Reprogramming the lung microenvironment by inhaled immunotherapy fosters immune destruction of tumor

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\textbf{ABSTRACT}

Due to their constant exposure to inhaled antigens, lungs represent a particularly immunosuppressive environment that limits excessive immune responses; however, cancer cells can exploit this unique environment for their growth. We previously described the ability of aerosolized CpG-ODN combined with Poly(I:C) (TLR9 and TLR3 agonists, respectively) to promote antitumor immunity in a B16 melanoma lung metastasis model. Here, we explored the possibility of improving the therapeutic efficacy of TLR9/TLR3 agonist combinations by including in the inhalant either an antibody directed to both Ly6G and Ly6C markers to locally deplete myeloid-derived suppressive cells (MDSCs) or IFN\textalpha to directly activate the natural killer (NK) and macrophage innate immune cells in the lung. Addition of nebulized anti-MDSC antibody RB6-8C5 to aerosolized CpG-ODN/Poly(I:C) resulted in reduced mRNA levels of immunosuppressive molecules (IL10, Arg-1, and Nos2), increased activation of resident NK cells and improved treatment outcome, with a significant reduction in established B16 melanoma lung metastases compared to treatment with CpG-ODN/Poly(I:C) alone. Likewise, addition of aerosolized IFN\textalpha led to increased mRNA levels of proinflammatory cytokines (IL15 and IFN\gamma) in the lung and recruitment of highly activated NK cells, with no evident signs of toxicity and with a significantly improved antitumor effect as compared with aerosolized CpG-ODN/Poly(I:C). Combining both IFN\textalpha and RB6-8C5 with CpG-ODN/Poly(I:C) did not produce an additive effect compared to IFN\textalpha + CpG-ODN/Poly(I:C) or RB6-8C5 + CpG-ODN/Poly(I:C). Our results indicate that the inhalation therapy is a feasible and non-invasive strategy to deliver immunodulatory molecules, including antibodies and cytokines that reprogram the lung tumor microenvironment to foster immune destruction of tumors.

\textbf{Abbreviations:} CpG-ODN, oligodeoxynucleotides containing CpG motifs; IFN, interferon; MDSC, myeloid-derived suppressive cells; Poly(I:C), polyinosinic-polycytidylic acid; TAM, tumor-associated macrophages; TLR, toll-like receptor

\section*{Introduction}

The recent clinical success of exploiting “immune checkpoint blockers” demonstrated that cancer can be efficiently treated by targeting immune rather than malignant cells. This change of perspective has spurred renewed interest in the immunosurveillance theory and implies that cancer therapy directed to definitive cures should consist in reinstating the immunological control of tumor growth, using strategies that potentiate immune effector activity or that remove inhibitory signals, such as immunosuppressive cells and immune checkpoints or both.

Due to constant exposure to the external environment, host immune components in the lung create a particular context in which carcinogenesis can proceed supported by a significant population of immunosuppressive cells, which engage in cross-talk with each other and with malignant cells. In the innate immune system, the main immunosuppressive cells enclose both resident pulmonary macrophages, which play a physiological role in maintaining immunological homeostasis by downmodulating local immune responses to antigens deposited on respiratory epithelial surfaces, and myeloid-derived suppressor cells (MDSCs), a heterogeneous population of immature bone marrow-derived cells that progressively accumulate within the tumor mass and suppress effector cell function. Studies have demonstrated the suppressive effect of MDSC both on T cells, involving mechanisms such as amino acid depletion, induction of oxidative stress, interference with their trafficking and expansion of T regulatory cells,\textsuperscript{1} and on NK cells, mostly through cell contact-dependent mechanisms involving membrane-bound TGF-\textbeta1 and recognition of NKG2D, the primary activating receptor for NK cells.\textsuperscript{2-5} MDSC and macrophages reportedly establish a continuous bidirectional cross-talk through soluble mediators and cell contact, resulting in increased production of IL10, decreased...
production of IL12 and IL6, downregulation of macrophage MHC II expression, and amplified macrophage immunosuppressive activity.6 This highly immunosuppressive environment counters immune effector cytotoxicity and strongly reduces the efficacy of immunotherapeutic approaches. Thus, successful immunotherapy, especially in the lung, depends on modification of both the effector cell-refractory tumor microenvironment and the potency of the effector cells themselves. In a model of B16 murine melanoma lung metastases in which protection primarily requires activation of NK effector cells, we showed that aerosolized Poly(I:C), a TLR3 agonist able to convert tumor-associated macrophages (TAM) from tumor-supporting (M2) to tumor-counteracting (M1) function, combined with aerosolized CpG-ODN, a TLR9 agonist able to activate NK cells, significantly improved antitumor effects.7 Our data suggested that aerosolization represents a non-invasive therapy to improve tissue concentration and exposure to immunotherapeutic agents, while limiting exposure and potential adverse effects in healthy organs.

