristics of the marginating-pulmonary (MP) leukocyte population have been recently reported by our group in rats [1–3]. These leukocytes reside in the micro-vasculature of the lungs and are thus located in a strategic position to physically interact with all circulating aberrant cells. Compared to circulating leukocytes, MP leukocytes and MP-natural killer (NK) cells from several strains of rat were found to exhibit numerous morphological and molecular unique characteristics and to show superior cytotoxicity against several syngeneic and allogeneic tumor lines [2]. MP-NK cells resemble yet another unique population of NK cells, known as pit cells, which reside in the liver sinusoids and exhibit high cytotoxic activity against the standard YAC-1 tumor line and against the “NK resistant” P815 cell line [4]. Similarly to the liver, the lungs are a major site for cancer metastasis, which can originate from any organ through its hematogenic drainage. Thus, the study of this unique leukocyte population in animals and in cancer patients may bare clinical implications.

The potential biological significance of the MP leukocyte population is suggested in our recent animal studies assessing resistance to MADB106 experimental metastasis in F344 rats. First, MP-NK cells, but not circulating NK cells, were shown to lyse MADB106 cells in vitro. Second, the lung retention of these syngeneic tumor cells following their intravenous inoculation is determined, by and large, by the numbers and cytotoxicity levels of MP-NK cells [5–9]. Last, MP-NK cytotoxicity was found to be a mirror image of MADB106 lung tumor retention under various conditions that enhanced or suppressed MP-NK cytotoxicity [1, 2, 10].

The herein studies aim at characterizing different MP cell subpopulations, NK cells in particular, by using both in vitro and ex vivo measures in a different species, the C57BL/6 mouse. To the best of our knowledge, the MP compartment in mice has never been studied, and to this end, we have developed a method to harvest MP leukocytes in this smaller animal. We used several tumor lines in order to assess NK cytotoxicity, including the allogeneic YAC-1, and the syngeneic B16F10 and 3LL cell lines, as these cell lines are susceptible to different NK-lysing mechanisms [11, 12]. Additionally, we studied several surface markers that may contribute to potential differences in cytotoxicity between MP and circulating leukocytes, specifically NKG2D, CD11a, CD11b, CD69, IFN γ -R, and IL-12R; markers that were all implicated in determining or regulating NK cytotoxicity (see “Discussion” section). Last, IL-12 was used in vivo to induce immune stimulation, in order to test whether such a manipulation would differentially affect the two immune compartments, as is suggested by a recent animal study [13].

Materials and methods

Animals

Male and female C57BL/6 mice were housed 4 per cage with free access to food and water on a 12:12 light–dark cycle at 22–24 °C. Animals were acclimated to the vivarium for at least 3 weeks prior to experimentation. All studies were approved by the Institutional

Housing and Use Committee of Tel Aviv University, which also regularly monitors the vivarium.

Collecting MP leukocytes by forced lung perfusion with minimal contamination of circulating leukocytes

Mice were anesthetized with an overdose of isoflurane, chest cavities were opened, and blood was collected from the right ventricle of the heart (0.6 ml). The cardiopulmonary complex (including the heart, lungs, and pulmonary arteries and veins) is excised from the animal and briefly soaked in phosphate-buffered saline (PBS) to remove remaining blood. The cardiopulmonary complex is then placed in a 100-mm-diameter Petri dish to enable lung perfusion with heparinized PBS via the heart ventricles. Specifically, a 24-gauge Venflon (a plastic cannula connected to a silicon tube) is inserted through the heart wall into the right ventricle, and the wall of the left ventricle is incised to allow free outflow. Four microliters of room temperature heparinized PBS (30 units/ml) is passed through the Venflon along 15 s (first perfusion session). This procedure is repeated three times with a 5-s intersession interval. The first 0.2 ml of perfusate outflow, which is heavily contaminated with blood, is collected and discarded, and the following ~10 ml of perfusate is collected from the Petri dish and concentrated to a volume of 0.6 ml. Our preliminary experiments showed that when additional perfusion sessions are conducted, the number of additional harvested leukocytes is negligible. The 0.6 ml of concentrated perfusate contains approximately 10 % hematocrit and 10^6 leukocytes. This paradigm aims to optimize the yield of MP leukocytes while maintaining minimal contamination with circulating leukocyte, and the results indicate that it provides a leukocyte population that is clearly distinct from circulating leukocytes.

