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The Antitumor Function of Extrathymic T Cells with an NK Cell Marker in the Mouse Liver and their Putative Counterpart in Humans

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Summary. **Few T cells with NK cell markers are present in the periphery (including the peripheral blood), but mouse livers contain a remarkably high proportion of $\alpha\beta$ T cells with an NK cell marker, NK1.1 Ag (NK1). Interleukin 12 (IL-12), when administered into both euthymic and athymic B6 mice, endows a potent cytotoxicity to liver mononuclear cells (MNC) and inhibits liver metastases of i.v. injected liver metastatic tumors. NK1$^+$ $\alpha\beta$ T cells are the main antitumor cytotoxic effectors induced by IL-12 in the liver. Interestingly, although the hepatic vein blood and lung usually do not contain NK1$^+$ $\alpha\beta$ T cells, IL-12 administration induces these cells in both sites. The cells induced by IL-12 in the lung also show strong antitumor cytotoxicity and are antimetastatic effectors against i.v. injected lung metastatic tumors. *In vivo* IL-12 activated liver MNC (but not splenocytes), when adoptively transferred into other tumor preinjected mice, inhibited metastases in the lungs and liver. This antimetastatic effect was inhibited by the depletion of either NK1$^+$ cells or CD3$^+$ cells from IL-12 activated hepatic MNC before transfer. Depletion of NK cells *in vivo* by anti-asialo GM1 Ab did not reduce IL-12 induced cytotoxicity of the liver or lung MNC or antimetastasis in either organ. Depletion of both NK cells and NK1$^+$ $\alpha\beta$ T cells by anti-NK1 Ab greatly inhibited the augmentation of the cytotoxicity of liver and lung MNC induced by IL-12 as well as antimetastasis in either organ. These findings show that NK1$^+$ $\alpha\beta$ T cells in the mouse liver are antitumor and antimetastatic effectors which move to other organs to inhibit tumor metastases. Human livers also contain high proportions of T cells with an NK cell marker, CD56. Although human peripheral blood lymphocytes (PBL) contain a small percentage of CD56$^+$ $\alpha\beta$ T cells, when monocyte depleted PBL were cultured with a combination of IL-12 and IL-2, populations of either CD56$^+$ $\alpha\beta$ T cells or CD56$^+$ $\gamma\delta$ T cells with potent antitumor cytotoxicity were selectively expanded. These cells had a much stronger cytotoxicity than CD56$^-$ T cells activated IL-2 alone. Taken together, our findings demonstrate that T cells with NK cell markers likely represent a more potent antitumor and antimetastatic effector population than NK cells or conventional T cells in mice as well as in humans.**

Key words—NK1.1 Ag$^+$$\alpha\beta$ T cells, liver, intermediate TCR, Interleukin 12, tumor metastasis.

I. Introduction

T cells with NK cell markers were first identified in humans by Abo and Balch.[1] They found that CD57 (Leu 7) is distributed on NK cells and a small population of peripheral T cells.[1,2] Subsequently, Lanier et al. found that CD56 (Leu 19) is distributed on most NK cells and a small population (5% or lles) of T cells with NK-like activity in peripheral blood lymphocytes (PBL).[3] In mice, NK1$^+$ $\alpha\beta$ T cells are initially found in the thymus as CD4$^-$8$^-$ double negative (DN) $\alpha\beta$ T cells.[4-6] Ohteki et al.[7] and we[8] recently found that NK1$^+$ $\alpha\beta$ T cells are most abundant in the liver; 30 to 50% of mouse liver T cells are NK1$^+$ $\alpha\beta$ T cells. Several reports[9-11] demonstrated that the murine liver contains an $\alpha\beta$ T cell population with an intermediate level of TCR (int TCR), which is lower than that of mature thymocytes or most peripheral T cells but higher than the low TCR intensity of immature double positive thymocytes; it has also been shown that int TCR cells can develop extrathymically in the liver.[10,12] Thymic DN cells are

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also int TCR cells. A majority of these int TCR cells were found to express NK1, and NK1$^+$ $\alpha\beta$ T cells are exclusively int TCR cells.[13] These cells have several other unique characteristics which render them different from conventional T cells: 1) They have large granular morphology;[13] 2) They are composed mainly of CD4$^+$ cells and DN cells;[7,13] 3) They contain self-MIs (minor lymphocyte stimulatory) Ag specific Vβ T cells and show a preponderance of Vβ7 and Vβ8 T cells;[11] 4) They mainly, if not exclusively, use Vα14 for their TCR;[14,15] 5) A significant proportion have perforin granules in their cytoplasm;[13] 6) They are present in the liver of athymic nude mice, although in smaller numbers;[8] 7) Although they contain CD4$^+$ cells, they are perhaps exclusively selected by MHC class I or related molecules but not by class II molecules.[7,16-18]

