Lung-resident natural killer cells control pulmonary tumor growth in mice

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Abstract

Accumulating evidences indicate an importance of NK cells in controlling tumor growth and metastasis. NK cell subsets display diversities in their function and tissue distribution and Mac-1^{hi} $CD27^{lo}$ NK cells are the predominant population of lung-resident NK cells. Although lung is a major organ where primary tumor develops and cancer cells metastasizes, there is no clear evidence whether circulating NK cells and/or tissue-resident NK cells control tumor growth in lung. In this study, we examined an anti-tumor function of lung-resident NK cells to control pulmonary tumor growth. In an orthotopic lung tumor model, NK cells controlled the pulmonary tumor growth, and mature circulating NK cell subsets were increased in tumor-bearing lungs through CXCR3-dependent mechanism. Although such increase of migratory NK cell subsets can be blocked by anti-CXCR3 treatment, there was no difference in the pulmonary tumor growth in anti-CXCR3-treated mice compared with control mice. In addition to pulmonary tumor growth, lung-resident NK cells, but not migratory NK cells, play a dominant role for controlling metastatic growth of cancer cells in lung. These results strongly indicate an importance of lung-resident NK cells for controlling pulmonary tumor growth.

Introduction

NK cells can be dissected into functionally distinct subsets by the expression of Mac-1 and CD27 (1-4). Along with phenotypically immature Mac-1^{lo} $CD27^{hi}$ NK cell subset (Mac-1^{lo}), we showed that CD27 expression further dissects the mature Mac-1^{hi} NK cell population into $CD27^{hi}$ and $CD27^{lo}$ subsets (1, 2). Such NK cell subset shows distinct tissue distribution as well as their functional diversity. Interestingly, the $CD27^{lo}$ subset is comparatively excluded from BM and LN, and is the dominant in blood circulation or in peripheral tissues (1, 2). In addition, NK cells are rapidly recruited to tissues at the site of immune responses including tumor, and the chemokine receptor CXCR3 has been known to play an important role in NK cell recruitment to tumor microenvironment (5-8). Amongst NK cell sub-populations, Mac-1^{lo} and $CD27^{hi}$ NK cell subsets constitutively express CXCR3, therefore those NK cell subsets are dominantly recruited into tumor (1, 6, 7).

Pulmonary NK cells play an important role in pathology of respiratory diseases, including infectious diseases, allergy and cancer (9-11). Accumulating evidences suggest that lung tissue-specific NK cells can be functionally and phenotypically distinct from other NK cells (1, 12). Under steady-state conditions, NK cells account for about 10% of total lymphocyte population in the lung, and those lung-resident NK cells are mostly $CD27^{lo}$ subset. The $CD27^{lo}$ NK cell subset express highly mature phenotype bearing with self-recognizing inhibitory

receptors and being tightly regulated in their responsiveness (1, 13). Although the importance of NK cells in tumor surveillance has been widely appreciated, a contribution of such tissue-resident NK cells is not understood. While lung is a major organ where primary tumors develop and cancer cells metastasize, it is not clear the role of lung tissue-resident NK cells for tumor control. In this study, we examined the role of lung resident NK cells in controlling primary lung tumor growth by using an orthotopic lung tumor model. We demonstrated that the lung-resident NK cells, but not migratory NK cells, play a dominant role in controlling pulmonary primary and metastatic tumor growth.

Materials and Methods

Mice

Wild-type C57BL/6 (B6) mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). IFN-$\gamma^{-/-}$ (IFN-$\gamma^{-/-}$) mice on B6 background were kindly provided by Dr. Y. Iwakura (Tokyo University of Science, Chiba, Japan) and maintained at Laboratory Animal Research Center, Institute of Medical Science, The University of Tokyo. In some experiments, groups of mice were treated with either anti-asialo-GM1 antibody (150 μg/mouse, Wako Chemicals, Osaka, Japan) on Day -3 and -1, anti-CXCR3 mAb (500 μg/mouse, clone CXCR3-173, Bio X Cell, NH, USA) on Day -1, 0, 2, 4 and 6, or FTY720 (1 mg/kg, Sigma, MI, USA) daily from Day 0 to 9 (where day 0 is the day of primary tumor inoculation). All experiments were approved and performed according to the guidelines of the Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences of The University of Tokyo, the Care and Use of Laboratory Animals of University of Toyama and the Animal Care and Use Committee of Institute of Medical Science of the University of Tokyo.

