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# Polyinosinic-Polycytidylic Acid Limits Tumor Outgrowth in a Mouse Model of Metastatic Lung Cancer

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**Polyinosinic-polycytidylic acid (poly I:C), a TLR3 ligand, is currently being tested in human clinical trials as an adjuvant to anti-cancer vaccines and in combination with other therapies. However, little is known about its activity in established pulmonary metastasis. The aim of our study was to elucidate the effect of poly I:C (1, 10, or 100  $\mu\text{g}/\text{mouse}$ ) in a mouse model of B16-F10–induced metastatic lung cancer. Lung tumor growth was arrested after a single administration of poly I:C. This was associated with higher influx of mature dendritic cells (DCs), which drove toward a Th1-like, Th17-like, and cytotoxic immune environment. The interference with IFN type I receptor signaling by means of a specific mAb reversed poly I:C-mediated tumor regression due to lower presence of myeloid DCs, cytotoxic DCs (CD11c<sup>+</sup>CD8<sup>+</sup>), NKT cells, CD8<sup>+</sup> T cells, and Th1-like cytokines. Moreover, the adoptive transfer of poly I:C-activated bone marrow-derived DCs into tumor-bearing mice resulted in activities similar to those of the systemic administration of poly I:C on lung tumor burden. In conclusion, our data prove that poly I:C has potential anti-tumor activity in a mouse model of established pulmonary metastasis. The activation of DCs and the production of IFN type I are responsible for an effective T cytotoxic immune response against metastatic lung cancer progression after poly I:C treatment. *The Journal of Immunology*, 2012, 188: 5357–5364.**

**M**etastatic lung cancer is one of the leading causes of death worldwide. Despite advances in treatment, the prognosis remains poor, with only 15% of patients surviving more than 5 y from the time of diagnosis. It is a low immunogenic cancer, resistant to the surveillance of the immune system (1, 2), which should protect the body from tumor development by recognizing cancerous cells. The process of shaping the immunogenicity of tumors has been termed “cancer immune-editing,” which relies upon the activation of the adaptive immune system to recognize and eliminate “transformed” cells (3). Therefore, the activation of the immune system could represent a means to induce tumor regression (2, 3). Special attention is being given to adjuvants or enhancers of immunity for cancer therapy (4). Adjuvants activate the innate immune system and in particular induce the maturation of APCs such as dendritic cells (DCs) (4, 5). Therefore, the main activity of adjuvants is to stimulate the innate immunity via the stimulation of pattern recognition receptors, including TLRs (6). DCs express a repertoire of pattern recognition receptors, which engagement leads to the activation of the adaptive immunity. The type of stimulus conditions DCs to adopt a Th1 or Th2 polarization (7).

Several TLR agonists are currently being tested as adjuvants for anti-cancer vaccines and therapies (1, 4). This study is focused on the effect of the synthetic dsRNA polyinosinic-polycytidylic acid (poly I:C) in a mouse model of metastatic lung cancer. Poly I:C is recognized by TLR3 and melanoma differentiation-associated protein-5 (MDA5), which induce the activation of transcription factors such as IRF3, IRF7, and NF- $\kappa$ B that promote the release of IFN type I (IFN- $\alpha$  and IFN- $\beta$ ; proinflammatory cytokines involved in Ag presentation) (6). Because poly I:C promotes the activation of the innate immunity and thus of long-lasting T cell immunity (8), it is of great interest as a potential anti-cancer agent (9). Indeed, the administration of poly I:C in mice that lack TIR domain-containing adapter inducing IFN- $\beta$ , a downstream adapter of TLR3 signaling, facilitated tumor progression in melanoma-bearing mice (10). Similarly, TLR3 knockout mice showed an increased tumor progression in a mouse model of prostate cancer (11). However, little is known about poly I:C anti-tumor activity in the lung. It was reported that the activation of TLR3 in the lung can increase IL-5, IL-13, and IgE (12), which are highly implicated in lung Th2-like pathologies, such as cancer (1). Yet Lowe et al. (13) demonstrated that repeated administrations of poly I:C together with tumor-specific Ag SV40 induces lung tumor regression in mice injected with mKSA cells, although this study was based on experimental vaccine design. Similarly, Jiang et al. (14) proved that repeated injections of poly I:C and in an experimental anti-cancer vaccine design contributed to pulmonary metastases regression. In contrast, the aim of our study was to determine whether the sole administration of poly I:C could affect lung tumor outgrowth in an established pulmonary metastasis mouse model. In this study, we show that poly I:C treatment reduced lung tumor growth in tumor-bearing mice because of an increased Th1- and Th17-like environment. The interference with IFN type I receptor (IFNAR) signaling by means of a specific mAb reversed this tumor regression phenotype because of a lower presence of DCs and Th1-like cytokines. The adoptive transfer of poly I:C-activated DCs into tumor-bearing