Here, we used aerosolization to locally shape a lung microenvironment that favors NK cell cytotoxicity against B16 melanoma lung metastases, characterized by low immunogenicity. Two inhalation-based immunotherapies directed both at blocking the inhibitory environment and at promoting effector cell activation were used: 1) CpG-ODN/Poly(I:C) combined with an antibody to selectively deplete lung MDSC and therefore enhance NK cell activation directly2-5 and through a reduced cross-talk with macrophages6; and 2) TLR agonists combined with aerosolized IFNα, essential for NK cell priming in response to cancer,8,9 and able to induce the M2 to M1 phenotypic shift in the lung microenvironment.10

Results

Local depletion of MDSC by RB6-8C5 antibody nebulization in the lung increases CpG-ODN/Poly(I:C)-induced NK cell activity

B16 murine melanoma cells reportedly induce high accumulation of MDSC.11-13 Studies have demonstrated a suppressive effect of MDSC on NK cells through cell contact-dependent mechanisms3-5; moreover, MDSC might play an immunosuppressive role in NK cell activation, exacerbating macrophage polarization toward an M2 phenotype.6 To generate a more permissive microenvironment for NK cell activation by aerosolized TLR agonists, we tested the possibility that delivery of nebulized RB6-8C5 antibody, reported to reduce both monocytic and granulocytic subsets from circulating blood,14 locally depletes MDSC. Indeed, flow cytometry analysis of enzymatically digested lungs of C57BL/6 mice injected i.v. with B16 cells and treated 7 d later with RB6-8C5 antibody or saline revealed significant depletion of both granulocytic (Ly6GhighLy6Clow) and monocytic (Ly6ChighLy6Clow) MDSC 24 h after antibody nebulization (Fig. 1). Since both populations were observed to repopulate the lungs already at 96 h after nebulization, to maintain a prolonged depletion of these cells, antibody nebulization was repeated at 72–96 h intervals. Repeated treatment with RB6-8C5 antibody for 2 weeks induced a significant expansion of both CD11b+Ly6C+ and CD11b+Ly6G+ populations in bone marrow and spleen of treated mice (Fig. S1).

Flow cytometry of enzymatically digested lungs to test the effect of MDSC depletion on recruitment and activation of NK cells induced by CpG-ODN/Poly(I:C) aerosolization in mice injected i.v. with B16 cells and treated with aerosolized CpG-ODN/Poly(I:C) alone or combined with nebulized RB6-8C5 antibody revealed enhanced expression of the NK cell CD69 activation marker and of NKG2D activating receptor in the MDSC-depleted samples, but no increase in the recruitment of NK cells (Fig. 2A). No significant difference in the frequency of macrophages, dendritic cells (DCs), monocytes, and T lymphocytes was detectable in lung of mice treated with aerosolized CpG-ODN/Poly(I:C) and RB6-8C5 antibody compared to mice treated with TLR agonists alone (Fig. S2).