Cells and reagents

The mouse Lewis Lung carcinoma cell line (3LL) was kindly provided by Dr. Kazuyoshi Takeda (Juntendo University), and the luciferase-expressing 3LL cell line (3LL-Luc2) was prepared as previously described (14). pGL4.50

[luc2/CMV/Hygro] vector, and D-luciferin were obtained from Promega (WI, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). Hygromycin B was obtained from Nacalai Tesque (Kyoto, Japan).

Intra-pulmonary tumor cell implantation and bioluminescent imaging

3LL-Luc2 cells were suspended (5 x 10^5 cells/ml) in PBS containing 500 μg/ml of Matrigel (BD Bioscience). Intra-pulmonary implantation procedure was described before (14). Briefly, B6 mice were anesthetized with isoflurane and were made a small skin incision at left chest wall. On observing the motion of left lung, 20 μl of cell suspension were directly injected into the lung with a 29-gauge needle attached with 0.3ml of syringe (Beckton & Dickinson). The skin incision was closed with a surgical skin clip. To obtain bioluminescent images, mice were injected with D-luciferin (Promega, 150 mg/kg i.p.) and luminescence was measured with an in vivo imaging system (IVIS Lumina II, Perkin Elmer, MA, USA) 10 minutes after the D-luciferin injection.

Flow cytometry

3LL-Luc2 cells were cells (10^4) were inoculated intra-pulmonary. Ten days after the inoculation, lung tissues were dissected, minced and digested with 2 mg/mL collagenase (Roche Diagnostics GmbH, Mannheim, Germany) and 0.1 mg/mL DNase I (Roche Diagnostics GmbH) in serum-free RPMI 1640 for 1 hr at 37 ^{o}C. Samples were further homogenized through wire mesh. For flow cytometry

analysis, cells were first pre-incubated with anti-CD16/32 (2.4G2) mAb to avoid non-specific binding of antibodies to FcγR. The cells were then incubated with a saturating amount of fluorophore-conjugated mAb. Antibodies against CD3e (2C11), NK1.1 (PK136), CD11b (M1/70), CD27 (LG.3A10) were purchased from Biolegend (San Diego, CA, USA), eBioscience (San Diego, CA, USA) or Tombo Bioscience (San Diego, CA, USA). Flow cytometry analysis was performed with a FACS Canto (BD Bioscience, San Jose, CA, USA) and the data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Statistical analysis

All data were obtained from a group of 6 to 9 mice and are representative of at least two independent experiments. Data were analyzed for statistical significance using the Student's *t*-test. *P* values less than 0.05 were considered significant.

Results

Characterization of NK cells in pulmonary tumor

To understand the role of NK cells in controlling lung cancer, we first analyzed NK cells in primary lung tumors by implanting orthotopicaly murine 3LL-Luc2 cells(n=9). Either tumor-bearing or control lung lobes were harvested 10 days after the tumor inoculation and the NK cell population were analyzed by flow cytometry. While there was no significant difference in the total NK cell population, the subsets of NK cells showed a significant difference between control and tumor-bearing lungs (Fig. 1). The subsets of mouse NK cells can be determined by the expression of two markers Mac-1 (CD11b) and CD27, and the functional and phenotypic differences in those NK cell subsets are well characterized (1). In tumor-bearing lungs, the population of migratory Mac-1^{lo} and $CD27^{hi}$ NK cell subsets were significantly increased whereas no obvious change was observed in tissue-resident $CD27^{lo}$ NK cell subset (Fig. 1). There was no difference in $CD3^{+}$ T cell population between control and tumor-bearing lungs (data not shown).

Next, we examined whether NK cells can be recruited from circulation into the tumor-bearing lung by CXCR3- or S1P-dependent mechanism, that are known as important for in vivo NK cell trafficking (1, 5, 6, 15, 16). As shown in Figure 2A, the population of migratory Mac-1^{lo} and $CD27^{hi}$ NK cell subsets in the

tumor-bearing lungs were significantly decreased in mice treated with anti-CXCR3 (aCXCR3: n=9). Contrary, there was no such difference in NK cell subsets of tumor-bearing lungs in FTY720-treated mice (n=7). Considering a significant reduction of $CD3^+$ T cells in the FTY720 treated tumor-bearing lungs was observed (data not shown), the trafficking of Mac-1^{lo} and $CD27^{hi}$ NK cells to primary lung tumor should be dependent on CXCR3, but not S1P, as similar to that to subcutaneous tumor (6, 7).