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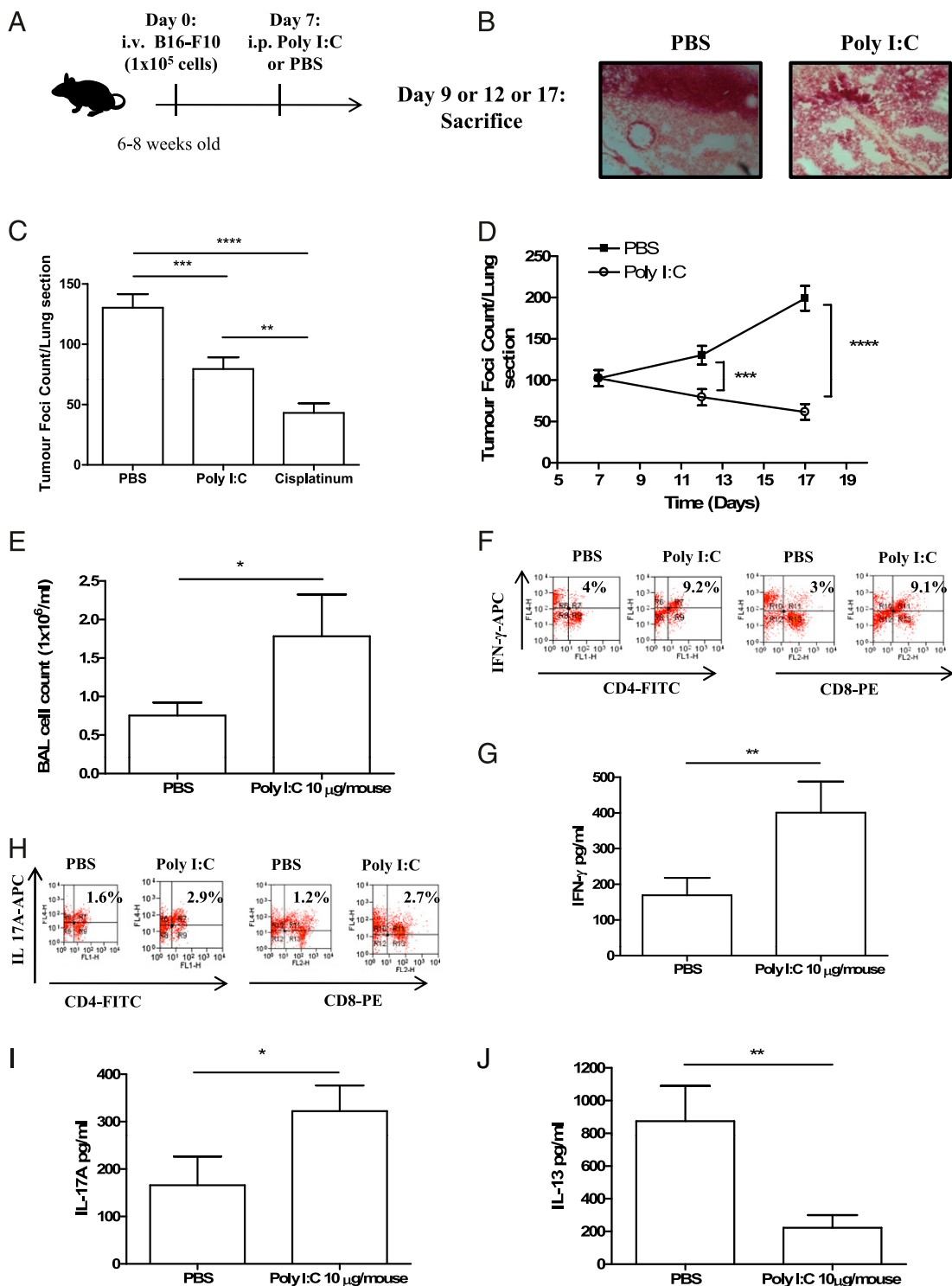
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Abbreviations used in this article: BAL, bronchoalveolar lavage; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; IFNAR, IFN type I receptor; MDA5, melanoma differentiation-associated protein-5; poly I:C, polyinosinic-polycytidylic acid.

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mice resulted in activities similar to those of the systemic administration of poly I:C on lung tumor burden. In conclusion, our study proves poly I:C therapeutic efficacy via the activation of

DCs and the production of IFN type I, which in turn facilitates an efficient anti-cancer adaptive immunity in a mouse model of metastatic lung cancer.



**FIGURE 1.** Poly I:C decreases lung tumor growth in a dose-dependent manner. **(A)** Poly I:C (10 µg/mouse) was administered i.p. 7 d after i.v. injection of B16-F10 melanoma metastatic cells ( $1 \times 10^5$  cells/mouse). Mice were sacrificed 2 d and/or 5 d after poly I:C or PBS injection. **(B)** Representative H&E panels for lungs derived from PBS- or poly I:C-treated mice. Original magnification  $\times 10$ . **(C)** Lung tumor foci count was assessed by counting the brown spots in the lung of tumor-bearing mice after PBS or poly I:C or cisplatin treatment. **(D)** Poly I:C administration had long-lasting anti-tumor activity in our mouse model. **(E)** The administration of poly I:C increased inflammatory cell count in the BAL fluid of tumor-bearing mice compared with that in the BAL fluid of PBS-treated tumor-bearing mice. Moreover, poly I:C treatment increased the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T cells **(F)** as well as CD4<sup>+</sup> and CD8<sup>+</sup> IL-17A<sup>+</sup> T cells **(H)**. IFN- $\gamma$  **(G)** and IL-17A **(I)** levels were significantly increased in the BAL fluid of poly I:C-treated lung tumor-bearing mice. **(J)** BAL fluid levels of IL-13 were reduced in poly I:C-treated mice compared with those of PBS-treated mice. Data represent mean  $\pm$  SEM,  $n = 13$ . Experiments were performed on three different experimental days. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$ , \*\*\*\* $p < 0.0001$  (Student  $t$  test and two-way ANOVA).

## Materials and Methods

### Mice

Female specific pathogen-free C57BL/6J mice (6–8 wk; Harlan Laboratories, Udine, Italy) were fed a standard chow diet and housed under specific pathogen-free conditions at the Istituto Nazionale Tumori, Fondazione “G. Pascale.” All animal experiments were performed under protocols that followed the Italian and European Community Council for Animal Care (DL no. 116/92).

### Cell culture

B16-F10 metastatic melanoma cells were purchased from American Type Culture Collection and cultured in DMEM supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml) (Sigma-Aldrich, Milan, Italy) in an atmosphere of 5% CO<sub>2</sub> at 37°C.

### Experimental protocol

Mice were injected i.v. with  $1 \times 10^5$  B16-F10 cells (day 0), and 7 d later poly I:C (1, 10, or 100  $\mu$ g/mouse; Vincibiochem, Milan, Italy) was administered by the i.p. route. In a first set of experiments, PBS or poly I:C (20  $\mu$ g/mouse) or cisplatin (20  $\mu$ g/mouse; Sigma-Aldrich, Rome, Italy) was injected once, and mice were sacrificed at day 2 or 5. These time points were chosen to evaluate innate immune cell infiltration into the lung of tumor-bearing mice at day 2 and to correlate adaptive immune cell recruitment to tumor outgrowth at day 5. In a second set of experiments, poly I:C was injected twice every 5 d, and mice were sacrificed at day 17 to evaluate long-term activity of poly I:C. Lung, spleen, and mediastinal lymph nodes were isolated. Our preliminary data showed a dose-dependent reduction of tumor burden in the lung of tumor-bearing mice after poly I:C administration (data not shown). We decided to use poly I:C at the dose of 10  $\mu$ g/mouse rather than of 100  $\mu$ g/mouse because there was no significant difference between these two doses and to rule out any specific activity of poly I:C.