Interleukin-12 (IL-12) administration

Lyophilized recombinant murine IL-12 (1×10^7 units/mg, purchased from Cytolab Ltd. Rehovot, Israel) was reconstituted in PBS according to manufacturer's instructions. Mice were injected with either a single administration of 0.1 or 0.5 $\mu\text{g}/200 \mu\text{l}$ /mouse, or with a regimen of three injections of 0.1 $\mu\text{g}/200 \mu\text{l}$ /mouse, given 48 h apart. In all three paradigms, the IL-12 injection was given 24 h before blood and lung perfusate was collected.

Tumor lines and their maintenance

All cell lines (YAC-1, B16 and 3LL) were maintained in monolayer cultures or in suspension in complete media (RPMI-1640 media supplemented with 10 % heat-inactivated fetal calf serum (FCS), 50 $\mu\text{g}/\text{mL}$ of gentamicin, 2 mM of L-glutamine, 0.1 mM of non-essential amino acids, and 1 mM of sodium pyruvate, Biological Industries, Kibbutz Beit Haemek, Israel) in 100 % humidity, 5 % CO_2 at 37 °C. Adherent lines were separated from the flask using 0.25 % trypsin.

Ex vivo assessment of NK cytotoxicity

Target cells and their radiolabeling—Cell lines were washed with complete media. For all cell lines, 5×10^6 cells were incubated for 1 h with 100 μCi chromium (^{51}Cr) (Danyel Biotech, Rehovot, Israel) in 100 μl saline, 100 μl fetal calf serum (FCS), and 275 μl

complete media. Following incubation, cells were washed three times (300 g for 10 min) and adjusted to the concentration of 2.5×10^4 /ml in complete media.

Measurement of NK cytotoxicity—We used a 4-h ^{51}Cr release assay to assess NK-mediated lysis of the three cell lines, as detailed elsewhere [3]. Shortly, effector cells (i.e., circulating or MP leukocytes) were washed three times (400 g for 10 min) with complete media and serially diluted by twofold and co-incubated with 2,500 radiolabeled target cells, yielding 6–8 different effector to target (E:T) ratios. Our earlier studies indicated that cytotoxicity in this assay is attributed to NK cells, since their selective depletion nullified all specific killing [14, 15].

Flow cytometry analysis

FACS analysis was used to assess the number of different leukocyte subpopulations and their expression levels of specific markers. Standard procedures were used to prepare cells for FACS analysis (described in [3]). Lymphocytes and granulocytes were identified based on forward and side scatters. NK, T, and NKT cells were identified within the lymphocyte cell population using FITC-conjugated anti-mouse NK1.1 (eBioscience, San Diego, CA) and PE-Cy5 anti-mouse CD3 (eBioscience, San Diego, CA) (NK are identified as NK1.1⁺CD3⁻, T cells as CD3⁺NK1.1⁻, and NKT as NK1.1⁺CD3⁺). Additional antibodies were used to characterize identified populations: PE Cy5-conjugated anti-mouse CD69 (Biolegend, San Diego, CA), APC-Alexa Fluor 750-conjugated anti-mouse CD11b, PE-conjugated anti-mouse CD11a, PE-conjugated anti-mouse NKG2D (eBioscience, San Diego, CA), rabbit anti-mouse IFN γ R, and rabbit anti-mouse IL-12R (Santa Cruz Biotechnologies, CA). Flow cytometry analysis was conducted using a FACScan (Becton Dickinson) upgraded to two steady-state lasers. To assess the total absolute numbers of cells or cell subpopulations per μl blood or perfusates, 300 standard polystyrene microbeads per microliter of sample (20 μm in diameter, Duke Scientific, Palo Alto, CA) were added to each sample. Following cytometry, the formula: (# events per cell population/# microbead events) $\times$ 300 was used to calculate the absolute numbers of cells per μl sample. The coefficient of variation for this method in our laboratory was found to be 6 % or less for identical samples.