Lung-resident NK cells control pulmonary tumor growth and metastasis

To determine the importance of NK cells for controlling primary lung tumor, we examined the growth of 3LL-Luc2 tumors in lung of NK cell-depleted mice (NK dep: n=9) treated with anti-asialo-GM1 antibody. In NK cell-depleted mice, the lung tumor growth was significantly enhanced compared with control B6 mice (Fig. 3A and B), indicating NK cells significantly contribute to anti-tumor immunity in controlling pulmonary tumor growth. Such NK cell-dependent anti-tumor immune response against primary lung tumor required IFN-γ because there was no difference in the presence or absence of NK cells for controlling primary lung tumor growth in IFN-γ-deficient mice (Fig. 3C: n=8). These results clearly indicate that NK cells control lung primary tumor growth in IFN-γ-dependent mechanism.

We next examined the contribution of migratory Mac-1^{lo} and $CD27^{hi}$ NK

cells and/or tissue-resident CD27^{lo} NK cells in controlling primary lung tumor. The trafficking of Mac-1^{lo} and CD27^{hi} NK cells to primary lung tumor was blocked by treating mice with anti-CXCR3 (Figure 2A). As shown in Figure 3D, there was no differences in the growth of lung tumor between control and anti-CXCR3-treated mice. There was also no difference in the growth of lung tumor with or without FTY720 treatment (Fig. 3E). Collectively, these results indicate the importance of lung-resident NK cells, rather than NK cells recruited from circulation, in controlling pulmonary tumor growth.

The importance of NK cells for protecting lung metastasis has been widely recognized, and we previously reported that IFN-γ production by lung NK cells is critical for such metastasis protection (11). As shown in Figure 4, NK cell depletion significantly enhanced the metastatic growth of 3LL-Luc2 cells in lung, whereas anti-CXCR3 treatment did not show any difference to control mice in lung metastasis of 3LL-Luc2 cells. Along with our presented finding in controlling pulmonary tumor growth, lung-resident NK cells, but not NK cells recruited by CXCR3, play an important role in controlling metastatic growth of 3LL-Luc2 cells. These results further emphasize the general importance of lung-resident NK cells for pulmonary immune-surveillance of cancer.

Discussion

It has been widely recognized an importance of NK cells in controlling tumor growth and metastasis (17-19). Although NK cell has been known to play a critical role in the resistance to lung metastases (11), it is not clear whether circulating NK cells and/or tissue resident NK cells control tumor growth in lung. While mature $CD27^{hi}$ and $CD27^{lo}$ NK cells reside in both lymphoid and non-lymphoid organs, $CD27^{lo}$ subset is a predominant NK cell population within lung (1, 2). In this study, we examined the role of lung resident NK cells in controlling primary lung tumor growth. In an orthotopic lung tumor model, NK cells controlled the pulmonary tumor growth, and mature circulating NK cell subsets, both Mac-1^{lo} and $CD27^{hi}$ subsets, were increased in tumor-bearing lungs through CXCR3-dependent mechanism. Although such increase of migratory NK cell subsets can be blocked by anti-CXCR3 treatment, there was no difference in the pulmonary tumor growth in anti-CXCR3-treated mice compared with control mice. In addition to pulmonary tumor growth, lung-resident NK cells, but not migratory NK cells, play a dominant role in controlling lung metastatic growth. These results strongly indicate an importance of lung-resident NK cells for controlling pulmonary tumor growth.