In some experiments, an anti-IFNAR mAb (mouse IgG, 1  $\mu$ g/mouse, i.p.) was used to interfere with IFN type I signaling after poly I:C or PBS treatment. The anti-IFNAR mAb was injected every day starting from the same day poly I:C or PBS was administered (day 7) before mice were sacrificed.

Lungs, lymph nodes, and spleens were isolated and digested with 0.5–1 U/ml collagenase (Sigma-Aldrich, Milan, Italy). Cell suspensions were passed through 70- $\mu$ m cell strainers, and RBCs were lysed. Cell suspensions were used for flow cytometric analysis of different cell subtypes. Bronchoalveolar lavage (BAL) fluid was collected using 0.5 ml PBS containing 0.5 mM EDTA and cell counts performed. In addition, lungs were homogenized and cytokines measured.

**Bone marrow-derived DCs and adoptive transfer experiments.** Mice were anesthetized with isoflurane prior to adoptive transfer experiments. Bone marrow-derived dendritic cells (BMDCs) were previously isolated from the femurs and tibias and cultured with GM-CSF (10 ng/ml; Biosource, San Francisco, CA) for 6–7 d as previously described (15). The purity of BMDCs was >95% as detected by flow cytometry analysis. These cells were CD11c<sup>+</sup> (DCs marker) and F4/80<sup>−</sup> (specific macrophage marker).

Cells were then pulsed overnight with poly I:C (10  $\mu$ g/ml) or PBS before being adoptively transferred into mice. Intravenous application of treated or PBS-treated CD11c plus BMDCs ( $5 \times 10^5$  cells/mouse) was performed (100  $\mu$ l) into recipient mice. Inoculation of cells (in PBS), extensively washed, was performed only one time. Seven days after the adoptive transfer experiments, mice were sacrificed. BAL fluid was collected and cell counts performed. In addition, lungs were homogenized and cytokines measured.

### Flow cytometry analysis

The composition of lung inflammatory cells was determined by flow cytometry (FACS Calibur; BD Biosciences, Milan, Italy) using the following Abs: CD11c–FITC, CD11b–PeCy5.5, Gr1–PE, CD20–PE, CD3–PeCy5.5, CD8–PE, NK1.1–PE, B220–PE, CD80–PE, MHC class II–PE, and MHC class I–FITC (eBioscience, San Diego, CA). Intracellular staining was performed by using the following Abs: CD4–FITC, CD8–PE, CD3–PeCy5.5, IFN- $\gamma$ –allophycocyanin, and IL-17A–allophycocyanin (eBioscience). Isotype control (mouse IgG) was used.

### ELISA

IL-12p40, IFN- $\gamma$ , IL-17A, and granzyme B were measured in lung homogenate and BAL fluid by use of commercially available ELISAs (R&D Systems and eBioscience, London, U.K.).

### Immunohistochemistry

Left lung lobes were fixed in OCT medium (Pella, Milan, Italy) and 7- $\mu$ m cryosections were cut. H&E staining was performed and used to measure the tumor burden. Lung metastasis foci were counted by using serial lung cryosections under H&E staining in a blinded fashion. Anti-TLR3 and/or MDA5 or rat IgG isotype control (eBioscience) were used. The diaminobenzidine acid system was used to detect complexes.

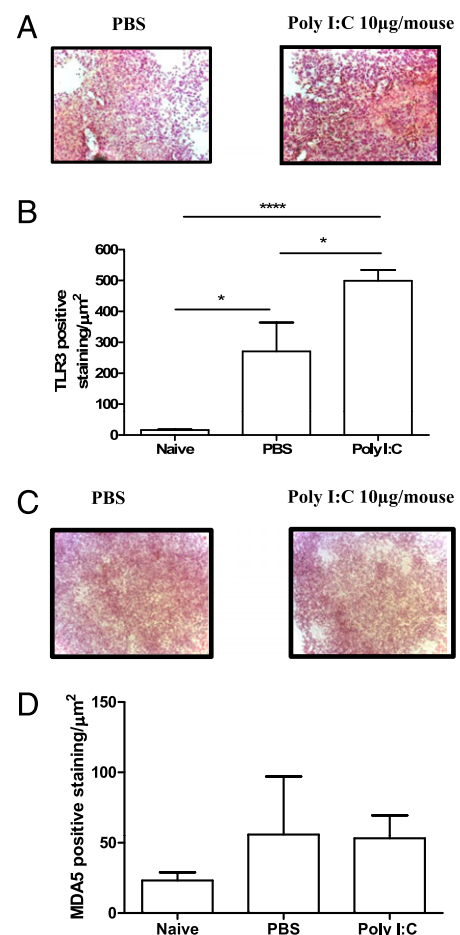
### Statistical analysis

Results are expressed as means  $\pm$  SEM. Changes observed in treated groups compared with controls were analyzed using one-way ANOVA followed by Bonferroni's post test, Student *t* test, and/or two-way ANOVA where appropriate. The *p* values <0.05 were considered significant.

## Results

### Poly I:C decreases lung tumor growth

To investigate the role of poly I:C in pulmonary metastasis, we used a mouse model by which lung metastatic B16-F10 cells were i.v. injected into the tail vein of C57BL/6J mice. As previously described (16), 7 d after B16-F10 cell inoculation, poly I:C (10  $\mu$ g/



**FIGURE 2.** TLR3 expression in the lung of tumor-bearing mice. Immunohistochemical analyses (**A**) revealed that TLR3 was expressed more highly in the lung of tumor-bearing mice compared with that in naive mice (**B**). Poly I:C treatment further increased the expression of TLR3 in the lung of tumor-bearing mice (**B**). In contrast, the expression of MDA5 (**C**, **D**) was not altered by poly I:C treatment compared with that in PBS-treated tumor-bearing mice. The isotype control (rat IgG) did not show any positive staining (data not shown). Rat anti-mouse TLR3 and rat anti-mouse MDA5; original magnification  $\times 10$ . Data represent mean  $\pm$  SEM, *n* = 4. Experiments were performed on two different experimental days. \**p* < 0.05, \*\*\*\**p* < 0.0001 (one-way ANOVA and Student *t* test).

mouse) was administered i.p. Mice were sacrificed 2 and/or 5 d after the single administration of poly I:C at day 7 (Fig. 1A).

