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Antimetastatic effect of NK1$^+$ T cells on experimental haematogenous tumour metastases in the liver and lungs of mice

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SUMMARY

Depletion of both natural killer 1.1$^+$ (NK1$^+$) intermediate αβ T-cell receptor (int T) cells and NK cells by *in vivo* treatment with anti-NK1 antibody greatly increased hepatic metastases of intravenously injected EL4 cells as well as pulmonary metastases of 3LL cells in C57BL/6 mice. However, depletion of NK cells alone by anti-asialo GM1 (AGM1) antibody treatment did not increase the metastases in either organ. Interleukin-12 (IL-12) administration into mice induced strong cytotoxicities of NK cell-depleted liver and lung mononuclear cells (MNC) comparable to those without NK-cell depletion and inhibited metastases in either organ. In contrast, in both NK cell- and NK1$^+$ int T-cell-depleted mice, IL-12 could not induce cytotoxic activity of liver and lung MNC and metastases in both organs increased with or without IL-12 treatment. These results confirmed the fact that NK1$^+$ int T cells are more potent antitumour effectors than NK cells against experimental haematogenous tumour metastases.

INTRODUCTION

A number of reports dealing with interleukin-2 (IL-2) receptor β (IL-2Rβ)$^+$ intermediate (int) T cells or natural killer 1.1$^+$ (NK1$^+$) T cells of mice have appeared in the literature.[1–18] NK1$^+$ T cells are exclusively confined to IL-2Rβ$^+$ int T cells, and a majority of IL-2Rβ$^+$ int T cells in the liver, along with a considerable proportion of these cells in the thymus, are NK1$^+$, while a majority of IL-2Rβ$^+$ int T cells in other organs are NK1$^-$.[17] NK1$^+$ int T cells produce the Th2-type cytokine, IL-4, via CD3 stimulation[7,18] but these cells also produce a Th1-type cytokine, interferon-γ (IFN-γ).[7,18] We recently found that one of the Th1-type cytokines, IL-12, induces activation of NK1$^+$ int T cells in the liver of mice and inhibits hepatic metastases of intravenously administered liver metastatic tumours.[16] In addition, although hepatic venous blood and lungs of mice normally have virtually no NK1$^+$ int T cells[19,20] IL-12 leads to the presence of these cells in the hepatic vein[19] and the presence of NK1$^+$ int T cells with a potent antitumour cytotoxic effect in the lung.[20] Transfer of liver mononuclear cells (MNC), but not splenocytes, of IL-12-injected mice into other tumour-preinjected mice exerted an antimetastatic effect in both liver and lung,[19] whereas the effect was inhibited by the depletion of either CD3$^+$ cells or NK1$^+$ cells of the transferred MNC, suggesting that the liver is the source of most (if not all) antimetastatic NK1$^+$ int T cells in the lung.[19,20] However, demonstrating the antimetastatic effect of NK1$^+$ int T cells *in vivo* is hampered by the fact that NK1$^+$ int T cells are often resistant to *in vivo* anti-CD3 antibody treatment [probably because of their weak T-cell receptor (TCR)/CD3 expression] and that anti-NK1 antibody treatment *in vivo* depletes both NK cells and NK1$^+$ int T cells. Therefore, direct comparison between the antimetastatic effects of NK cells and NK1$^+$ int T cells is not possible. Nevertheless, we recently noticed that a certain amount of anti-asialo GM1 (AGM1) antibody depletes only NK cells but does not affect NK1$^+$ int T cells *in vivo*, in spite of the fact that a significant proportion of these cells are AGM1 dim$^+$. In the present study, we present evidence showing that NK1$^+$ int T cells are more potent antimetastatic effectors than NK cells, even without IL-12 treatment.

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Abbreviations: AGM1, asialo GM1; IL-2Rβ, interleukin-2 receptor β; int, intermediate; MNC, mononuclear cells; NK1, natural killer cell 1.1 antigen.