Determining cytotoxicity level per MP-NK cell and per circulating NK cell, based on mathematical shifting of cytotoxicity curves

Cytotoxicity levels of the 6–8 effector cell concentrations were used for each sample to generate a cytotoxicity curve, based on the regression exponential fit method (see [10]). Only the monotonically increasing portions of the cytotoxicity curves were used for the extrapolation procedure. Then, based on the number of MP or circulating NK cells unique to each sample (based on FACS analysis), each curve was positioned in a cytotoxicity (y) by E:T ratio (x) plot, in a horizontal location based on its individual NK–target ratio. Thus, given that the same # of target cells was always used, cytotoxicity in different samples is compared based on the same number of NK cells and indicates cytotoxicity per NK cell in the context of the whole-lung perfusate or circulating leukocytes. In our experience, this approach yields a range of at least 4 NK–target ratios that are common to the great majority of samples in each study.

Statistical analysis

One- or two-way factorial analysis of variance (ANOVA) with a predetermined significance level of 0.05 was conducted. When NK cytotoxicity was studied, repeated-measures ANOVA was conducted (repeated measures of E:T ratios). Provided significant group differences were found, Fisher's protected least significant differences (Fisher's PLSD) contrasts were performed to compare specific pairs of groups, based on a priori hypotheses. Relationships between NK cytotoxicity and NKG2D and IL-12R expression on NK cells were assessed by the use of Pearson's correlation coefficients. All statistical analyses were conducted using StatView software (SAS Institute, San Francisco, CA).

Results

Comparing NK cytotoxicity of MP leukocytes to circulating leukocytes using syngeneic and allogeneic target cells

Procedure: In three study replicates, circulating and MP leukocytes were harvested from a total of 48 C57BL/6 mice as described above ("Materials and methods" section). In each study replicate (16 animals), perfusate or blood samples from 8 mice were combined (forming 2 combined samples of perfusate and two of blood), centrifuged, and the buffy coat containing leukocytes was harvested, together with a similar volume of red blood cells, and brought to a final volume of 1 ml. This was done in order to achieve an MP cell concentration of approximately 5×10^6 leukocytes per ml, similarly to blood leukocyte concentration.

Each of the combined samples served for three assessments of NK cytotoxicity: against allogeneic YAC-1 target cells, and against syngeneic B16 and 3LL target cells (the 3LL tumor line was used only in the two last replicates).

Results: We compared circulating to MP-NK cytotoxicity of the entire compartment (containing similar # of leukocytes), as well as cytotoxicity per NK cell, as described above. Repeated-measures ANOVA (for E:T ratios and compartment) was performed for each tumor line separately.

Against YAC-1 target cells, no difference in NK cytotoxicity per NK cell and per the entire immune compartment was evident (Fig. 1). However, against both syngeneic target tumor lines, B16 and 3LL, MP leukocytes showed significantly higher cytotoxicity than circulating leukocytes, both per NK cell ($F_{(1,10)} = 10.049$ for B16 target cells, and $F_{(1,6)} = 8.072$ for 3LL target cells, $p < 0.05$) and per the entire immune compartment ($F_{(1,10)} = 7.711$ for B16 and $F_{(1,6)} = 17.99$ for 3LL, $p < 0.05$ for both).

Comparing lymphocyte composition between the MP and circulating leukocytes populations

Blood and perfusates were collected from sixteen C57BL/6 mice as described in the "Materials and methods." The ratios of different leukocyte subpopulations, including lymphocytes, granulocytes, NK, NKT, and T cells, were determined based on FACS analysis (see "Materials and methods").

Results: ANOVA revealed significant differences between the two compartments in the percentage of NK and T cells, but not NKT, within the total lymphocyte population (see Table 1). ($F_{(1,15)} = 6.57$ for %NK, $F_{(1,15)} = 4.96$ for % T cells, $p < 0.05$ for both). No other differences were evident.

Effects of immunostimulation with IL-12 on NK cytotoxicity and on expression of cell surface markers

IL-12 administration A total of 45 C57BL/6 mice were treated with IL-12 as follows: in one study, the effect of a single administration of one of the two doses of IL-12 (0.1 or 0.5 µg/mouse) was evaluated. In the second study, a single administration of IL-12 was compared to a three-injection regimen (every other day). In both paradigms, the last IL-12 injection was given 24 h before blood and lung perfusate were collected and processed as described in “Materials and methods.”