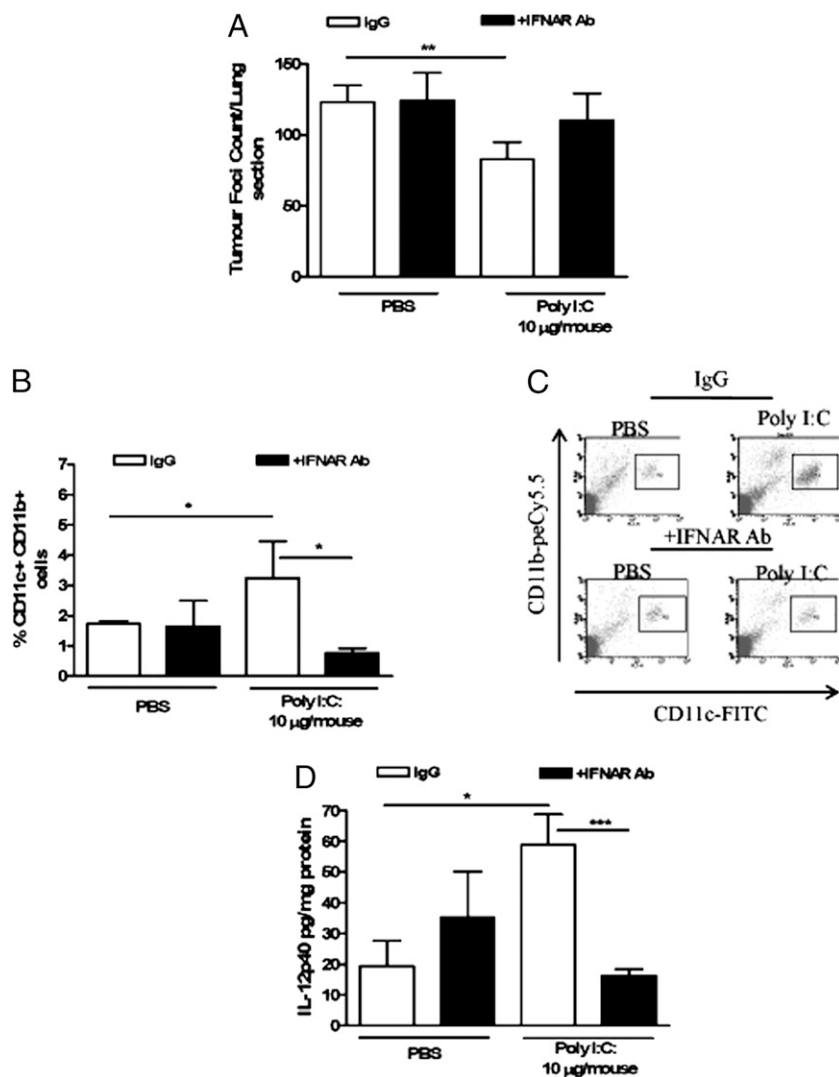
The systemic administration of poly I:C induced a significant decrease in the growth of pulmonary metastases in tumor-bearing mice compared with PBS (control) (Fig. 1C). Fig. 1B shows representative pictures of lungs from mice treated with PBS or poly I:C. Moreover, to understand the anti-tumor activity of poly I:C compared with what is actually used as anti-cancer therapeutic, we i.p. administered a well-known anti-cancer agent, cisplatin, into lung tumor-bearing mice. The amount of lung foci in cisplatin-treated tumor-bearing mice was reduced to  $\approx 67\%$  (Fig. 1C), whereas the amount of foci in the lung of poly I:C-treated tumor-bearing mice was reduced to  $\approx 40\%$  (Fig. 1C) compared with that of PBS-treated mice. In addition, to corroborate our previous data on the anti-tumor activity of poly I:C in our mouse model, we performed long-term experiments where poly I:C or PBS were injected twice every 5 d and mice sacrificed at day 17. The repeated administration of poly I:C further reduced lung tumor foci count compared with that of PBS-treated tumor-bearing mice in our model of established pulmonary metastases (Fig. 1D).

To determine whether poly I:C could induce lung inflammation in these mice as described for asthma, a Th2-like pathology (17), we evaluated the number of cells in the BAL fluid of PBS- and poly I:C-

treated mice. BAL fluid cell numbers were significantly increased in poly I:C-treated tumor-bearing mice ( $1.78.6 \pm 0.5$ ) compared with those in PBS-treated tumor-bearing mice ( $0.75 \pm 0.17$ ) (Fig. 1E). To confirm the anti-cancer activity of poly I:C, we analyzed the levels of IFN- $\gamma$ , a Th1-like cytokine, and IL-17A and IL-13, Th2-like cytokines. IFN- $\gamma$  (Fig. 1G) and IL-17A (Fig. 1I) levels were increased in the BAL fluid of poly I:C-treated mice compared with those in the BAL fluid of PBS-treated mice. Moreover, poly I:C treatment increased the percentage of CD4 $^{+}$  and CD8 $^{+}$  IFN- $\gamma$  $^{+}$  T cells (Fig. 1F) and of CD4 $^{+}$  and CD8 $^{+}$  IL-17A $^{+}$  T cells (Fig. 1H). The isotype control for the intracellular staining of IFN- $\gamma$  and IL-17A did not show positive staining (data not shown). In contrast, the levels of IL-13 were significantly reduced in poly I:C-treated mice compared with those in PBS-treated mice (Fig. 1J).

To understand better the effect of poly I:C on lung metastases growth, we continued by analyzing the expression of TLR3 and MDA5 in lung cryosections. Immunohistochemistry staining showed that TLR3 expression significantly increased in tumor-bearing mice compared with that in naive mice (Fig. 2A, 2B). Notably, administration of poly I:C further increased TLR3 expression in the lung of tumor-bearing mice (Fig. 2A, 2B). In contrast, the expression of MDA5 was not altered by poly I:C treatment in the lung of tumor-bearing mice compared with that in the lung of PBS-treated mice (Fig. 2C, 2D).