Analysis of transcript levels of immunosuppressive markers associated with MDSC suppressive activity3 in infiltrates obtained from digested lungs indicated that RB6-8C5 antibody combined with CpG-ODN/Poly(I:C) strongly induced decreased expression of IL10, Arg-1, and Nos compared to mice treated with CpG-ODN/Poly(I:C) alone (Fig. 2B). In light of the emerging role of macrophages as essential partners in promoting NK cell activation15 and due to their central role in responding to stimulation with TLR ligands,16 we tested whether MDSC depletion increases the ability of CpG-ODN/Poly(I:C)-activated lung macrophages to promote NK cell cytotoxic activity. Mice injected i.v. with B16 tumor cells were treated with aerosolized CpG-ODN/Poly(I:C) alone, or combined with nebulized RB6-8C5 antibody, or with RB6-8C5 antibody alone, or left untreated. At 3 weeks after tumor cell injection, lung suspensions obtained from enzymatic digestion were plated for 2 h at 37°C to separate adherent cells containing macrophages from non-adherent cells containing effector cells. Adherent cells were then cultured with splenic naïve NK cells obtained from healthy mice and 24 h later, cytotoxic activity of NK cells was assessed on YAC target cells. As shown in Fig. 2C, the in vivo combination of nebulized RB6-8C5 with TLR agonists significantly increased the ability of lung-derived immune adherent cells to activate NK cell cytotoxicity, increasing the stimulatory effect of CpG-ODN/Poly(I:C). Treatment with the RB6-8C5 antibody alone did not increase NK cell cytotoxic activity, suggesting that depletion of MDSC alone is not sufficient to promote macrophage-mediated NK cell activation.

Together, these results indicate that aerosolized delivery of RB6-8C5 antibody reaches the bronchoalveolar space, efficiently depletes MDSC from the tumor microenvironment, and enhances NK cell activation by aerosolized CpG-ODN/Poly(I:C).

Aerosolized IFNα reaches the lung and promotes NK cell activation

NK cell priming largely depends on the presence of IFNI( IFNα and β), which can enhance NK cell cytotoxicity and IFNγ production. Moreover, IFNα is the principal inducer of IL15 production, essential for NK cell accumulation and survival.17 Thus, we tested the possibility of delivering IFNα...
to the lung by aerosolization to increase its concentration locally and limit the possible toxicity associated with systemic administration.  

Flow cytometry analysis of immune cells obtained from enzymatically digested lungs of healthy mice treated at 72 h intervals with aerosolized IFNα for 2 weeks revealed a significant increase in the percentage of NK cells and expression of activation markers, as indicated by upmodulation of CD69 on the cell surface, as compared to untreated mice, 24 h after the last aerosolization (Fig. 3A). An increased expression of NKG2D activating receptor on NK cells of IFNα-treated mice was also observed. Moreover, IL-15Ra expression was increased on the NK cell surface (Fig. 3A) and in RNA extracted from lung infiltrates of IFNα-treated mice, transcript levels of IL15 and IFNγ were also significantly upregulated (Fig. 3B). Upmodulation of CD69 marker on NK cells was maintained up to 48 h after aerosolization and returned to level comparable to those of untreated mice 96 h after, enlightening the need for repeated aerosol treatments to maintain local effects of IFNα administration. Treatments with aerosolized IFNα were well-tolerated, as indicated by the absence of overt signs of toxicity, such as weight loss, hunching, ruffled fur or difficulty breathing, and of histological changes in lung structure (Fig. S3).

Based on the reported ability of IFNα to stimulate cells critical for NK effector activation, such as macrophages and DCs, and to promote macrophage-related tumoricidal effects through their polarization to the M1 phenotype, we tested whether IFNα combined with aerosolized CpG-ODN/Poly(I:C) in mice bearing B16 melanoma metastases increases the ability of lung-derived macrophages to promote NK cell cytotoxic activity. As shown in Fig. 3C, adherent cells obtained from lung suspensions of mice injected i.v. with B16 cells and treated with aerosolized IFNα plus CpG-ODN/Poly(I:C) induced a significantly increased cytotoxic activity of naive NK cells against YAC target cells as compared to adherent cells from lungs of mice treated with aerosolized CpG-ODN/Poly(I:C) alone.