Results

a. NK cytotoxicity per NK cell against YAC-1 target cells

For the following presentation and statistical analysis, we combined the two studies, as they both had the control group and the conditions of one injection of 0.1 µg/mouse. The studies also showed similar pattern of results. A two-way ANOVA indicated that IL-12 administration elevated NK cytotoxicity in all groups in both compartments (for circulating leukocytes: $F_{(3,23)} = 44.914$, for MP leukocytes: $F_{(3,33)} = 13.585$, $p < 0.05$ for both). No significant differences were observed between groups that received the different doses of IL-12 (first study) or different numbers of injections (second study). No differences were evident between circulating and MP leukocytes in the above indices (Fig. 2), although when analyzing only the highest three E:T ratios, MP-NK cytotoxicity is higher than circulating NK cytotoxicity ($F_{(1,48)} = 7.703$, $p < 0.01$).

b. NK cells and cell surface markers expression

- *%NK cells:* A two-way ANOVA yielded significant main effects for IL-12 treatment ($F_{(3,38)} = 11.869$, $p < 0.0001$) and for compartment ($F_{(1,38)} = 4.575$, $p < 0.05$) on the percentage of NK cells within the lymphocyte population (Fig. 2), without significant interaction. Analyzing each compartment separately, in the circulation, all doses of IL-12 caused a significant elevation (1.7-, 2.45, and 1.8-fold) of %NK ($F_{(3,38)} = 5.833$, $p < 0.05$, Fisher's PLSD, $p < 0.05$). In the MP compartment, the 0.1 and 0.5 µg/mouse administrations significantly elevated % NK cells (1.7-fold or 2.59-fold, respectively, $F_{(3,38)} = 10.571$, $p < 0.0001$, Fisher's PLSD, $p < 0.05$), but not the repeated administration.
- *NKG2D expression level on NK cells:* Two-way ANOVA revealed a main effect for IL-12 treatment ($F_{(3,38)} = 29.638$, $p < 0.0001$), but not for compartment, nor a significant interaction. NKG2D expression levels in circulating NK cells were significantly elevated after all IL-12

administrations, and only after the acute IL-12 administrations in MP-NK cells ($F_{(3,38)} = 32.535$ for circulating NK cells, $F_{(3,38)} = 14.529$ for MP-NK cells, $p < 0.05$, Fisher's PLSD $p < 0.05$ in both) (Fig. 3a). In both immune compartments, the administration of 0.5 µg/mouse IL-12 increased NKG2D expression more than the administration of 0.1 µg/mouse (Fisher's PLSD $p < 0.05$).

- *CD11a expression levels on NK cells:* no significant differences were observed between circulating and MP-NK cells in CD11a expression (Fig. 3b). On MP-NK cells, all IL-12 treatments caused an elevation in the CD11a expression levels ($F_{(3,38)} = 10.696$, $p < 0.05$, Fisher's PLSD, $p < 0.05$). In circulating NK cells, only the administration of 0.1 µg IL-12/mouse (both acute and repeated $\times 3$ administration) reached a significant elevation ($F_{(3,38)} = 4.317$, Fisher's PLSD $p < 0.05$). FACS analysis showed that all leukocytes expressed CD11a.
- *CD11a expression levels on NKT cells:* Interestingly, the expression levels of CD11a on NKT cells were significantly different when comparing the cells from the two compartments (Fig. 3c): two-way ANOVA revealed a main effect for the compartment and for IL-12 treatment. Circulating NKT cells expressed higher levels of CD11a than the MP-NKT population ($F_{(1,38)} = 20.976$, $p < 0.0001$), and IL-12 significantly elevated CD11a expression levels ($F_{(3,38)} = 8.594$, $p < 0.05$). In the MP compartment, IL-12 caused a dose-dependent elevation in CD11a level of expression on NKT cells ($F_{(3,38)} = 4.333$, $p < 0.05$, Fisher's PLSD, $p < 0.05$). In the circulation, only the chronic administration and the acute 0.1 µg/mouse dosage caused a significant effect ($F_{(3,38)} = 4.517$, $p < 0.05$, Fisher's PLSD, $p < 0.05$, the 0.5 µg/mouse IL-12 treatment was not significantly different from the control group, PLSD, $p = 0.081$).
- *CD11a expression on T cells:* ANOVA showed no significant effects (not shown).