Despite the accumulation of reports dealing with NK1$^+$ $\alpha\beta$ T cells, functional characteristics of these cells *in vivo* and *in vitro* have been poorly defined. It has been reported that upon stimulation by CD3, these cells secrete IL-4 and IFN-γ but not IL-2.[19,20] We recently found that a cytokine, IL-12, which activates NK cells and pre-activated T cells and is now known as an antitumor cytokine,[21-24] activates NK1$^+$ $\alpha\beta$ T cells in the liver and strong MHC unrestricted antitumor cytotoxicity to these cells.[8] However, although it was reported that[25] IL-12 administration in mice increases the survival time of tumor bearing mice and CD8$^+$ cells with the possible help of CD4$^+$ cells as antitumor effectors against subcutaneously innoculated tumors,[25,26] antimetastatic effectors induced by IL-12 in the liver and lung have not been determined. We have recently revealed that IL-12-activated hepatic NK1$^+$ $\alpha\beta$ T cells (but not NK cells nor conventional T cells) are precise antimetastatic effectors not only in the liver but also in the lung of i.v. injected tumors.[8,27,28] In the present report, we present further details on the antitumor function of hepatic NK1$^+$ $\alpha\beta$ T cells and consider why these cells are abundantly and preferentially present in the liver.

II. IL-12 induces potent cytotoxic NK1$^+$ $\alpha\beta$ T cells in the mouse liver

Administration (i.p.) of 0.5 μg murine IL-12 induces enhancement of the NK1 expression of NK1$^+$ $\alpha\beta$ T cells and an increase in the number of these cells in the mouse liver within 24 h (Fig. 1). Depletion of either CD3$^+$ cells or NK1$^+$ cells by respective Ab and C *in vitro* abolishes IL-12 induced cytotoxicity of hepatic mononuclear cells (MNC) against NK-resistant liver metastatic EL4 cells and lung metastatic 3LL cells (Table 1), suggesting that both CD3 and NK1 positive cells, namely, NK1$^+$ $\alpha\beta$ T cells, are cytotoxic effectors. This also holds for athymic nude mice. It was confirmed by cytotoxic assays after sorting that IL-12 activated NK1$^+$ $\alpha\beta$ T cells are the main cytotoxic effectors (Table 1).[28] Although NK cells stimulated by IL-12 also acquired a moderate measure of cytotoxicity, NK1$^+$ $\alpha\beta$ T cells were much more cytotoxic than NK cells. Bright TCR cells of thymic origin had virtually no cytotoxicity (Table 1).

III. IL-12 administration into mice inhibits liver and lung metastases of i.v. injected tumors

Administration of 0.5 μg IL-12 into mice (3$\times$weekly) began 1 day after i.v. injection of 5×10^4 liver or lung metastatic tumor cells. The mice were sacrificed 2 weeks after tumor inoculation and the number of tumor foci in the liver and lung were counted. As shown in Table 2, metastases of various liver and lung metastatic tumors were greatly inhibited in all strains of mice, including NK-deficient bg/bg mice (which have NK cells, though these have no or very weak NK activity) and athymic nude mice.[27] These findings also support the notion that NK cells or thymus derived T cells do not play a significant role in inhibiting metastases in either organ.

IV. Transfer of IL-12-activated hepatic MNC (but not splenocytes) into other mice with pre-injected tumors suppresses metastases

The effect of the adoptive transfer of *in vivo* IL-12 activated hepatic MNC, splenocytes and PBL on tumor metastases in the livers and lungs was examined. IL-12-activated hepatic MNC, splenocytes and PBL isolated from IL-12 treated (24 h previously) mice were adaptively transferred (i.v.) into mice that had been inoculated with tumors the day before. Transfers were carried out 4$\times$ more during the ensuing 2 weeks. The numbers of cells transferred each time were 2×10^6 liver MNC, 2×10^7 splenocytes, or 2×10^6 PBL. While the numbers of liver MNC and splenocytes transferred were adjusted approximately one third of the total MNC yield from the liver and spleen, the number of PBL transferred was adjusted to the number of hepatic MNC. In both organs, only the transfer of liver MNC was effective against tumor metastases (Fig. 2).[28]