These results indicate that aerosolized IFNα reaches the bronchoalveolar space and locally activates an innate immune response without apparent signs of toxicity, improving the macrophage-mediated activation of NK cells by aerosolized CpG-ODN/Poly(I:C).
Figure 2. Effects of myeloid derived suppressive cells (MDSC) depletion on lungs of mice treated with CpG-ODN/Poly(I:C) aerosol combined with nebulized RB6-8C5 antibody. (A) Representative dot plots and percentage (± SEM) of NK cells, evaluated as CD49b<sup>+</sup> cells gated on FSC<sup>low</sup>SSC<sup>low</sup>CD45<sup>+</sup>CD3<sup>-</sup> cells; of CD69<sup>+</sup>NK cells gated on CD49b<sup>+</sup>cells and NKG2D<sup>+</sup>NK cells gated on CD49b cells, in mice untreated or treated with CpG-ODN/Poly(I:C) alone or combined with nebulized RB6-8C5 antibody (5 mice/group); *p < 0.01, **p < 0.001. (B) mRNA levels (mean relative expression ± SEM) of IL10, Arg-1 and NOS2 evaluated by real-time PCR in mice treated with CpG-ODN/Poly(I:C) alone or combined with nebulized RB6-8C5 antibody from two independent experiments (4 mice/group in each experiment). Results are presented as 2<sup>−ΔΔCt</sup>; *p < 0.05. (C) Percent specific lysis of YAC-1 target cells by purified splenic naive NK cells cultured for 24 h with adherent cells obtained by plating lung suspensions from mice untreated or treated with CpG-ODN/Poly(I:C) alone or combined with nebulized RB6-8C5 antibody or with RB6-8C5 antibody alone. Box and whiskers: min to max represent data pooled from two independent experiments (3–4 mice/group); *p < 0.05, **p < 0.01.
Nebulized RB6-8C5 antibody and aerosolized IFNα potentiate the antitumor effect of aerosolized CpG-ODN/Poly(I:C)

To compare the effectiveness of local depletion of MDSC from the lung microenvironment by nebulized RB6-8C5 versus local delivery of IFNα in increasing the antitumor effect of CpG-ODN/Poly(I:C) aerosolization, the combined treatments were started one week after i.v. injection of B16 tumor cells, when multiple microscopic metastatic foci were clearly detectable in the lung (Fig. 4A). Tumor-bearing mice were divided into six groups and treated for 2 weeks at 72 h intervals with aerosolized CpG-ODN/Poly(I:C) alone or combined with RB6-8C5 or IFNα, or with RB6-8C5 or IFNα alone, or left untreated. No body weight loss was observed in mice exposed to aerosolization with both TLR agonists combined with RB6-8C5 or IFNα (data not shown). CpG-ODN/Poly(I:C) aerosol combined with RB6-8C5 or IFNα significantly increased antitumor activity as compared to Poly(I:C)/CpG-ODN aerosol alone (Fig. 4B), whereas no significant antitumor effect was observed after treatment with RB6-8C5 or IFNα alone.

To determine whether the reduced number of lung metastases reflected an increased ability of NK cells to promote antitumor activity, lungs were enzymatically digested and cytotoxic activity of non-adherent immune cells was evaluated using B16 tumor cells as target. As shown in Fig. 4C, the percent lysis was significantly higher in lung suspensions from mice treated with CpG-ODN/Poly(I:C) combined with RB6-8C5 antibody or IFNα than in those from mice treated with CpG-ODN/Poly(I:C) alone. Lysis of YAC-1 cells by non-adherent immune cells was similarly increased (data not shown), confirming the involvement and in vivo activation of NK cells in the antitumor response.

Analyses to test for a possible additive effect of CpG-ODN/Poly(I:C) aerosol combined with RB6-8C5 nebulization and IFNα aerosolization in mice injected with B16 cells and treated 1 week later or left untreated confirmed the reduction in the number of lung metastases after combining CpG-ODN/Poly(I:C) with RB6-8C5 antibody or IFNα, but revealed no additive effect in mice treated with CpG-ODN/Poly(I:C) combined with both reagents (Fig. 4D).

Together, these results indicate that an immunotherapeutic strategy for lung disease based on direct delivery of aerosolized CpG-ODN/Poly(I:C) combined with nebulized anti-MDSC antibody or aerosolized IFNα significantly increases the antitumor activity of these TLR agonists.