We also performed an analysis beyond dosage or mode of administration for IL-12 (presented in Fig. 4)

- CD69 expression on NK cells:* A two-way ANOVA showed that CD69 expression on NK cells was higher in the circulation than in the MP compartment ($F_{(1,15)} = 13.328$, $p < 0.05$). IL-12 administrations had no effect.
- CD11a expression on NK cells:* A two-way ANOVA showed main effects for compartment ($F_{(1,24)} = 8.546$, $p < 0.05$), IL-12 treatment ($F_{(1,24)} = 19.835$, $p < 0.001$), and their interaction ($F_{(1,24)} = 4.94$, $p < 0.05$). In the circulation, IL-12 treatment elevated expression levels by 1.36-fold ($F_{(1,24)} = 12.5$, Fisher's PLSD, $p < 0.05$) and in the MP compartment only by 1.08-fold ($F_{(1,24)} = 7.498$, Fisher's PLSD, $p < 0.05$).

- c. *IL-12 receptor expression on NK cells*: Two-way ANOVA showed that the expression levels of IL-12R were significantly higher on MP-NK cells than on circulating NK cells ($F_{(1,23)} = 28.625$, $p < 0.05$). ANOVA also showed a significant interaction for compartment and IL-12 treatment ($F_{(1,22)} = 4.844$, $p < 0.05$), such that IL-12 increased levels of IL-12R expression on MP-NK cells and decreased their levels in circulating NK cells. There was no main effect for IL-12 treatment.
- d. *IFN γ receptor expression on NK cells*: MP-NK cells significantly expressed higher levels of IFN γ receptor ($F_{(1,20)} = 46.122$, $p < 0.05$). No effect was evident for IL-12 administration.
- e. *IFN γ receptor expression on T cells*: Expression levels was higher on MP-T cells ($F_{(1,21)} = 29.125$, $p < 0.05$), and no significant effect was evident for IL-12 treatment.
- f. *IFN γ receptor expression on NKT cells*: Expression levels of IFN γ receptor were higher on MP-NKT cells than on circulating NKT cells ($F_{(1,21)} = 9.182$, $p < 0.05$). No effect for IL-12 administration was evident.
- g. *IL-12 receptor expression on granulocytes*: No difference between compartments or following treatment was evident (not shown in Fig. 4).
 - *Percentage of NK cells and of granulocytes expressing CD11b*: ANOVA showed no significant effect for compartment. IL-12 treatment elevated the percentage of NK cells and granulocytes expressing CD11b in both immune compartments ($F_{(1,15)} = 14.228$ for NK cells and $F_{(1,15)} = 8.477$ for granulocytes, $p < 0.05$ for both) (Fig. 5).

NKG2D and IL-12R expression on NK cells correlate with YAC-1 cytotoxicity in circulating but not in MP leukocytes

NK cells are known to employ various cytotoxicity pathways, relying on various membrane determinants, and therefore, we assessed correlations between the cytotoxicity in each compartment and the expressions levels of NKG2D and IL-12 receptors.

Results: The analysis below is conducted beyond dosage or mode of IL-12 administration, as there were no evident differences between these different conditions in NK cytotoxicity or in cell surface markers. IL-12R expression on circulating NK negatively correlated with circulating NK cytotoxicity (Pearson's correlation coefficient = 3.24, $p < 0.05$). The positive correlation between NKG2D expression on circulating NK and circulating NK cytotoxicity ($r = 0.587$) nearly reached significance levels (Pearson's correlation coefficient = 2.098, $p = 0.07$). In the MP compartment, no correlations between NK cytotoxicity and the expression of these markers were found.

Discussion

We previously showed in F334 rats that the MP immune compartment is characterized by elevated NK cytotoxicity against a syngeneic "NK resistant" tumor line (MADB106) [3].

We also reported differences in cellular composition and cell surface marker expression between the two compartments. The objectives of the current study were (1) to test whether similar differences in NK cytotoxicity between the MP- and the circulating leukocyte populations exist in mice and (2) to start studying specific molecular markers and potential mechanisms that could underlie differences in NK cell cytotoxicity between the two compartments. Overall, our current results in mice strengthen our findings in rats and provide initial indications for specific mediating molecular mechanisms that may underlie the improved MP-NK capacity to recognize, adhere, and initiate a cytotoxic response.