**FIGURE 3.** The blockade of IFNAR reverted poly I:C-mediated anti-tumor activity in the lung of tumor-bearing mice. Poly I:C (10  $\mu\text{g}/\text{mouse}$ ) was administered i.p. 7 d after i.v. injection of B16-F10 melanoma metastatic cells ( $1 \times 10^5$  cells/mouse). Lung histology was assessed 5 d after poly I:C administration. The anti-IFNAR mAb or the isotype control IgG was injected every day starting from the same day poly I:C or PBS was administered (day 7) before mice were sacrificed. **(A)** Lung tumor foci amount was similar in mice treated with IgG plus PBS (left white bar), IFNAR Ab plus PBS (left black bar), and IFNAR Ab plus poly I:C (right black bar) compared with IgG plus poly I:C (right white bar). **(B)** The blockade of IFNAR reduced the influx of CD11c $^{+}$ CD11b $^{+}$  cells into the lung of poly I:C-treated tumor-bearing mice. **(C)** Representative dot plots for CD11c $^{+}$ CD11b $^{+}$  cells. **(D)** IL-12p40 levels were reduced in lung homogenate of poly I:C-treated tumor-bearing mice after IFNAR Ab administration (black bar) compared with poly I:C (white bar). Data represent mean  $\pm$  SEM,  $n = 7$ . Experiments were performed on two different experimental days. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$  (one-way ANOVA and Student *t* test).



Altogether, these results indicate that the administration of poly I:C reduces lung tumor foci in association with increased lung inflammation in tumor-bearing mice.

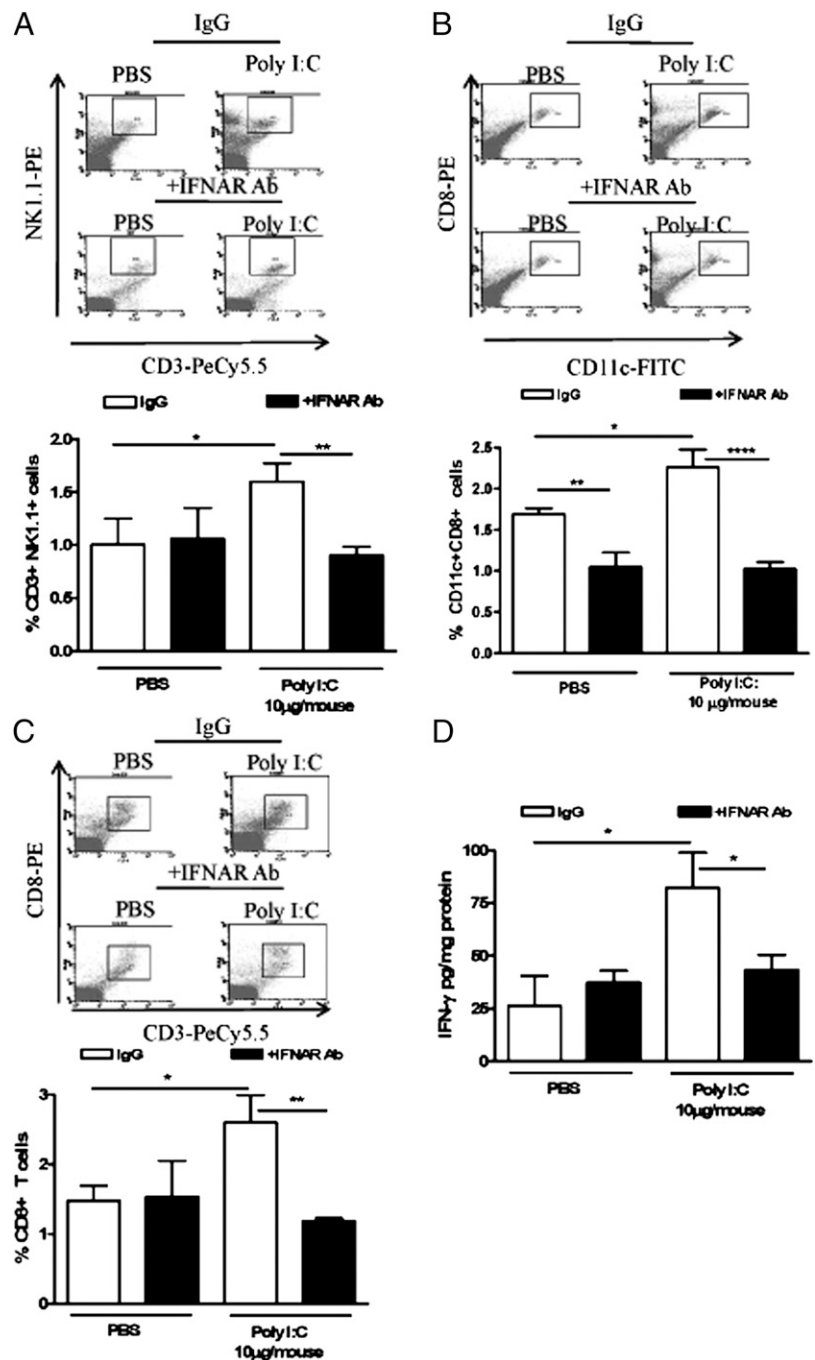
*Poly I:C-induced lung tumor reduction was partially mediated by IFN type I*

Upon poly I:C recognition, MDA5 and TLR3 initiate downstream signaling pathways that lead to activation of transcription factors, including IRF3 and NF- $\kappa$ B, which induce IFN type I production (9). To understand the role of IFN type I in poly I:C-induced lung tumor regression, we used a monoclonal blocking Ab for IFNAR (IFNAR Ab), which was given every day (i.p.) after poly I:C or PBS administration (day 7) in B16-F10–implanted mice. Mice were sacrificed 5 d after poly I:C or PBS treatment. The administration of isotype control IgG or IFNAR Ab (Fig. 3A) did not alter the amount of lung tumor foci in B16-F10–implanted mice

treated with PBS (Fig. 3A, white bar versus black bar) ( $123.2 \pm 11.7$  versus  $124.3 \pm 19.46$ ). In contrast, the blockade of IFNAR reverted the beneficial anti-tumor activity of poly I:C in lung tumor-bearing mice (Fig. 3A,  $82.75 \pm 12.2$  versus  $110.1 \pm 19.1$ ).