**Discussion**

Here, we show that an inhaled combination of immunotherapeutic agents can favor a lung micro-environment that permits effective immune destruction of tumors through restored activity of effector NK cells. Considering the particular environment in the lung, in which the local immunosuppressive milieu limits the potential immune-mediated tissue damage from constant exposure to inhaled environmental antigens/allergens, cancer arising in this organ easily promotes immune tolerance and escapes host immune surveillance.
Because immune checkpoint inhibitors are specifically directed to receptors expressed mostly by T cells, consistent with the absence of antitumor activity against B16 lung metastases upon treatment with anti-PD-1 antibody (unpublished data), we evaluated the usefulness of RB6-8C5 nebulized antibody, to deplete MDSC and thereby create a more permissive microenvironment for NK cell activation by aerosolized TLR agonists. Airway-delivered RB6-8C5 antibody, directed to both Ly6G and Ly6C, at 72–96 h interval effectively depleted MDSC recruited in the lung tumor microenvironment at a dose 4- to 8-fold lower than that used in systemic administration, improving antitumor effects of aerosolized TLR agonists. Although RB6-8C5 antibody is not only MSDCs specific, but can also target other Ly6G- or Ly6C-expressing cells, at a late time point of the experiment no differences in the percentage of macrophages, DCs, monocytes and CD8+ T cells were observed in the lungs. To note, an increased percentage of CD11b+Ly6C+ and CD11b+Ly6G+ cells was observed in bone marrow and spleen of RB6-8C5 treated mice, accordingly with the reported observation that the antibody treatment may expand bone marrow myeloid cells. The direct targeting of the affected organ is an attractive option to increase the benefits of treatment by enhancing tissue concentration and exposure to the agent, while limiting adverse effects and exposure of healthy organs/cells. The feasibility of using nebulized therapeutic antibodies was demonstrated in a study showing that airway-delivered cetuximab, an anti-EGFR antibody, accumulated rapidly in cancerous lung tissue at concentrations twice those achieved after i.v. delivery and in a study on an anti-influenza virus neutralizing monoclonal antibody (MAb) that revealed higher concentrations of the antibody in the bronchoalveolar lavage fluid (BAL), with low penetration into the bloodstream upon direct delivery to the respiratory tract. Moreover, local delivery of an anti-VEGF MAb to K-ras-induced adenocarcinoma-bearing lungs efficiently reduced tumor burden at ~100-fold lower serum concentration of MAb than that after

**Figure 4.** Antitumor effects of nebulized RB6-8C5 antibody and aerosolized interferon α (IFNa) combined with CpG-ODN/Poly(I:C) in mice bearing B16 melanoma lung metastases. (A) Representative images of multiple metastatic foci in hematoxylin and eosin-stained lung tissue sections from mice at day 7 after i.v. injection of B16 melanoma cells. Magnification 200x. (B) Mean number of B16 melanoma lung metastases in mice injected i.v. with B16 melanoma cells and untreated (7 mice) or treated starting at day 7 at 72-h intervals for 2 weeks with nebulized RB6-8C5 alone (6 mice), aerosolized IFNa alone (6 mice), aerosolized CpG-ODN/Poly(I:C) alone (8 mice), aerosolized CpG-ODN/Poly(I:C) combined with RB6-8C5 (8 mice) or IFNa aerosol (7 mice); "p < 0.05, ""p < 0.01. (C) Percent specific lysis of B16 target cells by non-adherent cells obtained by plating suspensions of digested lungs from untreated or treated mice after 24 h co-culture. Box and whiskers: min to max represent data pooled from two independent experiments (3–4 mice/group); "p < 0.05, ""p < 0.01. (D) Mean number of B16 melanoma lung metastases in mice injected i.v. with B16 melanoma cells untreated (6 mice) or treated starting at day 7 at 72-h interval for 2 weeks with aerosolized CpG-ODN/Poly(I:C) combined with RB6-8C5 (9 mice) or with IFNa (9 mice) or with RB6-8C5 and IFNa (9 mice).
systemic delivery.\textsuperscript{24} To our knowledge, ours is the first study to evaluate this route in administering an antibody to deplete a suppressive immune population. Our data indicate that while MDSC depletion by the nebulized antibody did not enhance NK cell recruitment induced by CpG-ODN/Poly(I:C), depletion did lead to increased NK cell activation, possibly by minimizing direct inhibition of these effector cells and/or by enhancing the ability of aerosolized CpG-ODN/Poly(I:C)-activated macrophages to promote NK cell cytotoxicity. Accordingly, Mattiola et al.\textsuperscript{15} have demonstrated \textit{in vitro} that LPS/IFNγ-polarized M1 macrophages, but not M0 or IL4-polarized M2 macrophages, can prime resting autologous NK cells. Thus, our \textit{ex vivo} results indicating that nebulized RB6-8C5 combined with TLR agonists significantly increases the ability of lung-derived immune adherent cells to activate cytotoxicity of naive NK cells suggest that MDSC depletion also favors reprogramming of macrophages to the M1 phenotype. Consistent with our results, systemic MDSC depletion by RB6-8C5 antibody in a model of murine Lewis lung carcinoma induced increased antitumor NK and T cell activity that was related to enhanced functional activity of antigen-presenting cells, such as macrophages and DCs, in the tumor.\textsuperscript{19}