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MATERIALS AND METHODS

Mice and preparation of MNC

Male C57BL/6 (B6) mice, 6 weeks of age, were purchased from Funabashi Farm Inc. (Tokyo, Japan). Mice were maintained and fed under specific pathogen-free conditions. Hepatic MNC were prepared essentially as previously described[16,19–22] Briefly, the liver was passed through a stainless steel mesh and suspended in Hanks' balanced salt solution. After one washing,

cells were resuspended in pH and osmolarity-adjusted 30% Percoll solution containing 100 U/ml heparin and were centrifuged at 2000 r.p.m. for 15 min at room temperature. The pellet was resuspended in red blood cell lysis solution, then washed twice in RPMI-1640 medium with 5% fetal calf serum (FCS). The yield of cells from normal mice was usually 3×10^6–4×10^6 cells/liver. Pulmonary MNC were prepared as described,[23] with a minor modification. The lungs were removed, and thymic and external bronchiolar tissue was dissected away. The remaining pulmonary tissue was minced and pressed through a stainless steel mesh and then washed twice by centrifugation in phosphate-buffered saline (PBS). Cells were resuspended in 30% Percoll solution and centrifuged as described above. The yield of cells from lung was usually 3×10^5–4×10^5 cells/mouse.

IL-12

Recombinant murine IL-12[24,25] with an activity of $4{\cdot}9 \times 10^6$ U/mg was kindly provided by Dr M. Kobayashi of the Genetics Institute (Andover, MA).

Cytotoxic assay

NK-sensitive Yac-1 cells, NK-resistant P815 mastocytoma cells of DBA/2 (H-2^d) origin and EL4 cells of B6 (H-2^b) origin were used as target cells. Each target cell group (3×10^6 cells) was labelled with 100 μCi $Na_2(^{51}Cr)O_4$ for 60 min at 37° in RPMI-1640 medium containing 10% FCS, washed three times with medium, and subjected to cytotoxicity assays. Labelled targets (10^4/well) were incubated in a total volume of 200 ml with effector cells in RPMI-1640 in 96-well round-bottomed microtitre plates. The plates were centrifuged after incubation for 4 hr and supernatant was harvested and counted with a gamma-counter. The cytotoxicity was calculated as the percentage of releasable counts after subtraction of spontaneous release. Spontaneous release was less than 15% of the maximum release.

Immunofluorescence analysis

Surface phenotypes of the liver MNC of control mice (injected with rabbit serum) and *in vivo* antibody-treated mice with or without IL-12 administration were identified by using monoclonal antibodies (mAb) in conjunction with two- or three-colour immunofluorescence tests. The mAb used included fluorescein isothiocyanate- (FITC) and phycoerythrin-(PE) conjugated anti-mouse CD3ε (145-2C11, hamster IgG), FITC- and PE-conjugated anti-mouse αβTCR (H57-597, hamster IgG), biotin-conjugated anti-NK1.1 antibody (PK-136, mouse IgG2a), biotin-conjugated anti-IL-2Rβ (TM-β1, rat IgG2b), all of which were purchased from PharMingen & Co. (San Diego, CA). Biotin-conjugated mouse IgG2a and FITC-conjugated hamster IgG (PharMingen) were used to rule out the non-specific binding of anti-NK1.1 antibody and anti-mouse CD3ε antibody. Anti-AGM1 antibody (WAKO, Tokyo, Japan) was developed with FITC-conjugated anti-rabbit immunoglobulin (Dakopatts, Glostrap, Denmark). Biotin-conjugated reagents were developed with PE-conjugated streptavidin (Tago, Burlingame, CA) or Red 613-conjugated streptavidin (Becton Dickinson & Co., Mountain View, CA). Before three-colour staining with anti-AGM1 antibody, anti-CD3 antibody and anti-IL-2Rβ antibody, hepatic MNC were incubated with Fc-blocker (2.4 G2, PharMingen). The presence of fluorescence-positive cells was analysed by fluorescence-activated cell sorter (FACScan; Becton Dickinson).