To explain why poly I:C treatment arrested tumor burden in our model, we determined the identity and numbers of leukocytes recruited to the lung 2 d after poly I:C treatment. We digested the lungs and performed flow cytometry analyses for DCs, identified as CD11c<sup>high</sup>, CD11b<sup>+</sup> and F4/80<sup>-</sup>. The percentage of DCs was increased in the lung of mice injected with poly I:C (Fig. 3B, white bar) compared with that in the lung of PBS-treated tumor-bearing mice (Fig. 3B, white bar). Moreover, the expression of the activation markers CD80 (PBS  $287.1 \pm 67.07$  versus poly I:C treatment  $416.5 \pm 29.15$ ), MHC class II (PBS  $238.7 \pm 61.07$  versus poly I:C treatment  $345.7 \pm 18.6$ ), and MHC class I (PBS  $26.47 \pm 1.47$  versus poly I:C treatment  $32.71 \pm 2.82$ ) was significantly

**FIGURE 4.** The blockade of IFN type I signaling interfered with the recruitment of cytotoxic immune cells to the lung of poly I:C-treated tumor-bearing mice. Flow cytometry analyses revealed that poly I:C increased the influx of NKT cells, identified as CD3<sup>+</sup> NK1.1<sup>+</sup> cells (A), CD8<sup>+</sup> DCs, identified as CD8<sup>+</sup> CD11c<sup>+</sup> cells (B), and CD8<sup>+</sup> T cells (C) to the lung of tumor-bearing mice compared with that of PBS-treated mice (right white bars). IFNAR Ab administration diminished the percentage of these cells to the lung of tumor-bearing mice after poly I:C injection (right black bars). Representative dot plots are shown. (D) IFN- $\gamma$  levels in lung homogenates. Data represent mean  $\pm$  SEM,  $n = 7$ . Experiments were performed on two different experimental days. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$  (one-way ANOVA and Student  $t$  test).



increased on these (CD11c<sup>+</sup>CD11b<sup>+</sup>) cells after poly I:C administration (data not shown). Furthermore, IL-12p40, a Th1-like cytokine, was significantly increased after 2 d of poly I:C treatment in the lung homogenates of tumor-bearing mice (Fig. 3D). In contrast, the administration of IFNAR Ab reduced the influx of CD11c<sup>+</sup>CD11b<sup>+</sup> cells to the lung of tumor-bearing mice after poly I:C treatment (Fig. 3B). Similarly, the levels of IL-12p40 were reduced after IFNAR blockade in poly I:C-treated lung tumor-bearing mice (Fig. 3D).

To confirm the role of IFN type I in poly I:C-mediated reduction of lung tumor growth, we went on by analyzing the cytotoxic immune cells recruited to the lung of tumor-bearing mice. Mice were sacrificed at 5 d after the injection of poly I:C or PBS. The percentage of NKT cells, identified as CD3<sup>+</sup>NK1.1<sup>+</sup> cells, was significantly increased in poly I:C-treated mice compared with that in mice treated with PBS (Fig. 4A, white bars). In contrast, the inhibition of IFNAR reduced the amount of NKT cells recruited to the lung after poly I:C treatment compared with that recruited to the lung in PBS-treated mice (Fig. 4A, black bar). The injection of IFNAR Ab did not alter the recruitment of these cells after PBS administration compared with that in mice that received IgG isotype control (Fig. 4A, black bar versus white bar).

Moreover, the recruitment of cytotoxic DCs (CD11c<sup>+</sup>CD8<sup>+</sup> cells) to the lung of tumor-bearing mice treated with poly I:C was greater than that to the lung of tumor-bearing mice treated with PBS (Fig. 4B, white bars). The injection of IFNAR Ab significantly reduced the percentage of these cytotoxic DCs in the lung of poly I:C-treated tumor-bearing mice (Fig. 4B, black bar) compared with that in the lung of mice treated with poly I:C alone (Fig. 4B, white bar).

To confirm further the relevance of cytotoxic immune cells in poly I:C-mediated reduction of lung tumor growth, we observed that CD8<sup>+</sup> T cells were significantly increased in poly I:C-treated lung tumor-bearing mice compared with that in PBS-treated mice (Fig. 4C, white bars). In contrast, the recruitment of these cells was significantly reduced when IFNAR Ab was injected in poly I:C-treated mice compared with that in PBS-treated mice (Fig. 4C, black bars and white bars).

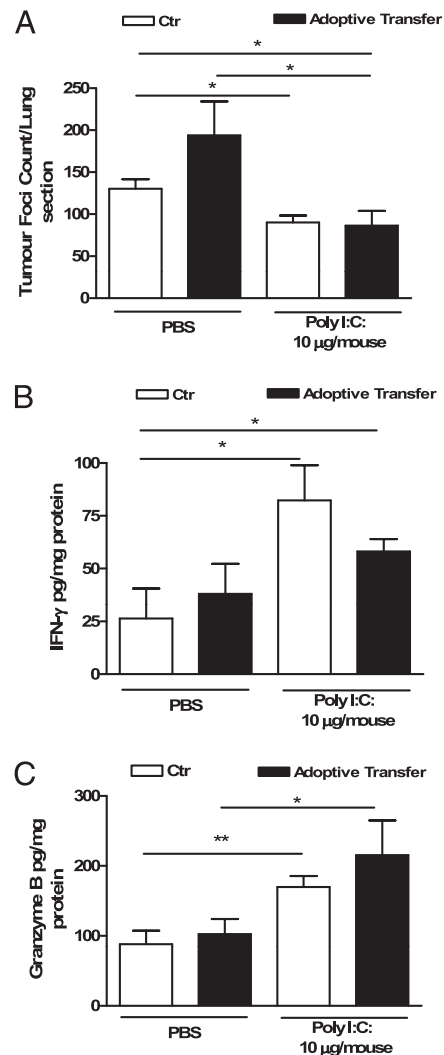
In support to the presence of cytotoxic immune cells, the levels of IFN- $\gamma$  were assessed. The administration of IFNAR Ab significantly reduced the production of this Th1-like and cytotoxic cytokine when poly I:C was administered (Fig. 4D, black bar).