Differences in cytotoxicity levels

NK cytotoxicity was higher in the MP compartment, both per leukocyte and per NK cells, when aliquots of the same samples were tested against the syngeneic cell lines (B16 and 3LL tumor lines), but not against the allogeneic YAC-1 target line. In addition, in all three target cell lines, cytotoxicity per ml blood decreased at the two highest E:T ratios, a common phenomenon in NK cytotoxicity assays. In the MP compartment, however, cytotoxicity continues to increase, or did not decrease, up to the highest E:T ratio, when tested against the syngeneic tumors, but not against the YAC-1 target line. Because the overall numbers of leukocytes tested were similar in the two compartments, these observations are most likely related to different activation levels of different leukocyte subpopulations (including NK cells), to differences in cellular composition between the two compartments, and/or to differences in cytotoxic mechanisms used by leukocytes in each compartment against the syngeneic tumor lines. The higher cytotoxicity exhibited by MP leukocytes against syngeneic cell lines also insinuates that MP leukocytes may lyse various aberrant self-cells more efficiently than circulating leukocytes, and thus, immune system may play a greater role in immunosurveillance than is often assumed based on testing circulating leukocytes.

The MP lymphocyte population in mice contains a higher percentage of NK cells than its counterpart in the circulation; however, this study also indicated increased MP-NK cytotoxicity per NK cell. As suggested in Melamed et al. [2], and in the current study, the other constituents of the immune microenvironment (e.g., macrophages, dendritic cells, granulocytes, T cells) differ in their composition between the two immune compartments. It is therefore possible that in mice, like in rats, a dominant prevalence of pro-inflammatory innate leukocytes contributes to the elevated MP-NK cell cytotoxicity.

When tested against YAC-1 target cells, circulating NK cytotoxicity, but not MP-NK cytotoxicity, positively correlated with NK cell expression of NKG2D. YAC-1 cells express NKG2D ligands constitutively [16, 17], as opposed to B16 cells [17], and are thus prone to NKG2D-mediated cytotoxicity. NK cells are known to employ additional cytotoxic pathways, including those dependant on natural cytotoxicity receptors (NCR) signaling (e.g., NKp44 and NKp46), which are important activating receptors for the anti-tumor and anti-viral activity of NK cells [12]. The different correlations exhibited between cytotoxicity and the cell surface markers expression in the two compartments suggest a different mechanism of NK versus YAC-1 cytotoxicity, such that in the MP compartment, the cytotoxicity exhibited is probably less NKG2D-dependent and more NCR-dependent.

Further evidence supporting different cytotoxic mechanisms stems from the differences in correlations of IL-12R expression and YAC-1 cytotoxicity between the two compartments (Fig. 6). IL-12R expression on NK cells correlates with YAC-1 cytotoxicity in circulating but not in MP leukocytes. Activation of NK cells by IL-12 is a major pathway for enhancing NK cytotoxicity. However, since IL-12 was administered 24 h before testing cytotoxicity, it is feasible that the IL-12R were blocked or internalized after binding to IL-12, possibly in a differential manner between the two immune compartments. Therefore, it is hard to interpret the significance of IL-12R expression in these circumstances, besides being immune compartment specific.

Markers of inflammation

Our results support the hypothesis that the MP immune compartment in mice is pro-inflammatory and immunologically activated. IFN γ -R expression levels by T and NK cells were higher in the MP compartment. It was found that several cytokines, including the proinflammatory IL-1 β and TNF α , can increase in IFN γ -R expression, resulting in an enhanced ability of the cells to respond to lower concentrations of IFN γ [18]. Additionally, secretion of IFN γ was found to be higher in lung mononuclear cells (MNC), compared to spleen MNC, and these increased levels were suggested to play a role in natural resistance against B16 lung metastasis [19]. Taken together, these observations indicate an elevation of IFN γ -R during inflammatory processes and enhancement of the inflammatory response. Therefore, in our study, the elevated MP IFN γ -R expression also strengthens our claim that the MP compartment is pro-inflammatory and immunologically activated.