NK cells account for about 10% of circulating lymphocytes, and also be found in the peripheral tissues including spleen, liver, gut, lung lymph node and uterus (1, 9, 20). The presence of NK cells in those peripheral organs has been

believed as a result of chemokine- and sphingosine 1-phosphate (S1P)-dependent distribution from the circulation (15, 16, 21-23). In lung tissue, NK cells play an important role in several pulmonary diseases other than cancer, including influenza infection, asthma, tuberculosis and others (24). It has been suggested that pulmonary NK cells are also recruited from the periphery (12, 25), however, the importance of lung-resident NK cells in those pathologies are not yet clear. We previously reported that the importance of liver-resident NK cells in controlling metastasis of cancer through TRAIL-dependent mechanism, and such NK cell surveillance in liver metastases was dependent on unique NK cell subset reside in liver (26, 27). In addition, we also reported that IFN-γ production by lung NK cells is critical for metastasis protection of murine melanoma cells (11). Further study is required for understanding whether lung-resident NK cells display any functional distinction to NK cells in other organs.

While NK cell trafficking under homeostatic condition are not clearly defined yet, the rapid accumulation of NK cells in the tumor microenvironment has been known to be controlled by CXCR3 (5) (6, 8). In contrast to the findings of subcutaneous tumors (6) (7), CXCR3 did not show any significant role in controlling tumor growth in lung. Moreover, the blocking of S1P pathway by treating with FTY720 did not affect NK cell-dependent tumor control in lung though S1P5-dependent NK cell trafficking was shown to be resistant to FTY720 (15), suggesting NK cell recruitment through either CXCR3 or S1P pathway are not involved in pulmonary tumor control. It has been reported that the

maintenance of lung NK cells is dependent on IL-15 (28) (29). In mice, lung-resident NK cells are mostly $CD27^{lo}$ NK cells subset which shows hypo-responsiveness to those homeostatic cytokines for NK cell including IL-15 (1) (3). The mature $CD27^{lo}$ NK cell subset expresses higher levels of inhibitory Ly-49 and CD94/NKG2 complexes (13), therefore lung-resident NK cells assumed to be tightly regulated for their responsiveness. Nevertheless, further study will be required to fully understand NK cell behavior in the lung tissue microenvironment for maximizing their anti-tumor function against lung cancer and developing new NK cell-targeted immunotherapy.

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Figure legends

Figure 1. Characterization of NK cells in pulmonary 3LL-Luc2 tumor.

B6 mice were inoculated intra-pulmonary with 3LL-Luc2 (10^4) and lungs were harvested 10 days after the inoculation. Mononuclear cells were isolated from control lung (Control) or tumor-bearing lung (Tumor) and then subjected to flow cytometry analysis. The representative FACS plots from the indicated groups of mice are shown and numbers represent percentage of cells in the different gates. The proportion of NK cells (A, $NK1.1^+CD3^-$ cells) or NK cell subsets ($Mac\text{-}1^{lo}$: $Mac\text{-}1^{lo}$ $CD27^{hi}$, $CD27^{hi}$: $Mac\text{-}1^{hi}$ $CD27^{hi}$, $CD27^{lo}$: $Mac\text{-}1^{hi}$ $CD27^{lo}$, electronically gated on $NK1.1^+CD3^-$ cells) from the indicated lung samples are presented. The data represent mean ± SEM and representative of two experiments. *$P < 0.05$ compared with control group.