Tumours used for experimental metastases and evaluation of tumour metastases

EL4 T-cell lymphoma cells, which are highly metastatic for liver, and 3LL cells of B6 origin, which are highly metastatic for lung, were used for the metastases experiments. Tumour cells in culture were washed three times with PBS and were suspended at $0{\cdot}2 \times 10^5$–1×10^5 cells/0·2 ml in PBS. Aliquots (0·2 ml) of tumour cell suspension were injected into mice through the tail vein. After 14 days, mice were killed and test organs were removed and fixed in Bouin's solution to facilitate visualization of tumour colonies prior to counting the number of colonies.

In vivo *cell depletion*

For flow cytometric analysis and cytotoxic assays, mice were injected intraperitoneally with 50 μg anti-AGM1 antibody or 200 μg anti-NK1 antibody 4 days before killing and 0·5 μg IL-12 was injected 1 day before killing. Anti-NK1 antibody was prepared from a PK136 hybridoma grown in our laboratory. Control mice received 0·3 ml of normal rabbit serum (or PBS) 4 days before killing because anti-AGM1 antibody is polyclonal rabbit IgG. Antibody treatment depletes the respective cell populations at least 5 days. For tumour metastasis experiments, mice were treated with antibodies 3 days before tumour inoculation and IL-12 was injected 1 day before tumour inoculation. After tumour inoculation on day 0, antibodies were additionally injected into mice on days 3 and 9. Control mice were injected with rabbit serum on the same days. Mice were killed 14 days after tumour inoculation and the numbers of metastatic foci were counted.

RESULTS

IL-12-activated NK1$^+$ int T cells and NK cells are distinct populations

Anti-AGM1 antibody treatment (50 μg/mouse) *in vivo* depleted NK cells in the liver but NK1$^+$ int T cells remained intact, while anti-NK1 antibody (200 μg/mouse) depleted both NK cells and NK1$^+$ int T cells in the liver of mice (Fig. 1, upper panels). Anti-AGM1 or anti-NK1 antibodies also depleted respective populations in the spleen and peripheral blood lymphocytes (data not shown). Since it has sometimes been observed that IL-12-activated NK cells and NK1$^+$ int T cells are close in appearance[16,17] (Fig. 1, left lower panel), we examined the effect of IL-12 in these antibody-treated mice. After IL-12 administration into NK cell-depleted mice, the NK1 expression of NK1$^+$ int T cells was slightly enhanced and their TCR intensity was broader (Fig. 1, lower middle panel). It is obvious that IL-12 could not induce a significant population of these NK1$^+$ int T cells in both NK cell- and NK1$^+$ int T cell-depleted mice (Fig. 1, right panels). Thus, NK cells and NK1$^+$ int T cells are distinct from each other and IL-12-activated NK1$^+$ int T cells are derived from a resting form of these cells but not from NK cells. However, it should be noted that enhancement of NK1 expression of NK1$^+$ int cells by IL-12 might be dependent on the anti-NK1 antibody batch and sometimes its enhancement is not so obvious (not shown).