Altogether, these data suggest that the systemic administration of poly I:C induced the activation of DCs and facilitated the recruitment of cytotoxic immune cells to the lung of tumor-bearing mice. Given that poly I:C induces the expression of IFN type I, the anti-tumor activity of poly I:C in lung tumor-bearing mice was IFN dependent.

#### *The adoptive transfer of poly I:C-pulsed DCs reduced lung tumor outgrowth*

Our previous data showed that poly I:C treatment increased the recruitment of Th1-polarizing DCs (Fig. 3B, 3C) to the lung of tumor-bearing mice. To determine whether DCs cells were responsible for poly I:C negative modulation of lung tumor growth, adoptive transfer experiments were performed. BMDCs were treated overnight with PBS or poly I:C and then adoptively transferred into C57BL/6J mice by i.v. injection on day 7 after inoculation of B16-F10 cells. Mice were sacrificed at day 12 (5 d after the adoptive transfer of BMDCs).

Lung tumor-bearing mice had a reduced number of metastatic foci when poly I:C-pulsed BMDCs were adoptively transferred (black bar:  $86.33 \pm 17.5$ ) compared with that of mice transferred with PBS-treated BMDCs (white bar:  $193.8 \pm 40.5$ ) (Fig. 5A).



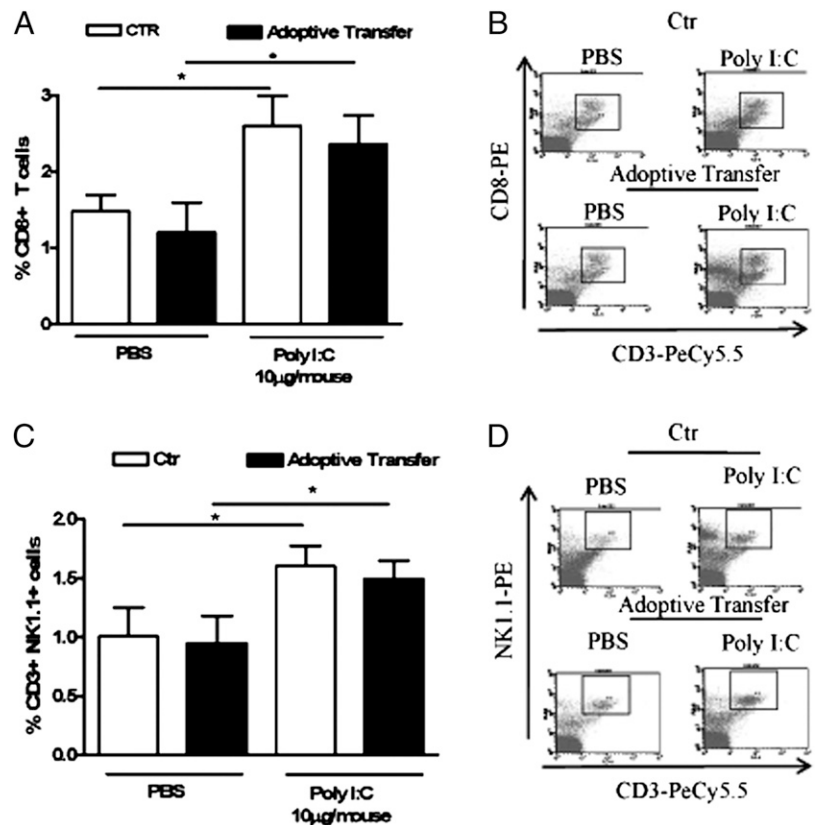
**FIGURE 5.** The adoptive transfer of poly I:C-pulsed BMDCs exerts anti-tumor activity in lung tumor-bearing mice. Poly I:C (10  $\mu$ g/mouse) or PBS (white bars) and poly I:C (10  $\mu$ g/ml)- or PBS-pulsed BMDCs (black bars) were administered 7 d after i.v. injection of B16-F10 melanoma metastatic cells ( $1 \times 10^5$  cells/mouse). Lung histology was assessed 5 d after each treatment. (A) Adoptive transfer of poly I:C-pulsed BMDCs revealed a similar lung tumor foci count compared with that of the i.p. administration of poly I:C. IFN- $\gamma$  (B) and granzyme B (C) levels detected in lung homogenates of adoptively transferred mice that received poly I:C-pulsed BMDCs were the same as those in mice that received poly I:C i.p. Data represent mean  $\pm$  SEM,  $n = 8$ . Experiments were performed on two different experimental days. \* $p < 0.05$ , \*\* $p < 0.01$  (one-way ANOVA and Student  $t$  test).

The adoptive transfer of PBS-pulsed BMDCs did not statistically alter the amount of lung tumor foci compared with that of PBS-treated mice (Fig. 5A). Notably, we did not observe any difference between mice treated with i.p. injection of poly I:C (white bar:  $90 \pm 8.34$ ) and mice adoptively transferred with poly I:C-pulsed BMDCs (black bar:  $86.33 \pm 17.5$ ), suggesting that the anti-tumor activity of poly I:C in the lung was mediated by the activation of DCs.

In addition, the production of the cytotoxic IFN- $\gamma$  (Fig. 5B) and granzyme B (Fig. 5B) in the lung of mice adoptively transferred with poly I:C-pulsed BMDCs was similar to that of mice treated with the systemic injection of poly I:C.

To confirm the role of DCs in our experimental design, we continued by analyzing the cytotoxic immune cells in the lung of

**FIGURE 6.** The adoptive transfer of poly I:C-pulsed BMDCs increased the influx of cytotoxic immune cells to the lung of tumor-bearing mice. Flow cytometry analyses revealed that the adoptive transfer of poly I:C-pulsed BMDCs increased the influx of CD8<sup>+</sup> T cells (A) and NKT cells (C) to the lung of tumor-bearing mice similarly to what happened to mice that received poly I:C via the i.p. route (right white bars). (B and D) Representative dot plots are shown. Data represent mean  $\pm$  SEM,  $n = 8$ . Experiments were performed on two different experimental days. \* $p < 0.05$  (one-way ANOVA and Student  $t$  test).



mice adoptively transferred with BMDCs. The percentage of CD8<sup>+</sup> T cells (Fig. 6A, 6B) and NKT cells (Fig. 6C, 6D) in the lung of mice adoptively transferred with poly I:C-pulsed BMDCs (black bars) was similar to that of mice i.p. injected with poly I:C (white bars).