To directly increase the potency of NK cells, we aerosolized CpG-ODN/Poly(I:C) together with IFNα, a proinflammatory cytokine essential for NK cell priming. Type I IFN has been used successfully to treat infections and several types of cancer, based on its ability to promote NK cell activation. For example, IFNα is used as an adjuvant therapy after surgery to prevent recurrence of melanoma, although side-effects upon systemic treatment are substantial.\textsuperscript{25} Our results indicate that aerosol-delivered IFNα reaches lung NK cells, promotes their recruitment and activation, and upregulates IL15/IL-15Ra expression necessary for NK cell priming. Notably, we detected no evident signs of toxicity on the structure of lung parenchyma or on the weight and behavior of mice, consistent with data indicating that aerosol-delivered IFNα in patients with pulmonary tuberculosis reaches the lower respiratory tract without systemic side-effects.\textsuperscript{26}

No significant antitumor effect was observed in mice treated with aerosolized IFNα alone, consistent with the limited antitumor effect reported in some pioneer studies in patients with advanced bronchio-alveolar carcinoma treated with inhaled IFNα alone.\textsuperscript{27,28} However, in combination with TLR9 and TLR3 agonists, which strongly activate an innate immune response while reducing M2 polarization, IFNα improved NK-mediated protective immunity, raising the possibility that TLR agonists create a microenvironment favorable for immune stimulation by IFNα. Additive immunostimulatory effects by IFNα and CpG-ODN were observed in an intraperitoneal model of melanoma systemically treated with IFNα and CpG-ODN,\textsuperscript{29} and a synergistic effect of CpG-ODN and IFNα on DC maturation was found in mice bearing MC38 colorectal cancer treated with DCs pre-incubated with IFNα-secreting cells and CpG-ODN.\textsuperscript{30} In addition to its direct effects on NK cells, IFNα is known to stimulate other cell types critical for NK effector activation, such as macrophages and DCs,\textsuperscript{17} and to promote a shift of macrophages to tumoricidal effectors, as shown in a model of lung metastases induced by hepatocellular carcinoma cells, wherein systemic IFNα treatment directly transformed the lung microenvironment by modulating macrophage polarization.\textsuperscript{10} Accordingly, we found that adherent cells from lung immune infiltrates of B16 metastases-bearing mice treated with CpG-ODN/Poly(I:C)/IFNα aerosol significantly increased cytotoxic activity of naive NK cells, as compared to cells from mice treated with CpG-ODN/Poly(I:C) alone.

The two different inhaled combinations showed a similar level of antitumor effect, i.e., up to 79% reduction in mean number of lung metastases. Unexpectedly, no additive effect was observed in mice treated with CpG-ODN/Poly(I:C) combined with both IFNα and MDSC-depleting antibody. Further experiments are needed to assess a possible interference between IFNα and the MDSC-depleting antibody and to evaluate other doses or time frames of administration.

Together, our results indicate that an effective antitumor response can be obtained against established lung multiple foci of a highly aggressive tumor using airway delivery as a convenient and non-invasive way to administer immunotherapy. This proof-of-principle study points to the promise of rational combinations of immunostimulants, cytokines and antibodies delivered locally by inhalation to reprogram the immune tumor microenvironment as a novel strategy to treat lung cancer patients.