Figure 1

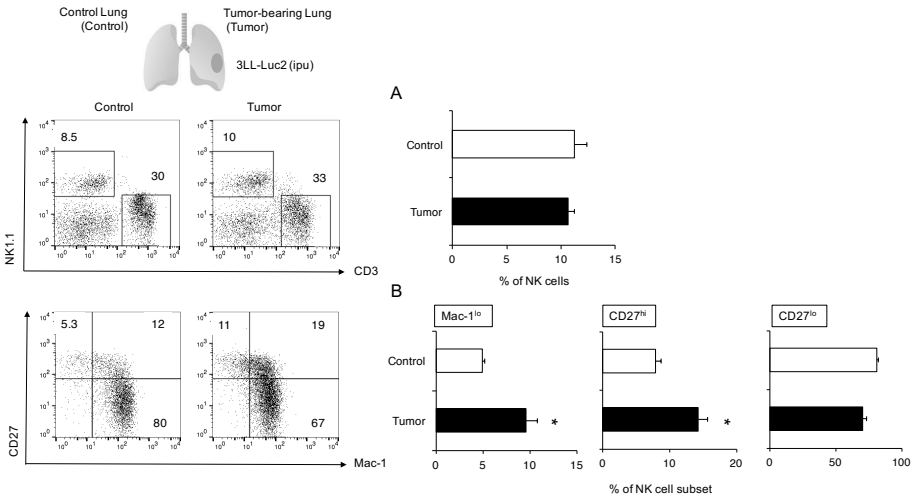
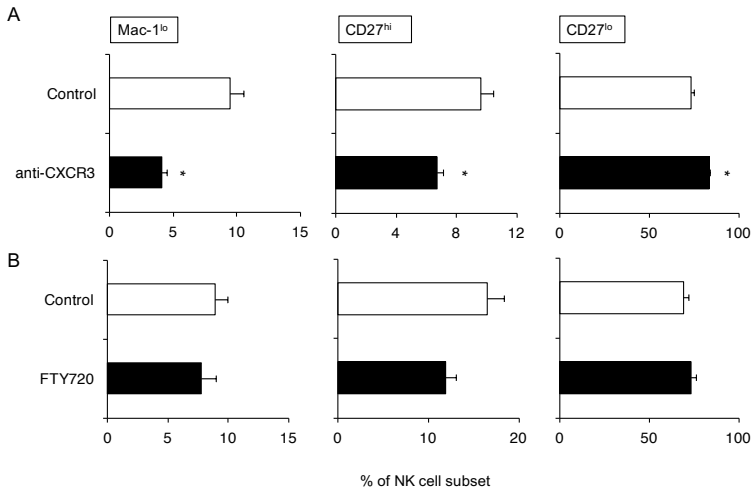

Control Lung
(Control)
Tumor-bearing Lung
(Tumor)
3LL-Luc2 (ipu)
Control
Tumor
8.5
30
10
33
NK1.1
CD3
5.3
12
11
19
80
67
CD27
Mac-1
A
Control
Tumor
0
5
10
15
% of NK cells
B
Mac-1lo
CD27hi
CD27lo
Control
Tumor
*
*
0
5
10
15
0
10
20
0
50
100
% of NK cell subset

Figure 2. CXCR3 controls migratory NK cell accumulation in pulmonary 3LL-Luc2 tumor.

3LL-Luc2 (10^4) were inoculated intra-pulmonary to B6 mice. (A) To block CXCR3, mice were treated with anti-CXCR3 (500 μg/mouse, i.p.) on day -1, 0, 2, 4 and 6. (B) FTY720 (1 mg/kg, i.p.) were treated daily from day 0 to 9 (day 0 = tumor inoculation). Mononuclear cells were isolated from tumor-bearing lung and then subjected to flow cytometry analysis. The proportion of NK cell subsets (Mac-1^{lo} : Mac-1^{lo} $CD27^{hi}$, $CD27^{hi}$: Mac-1^{hi} $CD27^{hi}$, $CD27^{lo}$: Mac-1^{hi} $CD27^{lo}$, electronically gated on $NK1.1^{+}CD3^{-}$ cells) from the indicated lung samples are presented. The data represent mean ± SEM and representative of two experiments. *$P < 0.05$ compared with control group.

Figure 2


A
Mac-1lo
CD27hi
CD27lo
Control
anti-CXCR3
*
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0
5
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4
8
12
0
50
100
B
Control
FTY720
0
5
10
15
0
10
20
0
50
100
% of NK cell subset

Figure 3. Lung-resident NK cells control pulmonary 3LL-Luc2 tumor growth.

3LL-Luc2 (10^4) were inoculated intra-pulmonary to B6 WT mice or IFN$\gamma^{-/-}$ mice. To deplete NK cells (NK dep), mice were treated with anti-asGM1 antibody (150 μg/mouse, i.p.) on day -3 and -1 (day 0 = tumor inoculation). To block CXCR3, mice were treated with anti-CXCR3 (500 μg/mouse, i.p.) on day -1, 0, 2, 4 and 6. FTY720 (1 mg/kg, i.p.) were treated daily from day 0 to 9 (day 0 = tumor inoculation). The representative bioluminescent images of mice bearing orthotopic 3LL-Luc2 tumor are shown (A). The bioluminescence of 3LL-Luc2 tumor was monitored in WT mice (B, D, E) or IFN$\gamma^{-/-}$ mice (B). Luminescence was normalized by that of the individual mouse on day 0. Data are presented as the mean ± SEM. *P < 0.05 compared with control group.