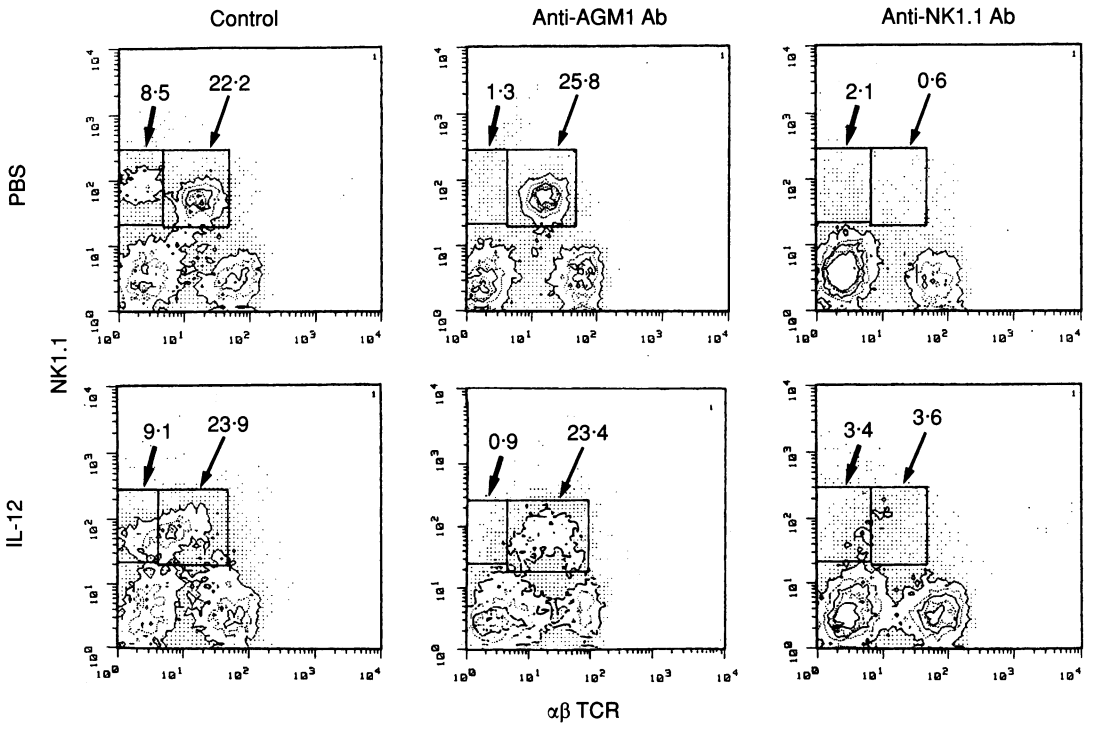

Figure 1. Anti-NK1 antibody but not anti-AGM1 antibody treatment *in vivo* depletes NK1$^+$ int cells. 4 days before killing, mice were injected intraperitoneally with the respective antibodies or rabbit serum (control). One day before sacrifice, mice were injected intraperitoneally with 0·5 μg of IL-12 or PBS. The numbers in each figure are the percentage of NK cells and NK1$^+$ int ↑ cells in total hepatic MNC (indicated by arrows).

AGM1 expression of liver NK cells and int T cells

As demonstrated in Fig. 2, NK cells are AGM1 bright positive, while a significant proportion of int T cells are AGM1 dim positive. This is probably the reason that *in vivo* anti-AGM1 antibody treatment depletes NK cells but does not affect int T cells. In contrast, most bright TCR cells are AGM1 negative.

NK1$^+$ int T cells are responsible for IL-12-induced cytotoxicity of liver and lung MNC

Although cytotoxicity of NK cell-depleted hepatic MNC (by anti-AGM1 antibody) was decreased (Table 1), IL-12 administration into NK cell-depleted mice endowed a cytotoxicity of hepatic MNC nearly comparable to that of mice without antibody treatment (Table 1). In contrast, depletion of both NK cells and NK1$^+$ int T cells in the liver abrogated cytotoxicity and IL-12 could not induce cytotoxicity of hepatic MNC (Table 1). We recently reported that although lungs of mice usually lack NK1$^+$ int T cells, IL-12 can induce NK1$^+$ int T cells.[20] Lung NK cells were also depleted by anti-AGM1 antibody, but while IL-12 could induce NK1$^+$ int T cells in the lung, it could not induce these cells after anti-NK1 antibody treatment (not shown). In addition, a substantial cytotoxicity was induced in NK cell-depleted lung MNC, whereas those of anti-NK1 antibody-treated mice did not show cytotoxicity (Table 2).

NK1$^+$ int T cells but not NK cells are main antimetastatic effectors both in the liver and lung

Consistent with these findings, EL4 cell metastases in the liver, as well as 3LL cell metastases in the lung, were greatly

Table 1. Cytotoxicities of liver MNC against tumours in response to various treatments