\textbf{Materials and methods}

\textbf{Mice and experimental protocols}

Experiments were conducted using 6–8-week-old female C57BL/6 mice (Charles River, Calco, Italy) maintained in laminar flow rooms at constant temperature and humidity, with food and water given \textit{ad libitum}. Experiments were approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale dei Tumori of Milan, according to the Declaration of Helsinki conventions for the use and care of animals. Mice were injected intravenously (i.v.) with $5 \times 10^5$ B16 melanoma cells and treated 7 d later at 72 h intervals for 2 weeks. CpG-ODN1826, Poly(I:C) and Intron A were aerosolized using a whole-body exposure system (EMMS, Edinburgh, Scotland, UK) as described.\textsuperscript{31} Briefly, Poly(I:C) (15 mg), CpG-ODN (1.5 mg) or Intron A (4000.000 IU) were dissolved in 5 mL of saline and placed in the nebulizer to treat up to 10 mice placed in the aerosol box and exposed to aerosol for 15 min. The MAb RB6-8C5 was administered endotracheally.\textsuperscript{22,24} Briefly, mice were anesthetized and given 25 μg/mouse of RB6-8C5 MAb using a Microsprayer Aerosolizer Model IA-1C connected to a FMJ-250 high-pressure syringe (Pen-Century, Philadelphia, PA, USA) introduced just before the first trachea bifurcation.\textsuperscript{32} In all experiments, mice were weighed twice weekly, euthanized at the end of experiments and macroscopic lung metastases counted. All \textit{in vivo} experiments were repeated at least twice.

\textbf{Cell lines and reagents}

B16 mouse melanoma cells and YAC-1 mouse lymphoma cells were routinely maintained at 37°C in a 5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium (DMEM, Gibco By Life technologies, 41965-039) and RPMI-1640
Lungs from healthy or tumor-bearing mice untreated or treated were digested in DMEM medium containing collagenase and hyaluronidase as described above. Cell suspensions obtained after lysis of red blood cells were incubated with QIAzol reagent (QIAGEN, 79306) and total RNA was isolated according to the manufacturer’s instructions. Reverse transcription was performed using the high capacity RNA-to-cDNA kit (Applied Biosystems By Life Technologies, P/N 4387406) and real-time PCR was performed with TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, P/N 4352042) and SDS 2.4 software with a 7900HT Fast Real Time PCR System (Applied Biosystems), using the following TaqMan® gene expression assays: Arg1 (assay Mm00475988_m1), Nos2 (assay Mm00440502_m1), IL10 (assay Mm01288386_m1), IFNγ (assay Mm01168134_m1), IL15 (assay Mm00434210_m1), and IL15Ra (assay Mm04336046_m1). Expression of each gene was normalized to ActB (TaqMan assay Mm00607939_s1) and B2M (TaqMan assay Mm00437762_m1).

In vitro cytotoxicity assays

The ability of adherent cells from lung immune infiltrates of mice to modulate activity of splenic naïve NK cells was evaluated by measuring cytotoxic activity on 51Cr-YAC-1 target cells. Lungs from tumor cell-injected mice untreated or treated were digested in DMEM medium containing collagenase/hyaluronidase and suspensions were plated for 2 h at 37°C to separate adherent cells containing mostly macrophages from non-adherent cells containing effector cells. Adherent cells were washed and cultured at a 1:1 cell ratio with naïve NK cells purified from spleen of C57BL/6 mice by NK Cell Isolation Kit II mouse (Miltenyi, 130-096-892). After 24 h, non-adherent cells were collected and incubated for 4 h at 37°C at an effector:target ratio of 50:1 in triplicate 96-well U-bottomed plates with YAC-1 target cells labeled with 100 μCi 51Cr (PerkinElmer) for 4 h at 37°C and washed three times with PBS-5% FCS. Radioactivity of the supernatant (80 μL) was measured with a Trilux Beta Scintillation Counter (PerkinElmer). Percent specific lysis was calculated as: 100 × (experimental cpm–spontaneous cpm)/(maximum cpm–spontaneous cpm). To assess cytotoxic activity of effector cells of untreated or treated mice, lungs were enzymatically digested as above and lung suspensions were plated for 2 h at 37°C to collect non-adherent cells containing effector cells. B16 melanoma and YAC-1 target cells were labeled with 51Cr as above and incubated for 4 h at 37°C with non-adherent cells at the effector:target ratio of 50:1 and percent specific lysis was calculated as above.

Statistical analysis

PCR data were analyzed using the 2^(-ΔΔCt) method. In all experiments, differences among groups were compared using two-tailed unpaired Student’s t-test and considered significant at p ≤ 0.05.
Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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