The IL-12 receptor (IL-12R) appears to be an essential component of activation in T and NK cells, as well as in macrophages and dendritic cells. IL-12 induces proliferation, the expression of cytotoxic mediators, and the production of cytokines, including IFN γ [20]. In the current study, we found that MP-NK cells of naïve mice express a higher level of IL-12R. Administration of IL-12 to mice increased both IL-12R and NK cytotoxicity in the MP compartment, more than in the circulation (as shown in Figs. 2a, 4). Taken together, it seems that the MP compartment is able to respond more efficaciously to IL-12 stimulation, perhaps even to lower levels of IL-12, compared to the circulation.

Interestingly, IL-12 treatment increased the proportion of CD11b^{bright} NK cells and granulocytes in both the MP compartment and the circulation. It was suggested by other studies that high levels of CD11b on NK and granulocytes might reflect a local chemotactic pro-inflammatory signal, or recruitment of activated cells to this region [21, 22]. NK cells expressing high levels of CD11b showed greater responsiveness to dendritic cells, IL-12, and IL-18 (resulting in increased IFN γ production) and were shown to be involved in the rejection of major histocompatibility complex (MHC) class I-negative tumors [23]. It is feasible, however, that if lower levels of IL-12 (perhaps physiological levels) were used, differential impacts on this marker would be evident. Indeed, looking only at the groups treated with IL-12, where the variance is smaller than in non-treated animals, the MP compartment showed a trend toward a higher level of this index (Fig. 5).

The interpretation of two findings in our study could be controversial and may or may not support our suggestion pertaining to the pro-inflammatory nature of the MP compartment.

CD11a is considered to be a marker of activation; however, its expression on MP-NKT cells was lower than on circulating NKT cells. It was reported that lack of CD11a on NKT, NK, and CTL might impair conjugate formation with target cells such as YAC-1, as it provides strong adhesion to target cells, which is required for lysis [24–26]. Nevertheless, it could be suggested that in the MP compartment, the role of NKT cells is to produce cytokines, rather than to lyse aberrant cells—a role reserved to MP-NK cells, which did show higher CD11a expression. Alternatively, CD11a expression was found to have a crucial role in Th-2-polarizing functions of NKT cells [27]. It is therefore possible that the reduced CD11a expression level on MP-NKT cells (compared to circulating NKT) indicates a Th-1-prone MP environment, which may actually foster a proinflammatory state.

The second finding of inconclusive nature is the lower expression levels of CD69 on MP-NK cells compared to circulating NK cells. Although previous reports suggested that CD69 is a stimulatory receptor, recent insights from CD69 knockout and CD69-blocked mice have shown that CD69 is actually a negative regulator of NK cell response, in part through the secretion of TGF- β [28, 29]. Furthermore, CD69 targeting by a non-depleting anti-CD69 antibody increased anti-tumor responses by enhancing NK cytotoxicity and IFN γ production [30]. In our study, the expression of CD69 in MP-NK cells is lower than in circulating NK cells and accordingly may contribute less inhibitory signals on this active population.

To better define and understand the MP population, one can compare it to leukocyte populations from other allegedly activated immune compartments, specifically the hepatic compartment and hepatic NK cells (Pit cells) in rats. In 1976, Wisse et al. [31] were the first to describe a unique population of NK cells, situated in the wall of rat liver sinusoids, which express high levels of natural cytotoxicity against a variety of tumor cell lines [32]. More recent studies suggested additional unique characteristics and several mechanisms for high hepatic rat NK cell cytotoxicity, including the involvement of NKG2D, CD11a, and type-1 IFN, and suggested pit cells as targets for liver immunotherapy against metastatic disease [33, 34]. In our ongoing studies in rats, we compare MP leukocytes to both circulating and hepatic leukocytes and measure mRNA and induced secretion of several pro- and anti-inflammatory cytokines in these three compartments (manuscript in preparation). Our results thus far demonstrate that the MP and marginating-hepatic immune compartments are similar in being more active and proinflammatory than circulating leukocytes in these indices.