		% cytotoxicity at different E/T ratio			
Treatment	Target	100:1	50:1	25:1	12·5:1
PBS	Yac-1	21±3	13±1	10±1	6±1
R.Serum		20±3	12±2	10±1	5±1
α AGM1 antibody		5±2	3±1	3±1	2±1
αNK1 antibody		1±1	1±0	<1	<1
IL-12		57±3	47±3	40±2	26±3
αAGM1+IL-12		45±3	37±1	26±2	19±2
αNK1+IL-12		2±1	1±1	<1	<1
PBS	P815	9±3	3±1	2±1	1±1
R.Serum		10±2	4±1	<1	<1
α AGM1 antibody		2±1	1±1	<1	<1
αNK1 antibody		0	0	0	0
IL-12		20±4	13±1	8±1	2±1
αAGM1+IL-12		16±2	10±2	7±1	3±1
αNK1+IL-12		1±0	<1	0	0
PBS	EL-4	12±2	10±2	8±3	5±1
IL-12		40±4	26±5	21±3	16±2
αAGM1+IL-12		36±5	20±4	18±2	15±3
αNK1+IL-12		3±1	<1	0	0

Three to six mice of each group were injected intraperitoneally with the respective antibodies or control reagents 4 days before killing and IL-12 or PBS were injected intraperitoneally 1 day before sacrifice. Data show percentage cytotoxicity at different E/T ratios and are representative from two independent experiments.