Figure 3

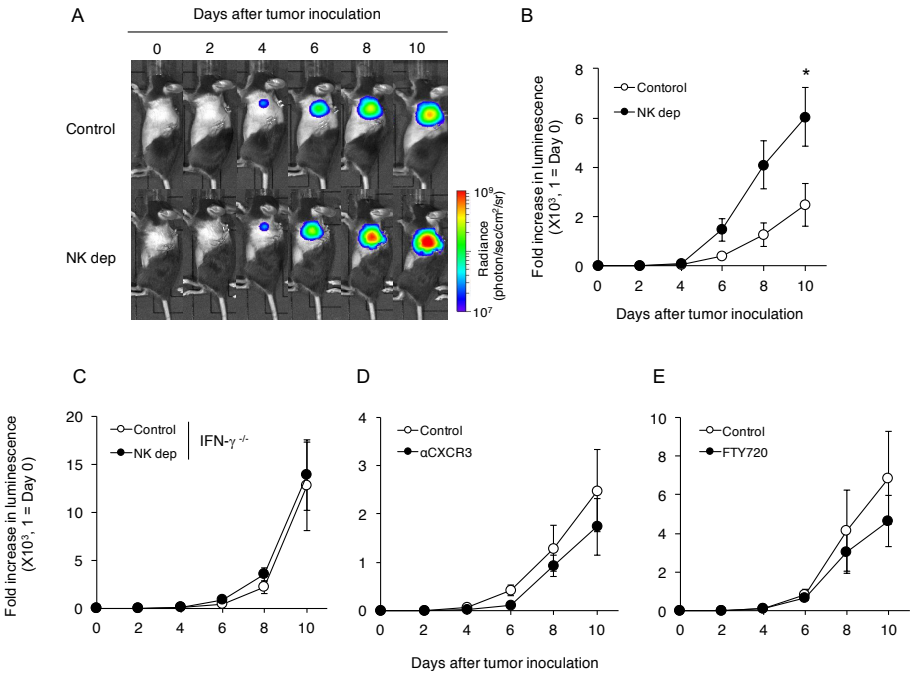
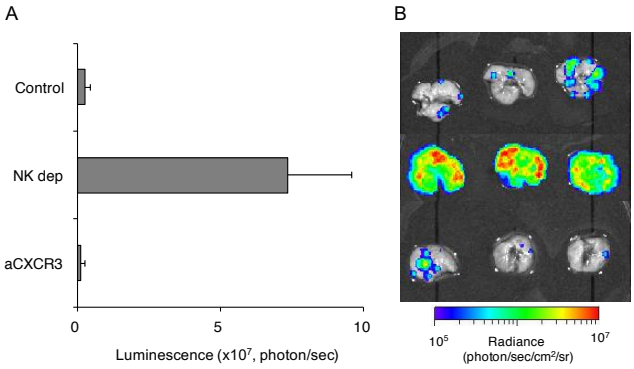

A
Days after tumor inoculation
0 2 4 6 8 10
Control
NK dep
10^9
Radiance (photon/sec/cm²/sr)
10^7
B
Contorol
NK dep
*
Fold increase in luminescence (X10³, 1 = Day 0)
Days after tumor inoculation
C
Control
NK dep
IFN-γ -/-
Fold increase in luminescence (X10³, 1 = Day 0)
D
Control
αCXCR3
Days after tumor inoculation
E
Control
FTY720

Figure 4. Lung-resident NK cells control pulmonary 3LL-Luc2 tumor metastases.

3LL-Luc2 (10^4) were inoculated intravenously to B6 mice. To deplete NK cells (NK dep), mice were treated with anti-asGM1 antibody (200 μg/mouse, i.p.) on day -3 and -1 (day 0 = tumor inoculation). To block CXCR3, mice were treated with anti-CXCR3 (500 μg/mouse, i.p.) on day -1, 0 and 2. The bioluminescence of lungs were measured at 4 days after the tumor inoculation. The representative bioluminescent images of lung inoculated (A) and summary of lung metastases (B) are shown. Data are presented as the mean ± SEM. *P < 0.05 compared with control group.

Figure 4


A
B
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NK dep
aCXCR3
0
5
10
Luminescence (x10^7, photon/sec)
10^5
Radiance
(photon/sec/cm^2/sr)
10^7