These data indicate that the anti-tumor effect of poly I:C was principally mediated by the activation of DCs, which facilitated the recruitment of cytotoxic immune cells to the lung of tumor-bearing mice, which were responsible for limiting tumor outgrowth after poly I:C injection.

## Discussion

In this study, we investigated the effect of poly I:C in a mouse model of pulmonary metastasis. We demonstrated that the treatment with poly I:C facilitated tumor regression in the lung of tumor-bearing mice. The anti-tumor activity of poly I:C was IFN-dependent, as the blockade of IFNAR reverted lung tumor regression associated with higher influx into the lung of cytotoxic immune cells such as CD11c<sup>+</sup>CD8<sup>+</sup>, NKT, and CD8<sup>+</sup> T cells. Moreover, DCs were primarily responsible for poly I:C-mediated lung tumor arrest/regression. The adoptive transfer of poly I:C-pulsed DCs reduced lung tumor burden in the same manner as when poly I:C was administered systemically to lung tumor-bearing mice.

Poly I:C is currently being tested in human clinical trials as an adjuvant to anti-cancer vaccines and in combination with other therapies (18). Basic research on animal models of prostate cancer (11) demonstrated that poly I:C could be an efficient chemotherapeutic because it reduced tumor volume. To our knowledge, our study is the first to demonstrate a beneficial anti-tumor activity of poly I:C in a mouse model of established pulmonary metastasis. Previous studies proved that poly I:C is a good adjuvant for anti-cancer vaccine against glioma (19) and lung metastasis (13, 14). The difference between our study and the latter studies is the

experimental protocol. We observed that only one administration of poly I:C was able to diminish lung tumor progression in mice implanted with B16-F10 cells. In support, Lowe et al. (13) and Jiang et al. (14) similarly observed that repeated poly I:C administrations reduced lung tumor metastasis in mice exposed to an anti-cancer vaccine experimental design (13, 14).

One of the potential explanations for poly I:C-derived anti-tumor activity is the induction of apoptosis. The ligation of TLR3 can lead to the apoptosis cascade (20), which can serve as a potential strategy for tumor regression as already demonstrated in a prostate cancer mouse model after poly I:C administration (11). In our study, the administration of poly I:C significantly increased the production of granzyme B in the lung of tumor-bearing mice. Granzyme B is an essential molecule that leads to apoptosis and is predominantly produced by cytotoxic immune cells such as CD8<sup>+</sup> T cells and NKT cells (21). In support, mice that received poly I:C had a higher influx into the lung of cytotoxic DCs (CD8<sup>+</sup>CD11c<sup>+</sup> cells), NKT cells, and CD8<sup>+</sup> T cells, all of which are described as granzyme B-producing cells.

The main feature of poly I:C as a potential adjuvant for anti-viral and anti-cancer vaccines is its capability to mount a long-lasting adaptive immunity, which in terms of tumor progression represents a way to fight tumor-immune escape (1, 22). Our data show that the injection of poly I:C facilitated a Th1- and Th17-like response in the lung of tumor-bearing mice. IFN- $\gamma$  is a Th1-like cytokine of which anti-tumor activity is well reported (1, 16, 22). Similarly, we observed higher production of IL-17A, which facilitates tumor regression as demonstrated with IL-17-deficient mice (23). In contrast, poly I:C reduced the levels of IL-13, a Th2-like cytokine that can contribute to tumor exacerbation in the lung (1). Moreover, poly I:C promoted the activation of cytotoxic DCs, NKT cells, and CD8<sup>+</sup> T cells, which were CD69<sup>+</sup> in the lung of poly I:C-treated tumor-bearing mice compared with control mice (data not shown). Thus, the polarization of T cells toward a Th1-



and Th17-bias, with concomitant reduction of a Th2-like environment, and the higher recruitment of cytotoxic immune cells collaborate to limit lung tumor outgrowth in poly I:C-treated tumor-bearing mice. It is to be noted, however, that the recruitment of these cytotoxic cells was secondary to the activation of DCs by poly I:C. Adoptive transfer experiments proved that the inoculation of poly I:C-pulsed BMDCs was still as efficient as the i.p. injection of poly I:C at reducing lung tumor foci. In support, the administration of poly I:C increased the influx of DCs (Fig. 3) to the lung of tumor-bearing mice and drove toward a Th1 polarization in the presence of IL-12p40. In support, TLR3 is highly expressed by DCs (24), especially by the cytotoxic CD8<sup>+</sup> DCs.

Although poly I:C induces the production of IFN type I in a TLR-dependent and -independent manner (25), IFN type I is essential for poly I:C-mediated tumor regression (22). In our study, the blockade of IFNAR, which disables any activity of IFN type I (21), reverted the anti-tumor activity of poly I:C in lung tumor-bearing mice. Indeed, the lower influx of DCs, CD8<sup>+</sup> DCs, NKT cells, and CD8<sup>+</sup> T cells in IFNAR Ab-treated tumor-bearing mice was associated with higher lung tumor burden even after poly I:C administration. These results confirmed what was already known about the anti-tumor effects of IFN type I (22). Moreover, given that DCs are responsible for poly I:C anti-tumor function, IFN type I is required by DCs to switch toward a mature phenotype and induce Th1 immunity and a CTL response in our mouse model. In vitro-stimulated BMDCs highly produced IFN type I after poly I:C treatment (data not shown).

In conclusion, our study demonstrates that poly I:C has anti-tumor, long-lasting activity in a mouse model of pulmonary metastasis. DCs mediate the activity of poly I:C to mount an effective immune response against lung tumor. The adoptive transfer of poly I:C-pulsed BMDCs into tumor-bearing mice resulted in reduced numbers of lung tumor foci and enhancement of the cytotoxic immune environment in the lung of tumor-bearing mice. The blockade of IFN type I function resulted in recovered tumor growth after poly I:C treatment. Thus, the activation of DCs and IFN type I production allowed an effective T cytotoxic immune response, demonstrating that poly I:C is a good adjuvant for lung cancer chemotherapy.

## Disclosures

The authors have no financial conflicts of interest.

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