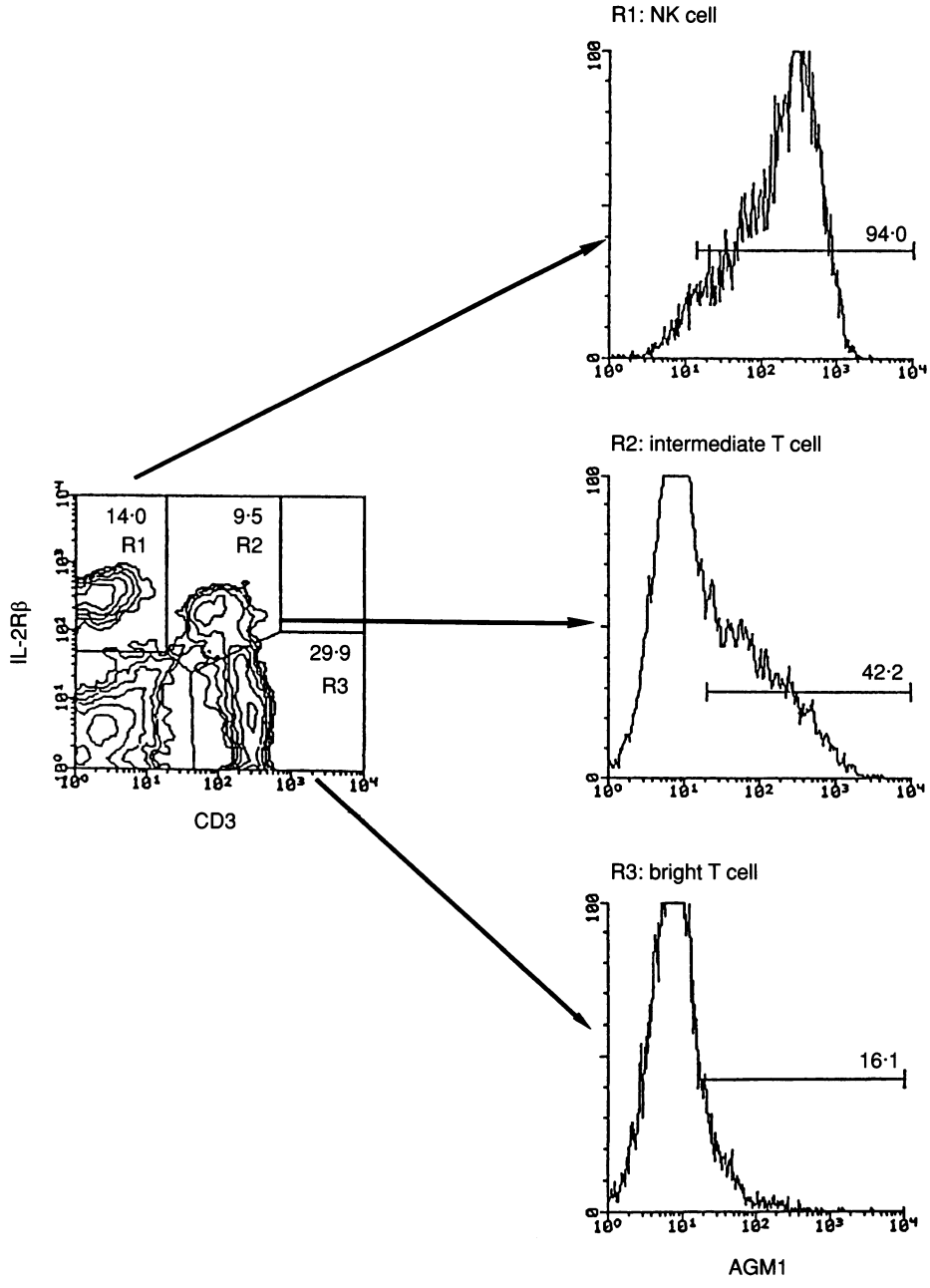

Figure 2. Three-colour flow cytometric analysis for AGM1 expression of liver MNC. NK cells (R1), int T cells (R2) and bright TCR cells (R3) were gated and AGM1 expression was examined.

increased by the depletion of both NK cells and NK1$^+$ int T cells in IL-12 untreated mice (Table 3) and IL-12 could not induce antimetastases. Furthermore, NK-cell depletion alone did not significantly increase metastases in either organ, even in IL-12 untreated mice, and antimetastases comparable to mice without NK-cell depletion could be induced by IL-12 (Table 3).

DISCUSSION

The present study shows that antitumour and antimetastatic functions are among the important physiological functions of NK1$^+$ int T cells. It is of considerable interest that although NK1$^+$ int T cells have less cytotoxic activity than NK cells in their resting states, NK1$^+$ int T cells seem to exert a stronger antimetastatic effect than do NK cells without exogenous IL-12. In addition, although one might argue that NK cells are precursors of NK1$^+$ int T cells or that NK cells could be a major antimetastatic effector, it was confirmed herein that both populations are distinct one from another and NK cells do not play an important antimetastatic role in our experimental model.

The int T cells (presumably NK1$^+$) in the liver of mice gradually emerge after birth and are abundant by adulthood.[26] We recently reported[27] that systemic lipopolysaccharide administration induces IL-12 production from Kupffer cells and activated NK1$^+$ int T cells, and suggested that these cells are specifically and abundantly present in the liver because bac-

Table 2. Cytotoxicities of lung MNC against tumours in response to various treatments

Treatment	Target	% cytotoxicity at different E/T ratio 100:1	50:1	25:1	12·5:1
PBS	P815	5±1	3±1	<1	<1
IL-12		15±3	10±3	6±2	1±0
αAGM1+IL-12		13±2	8±2	4±1	1±1
αNK1+IL-12		<1	0	0	0
PBS	EL-4	ND	8±2	5±1	3±1
IL-12		ND	24±3	18±3	10±1
αAGM1+IL-12		ND	21±2	16±3	8±1
αNK1+IL-12		ND	3±1	1±1	<1

Five to ten mice of each group were injected with the respective antibodies or PBS 4 days before killing and IL-12 or PBS were injected 1 day before killing. Data show percentage cytotoxicity at different E/T ratios; ND, not determined. Repeated experiments showed similar results.

Table 3. Anti-NK1 antibody treatment *in vivo* increases liver and lung metastases

Treatment	EL4 liver metastases	3LL lung metastases
R.Serum	96±18	122±26
αAGM1 antibody	102±24	128±32
αNK1 antibody	152±26	204±36
IL-12	15±4	25±5
αAGM1+IL-12	22±5	33±8
αNK1+IL-12	130±20	156±28

Data represented are mean±SD from six mice of each group. Mice were injected with antibodies or rabbit sera 3 days before tumour inoculation and injected with IL-12 or PBS 1 day before tumour inoculation. Antibodies or rabbit sera were additionally injected into mice on days 3 and 9. The numbers of metastases were examined 14 days after intravenous tumour inoculation. Anti-NK1 antibody treatment significantly ($P<0·001$, by Student's *t*-test) increased both in the liver and lung of IL-12 untreated mice. IL-12 decreased metastases in both organs with or without anti-AGM1 antibody treatment ($P<0·001$).

terial components (including lipopolysaccharide and peptidoglycan polysaccharide) are continuously brought from the intestine and systemic circulation to Kupffer cells.[28–32] Thus, it can be speculated that the population of NK1$^+$ int T cells expands, at least partly, as a result of the symbiotic relationship between the hosts and bacteria in the intestine and other sites. It is also noteworthy that not only Kupffer cells but also hepatocytes themselves play a role in the elimination of bacteria.[32] Since liver fibrosis decreases these cells,[33] there may then be a corresponding need for physiological expansion of NK1$^+$ int T cells.