Although this study suggests potential molecular mechanisms that are likely to underlie enhanced MP-NK cytotoxicity against syngeneic tumor cells, it does not unequivocally implicate any of these mechanisms. Another shortcoming of the study is that purified NK cells from the two compartments were not studied. However, the procedure of purification is long and commonly necessitates cell manipulations, which we found to suppress NK cytotoxicity and eliminate compartment differences [2].

Overall, this study extends the observations suggesting that the pulmonary immune compartment has unique characteristics of activated leukocytes, allowing MP-immunocytes to immediately and effectively react toward circulating pathogens or malignant or aberrant

cells. This assertion is based on our findings regarding expression levels of various membrane markers and NK cytotoxicity levels against syngeneic target tumor lines.

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Fig. 1. MP and circulating NK cytotoxicity against various target cell lines. The *left column* (a, b, and c) represents cytotoxicity per leukocyte against three different cell lines: the allogeneic YAC-1, and the syngeneic B-16 and 3LL. The *right column* (d, e, and f) presents extrapolated data of cytotoxicity per NK cells against these target lines, as described in the “Materials and methods” section. Data are presented as mean $\pm$ SEM

Fig. 2.

Effects of immunostimulation with IL-12 on NK cytotoxicity (per NK cell) and expressions of cell surface marker. Mice were treated with either a single dose of IL-12 (0.1 or 0.5 $\mu\text{g}/\text{mouse}$) or a three-injection regimen (every other day) of 0.1 $\mu\text{g}/\text{mouse}$. In both paradigms, the last IL-12/saline injection was administered 24 h before blood withdrawal and lung perfusate collection. **a** IL-12 administration elevated NK cytotoxicity per NK cell in all groups in both compartments. When analyzing only the highest three E:T ratios, cytotoxicity in MP-NK is higher than in circulating NK. **b** All doses of IL-12 caused a significant elevation in %NK of the lymphocyte population. In the MP compartment, the 0.1 and 0.5 $\mu\text{g}/\text{mouse}$, but not the repeated administration paradigm, significantly elevated % NK cells. Data are presented as mean $\pm$ SEM

Fig. 3.

Expression levels of NKG2D and CD11a following IL-12 administration. In both the MP compartment and the circulation, IL-12 administration caused an elevation in the expression levels of NKG2D (a) and CD11a (b) on NK cells, and of CD11a on NKT cells (c).

Additionally, MP-NKT cells exhibited lower expression levels of CD11a than circulating NKT cells (d). Data are presented as mean $\pm$ SE

Fig. 4.

Effects of IL-12 on various cell surface markers on MP and circulating leukocytes. **a** CD69 expression on NK cells was higher in the circulation than in the MP compartment and IL-12 administrations had no effect. **b** IL-12 administration elevated CD11a expression in circulating NK more than in MP-NK cells. **c** The expression level of IL-12R was significantly higher in MP-NK cells than in circulating NK cells. IL-12 increased levels of IL-12R expression on MP-NK and decreased their levels in circulating NK cells. There was no main effect for IL-12 treatment. **d**, **e**, and **f** IFN γ -R expression in MP-NK, MP-T, and MP-NKT cells were higher than in circulating NK, T, or NKT cells, respectively, with no effect for IL-12 administration. Data are presented as mean $\pm$ SE

Fig. 5.

Percentage of NK cells and of granulocytes expressing CD11b. IL-12 treatment elevated the percentage of NK cells **(a)** and percentage of granulocytes **(b)** which express CD11b, in both the circulating and MP immune compartments. No significant differences between the compartments were evident in either index. Data are presented as mean $\pm$ SEM

Fig. 6.

IL-12R and NKG2D expression on NK cells correlated with YAC-1 cytotoxicity in circulating leukocytes, but not in MP leukocytes. In the circulation, IL-12R (a) and NKG2D (c) expression levels on NK cells correlated with circulating NK cytotoxicity. In MP-NK cells (b + d), MP-NK cytotoxicity did not correlate with expression levels of these markers

Table 1

Difference in leukocyte composition between the circulating-and MP- immune compartments

	% NK	% NKT	% T
Circulating compartment	4.9 ± 0.3	1.54 ± 0.176	32.22 ± 1.32
MP compartment	6.7 ± 0.69 **	1.33 ± 0.169	29.5 ± 1.63 **

**
 $p < 0.05$