Wiltrout *et al.* previously reported[34] that AGM1$^+$ cells with potent cytotoxicity are induced in the liver and lung by biological response modifiers and these cells (called organ-associated NK cells) are relatively resistant *in vivo* to anti-AGM1 antibody treatment. These organ-associated NK cells in fact are probably identical to NK1$^+$int T cells. Lukomska *et al.* also reported[35] that rat liver contains a population of NK cells which is resistant to anti-AGM1 antibody treatment.

It is a rather unexpected finding that depletion of NK cells did not increase liver and lung metastases, because it is well known that IL-12 activates NK cells[36–38] and IL-12 certainly endows a significant augmentation of cytotoxicity to liver NK cells[19,27] In addition, both NK cells and NK1$^+$ int T cells are stimulated to produce IFN-γ by the stimulation via NK1 antigen or by IL-12.[39] Nevertheless, even in IL-12 untreated mice, NK1$^+$ int T cells seem to be more potent antimetastatic effectors, because NK-cell depletion did not result in an increase in metastases. However, this phenomenon can be explained by the fact that both EL4 cells and 3LL cells are NK-resistant tumours and may preferentially stimulate NK1$^+$ int T cells directly and/or through activation of Kupffer cells and alveolar macrophages. In fact, in spite of the transfer of 10-fold more splenocytes (as compared to hepatic MNC) from IL-12-treated mice, which apparently contain a larger number of NK cells than hepatic MNC, significant antimetastasis was not induced in the liver and lung of other mice which had been pre-injected with these tumours.[19] Although IL-12 could induce an anti-metastatic effect in NK-deficient *bg/bg* mice comparable to that of normal mice against EL4 metastases in the liver,[20] IL-12 induced only a mild elevation of antitumour cytotoxicity of hepatic MNC in β2-microguloblin-deficient mice[19] which lack most of the hepatic NK1$^+$ int T cells. It was also reported that hepatic int T cells are more cytotoxic than hepatic NK cells against syngeneic hepatoma MH134 (NK-resistant), especially when these cells are stimulated via CD3.[33] Furthermore, cytotoxic activity of hepatic MNC against tumour cells is greatly inhibited when int T cells are decreased by hepatic fibrosis, despite the presence of relatively intact hepatic NK cells.[33]

While we cannot absolutely rule out the possibility that anti-AGM1 antibody stimulates int T cells, it probably does not have much of an effect because *in vivo* anti-AGM1 antibody treatment indeed greatly reduced the cytotoxicity of liver MNC of IL-12 untreated mice, suggesting that anti-AGM1 antibody itself does not activate int T cells. Thus, NK1$^+$ int T cells are an important antitumour effector population in our experimental model. However, under certain conditions or by factors, NK cells can be more important antitumour effectors than NK1$^+$ int T cells. In fact, it was reported that NK cells appear early in peritoneal cavity of mice inoculated intraperitoneally with tumours suggesting that NK cells are important for the inhibition of tumour growth and for the induction of CD8$^+$ cytotoxic T cells.[40,41] It is also possible that NK cells and NK1$^+$ int T cells affect each other and the absence of NK cells may rather activate NK1$^+$ int T cells under certain conditions.

Since T cells with an NK-cell marker, CD56, are also abundant in the liver of humans[42] and CD56$^+$ T cells in the peripheral blood acquire a potent antitumour cytotoxicity *in vitro* by a combination of IL-12 and IL-2,[42] it is suggested that T cells with NK-cell markers in both species have an important role in monitoring intra- and perivascular areas. Details of the interaction between these T cells and conventional T cells or NK cells remain for further investigation.

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