V. CD3$^+$NK1$^+$ cells are antimetastatic effectors of transferred hepatic MNC

To investigate which population of hepatic MNC is

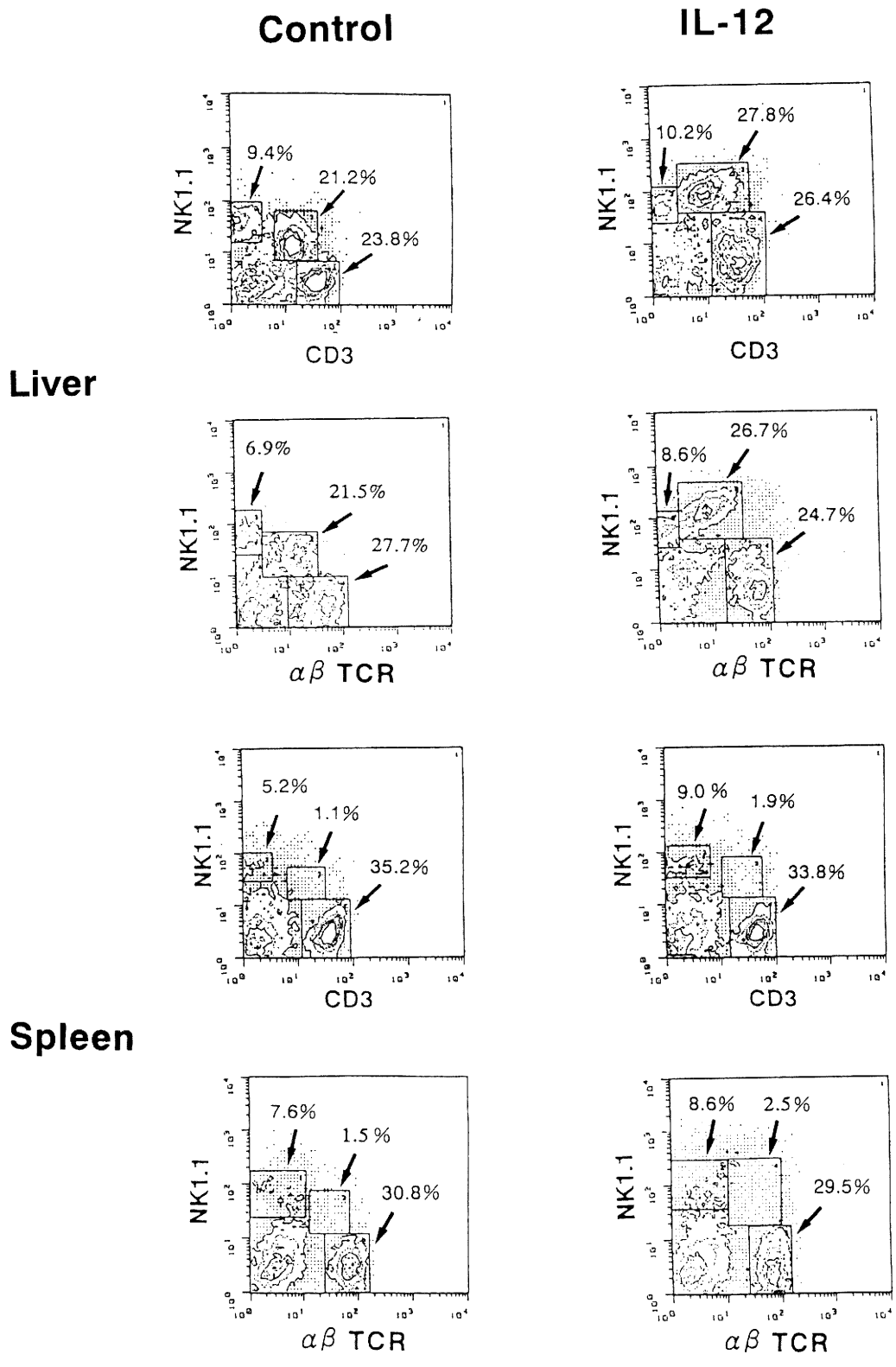

Fig. 1. Two-color immunofluorescence profiles of NK1 and either CD3 or $\alpha\beta$ TCR expressions of the liver and the spleen MNC of C57BL/6 mice and 24 h after injection of PBS (left panel) or 0.5 μg of IL12 (right panel).

Table 1. $CD4^+NK1^+TCR/CD3^{int}$ cells are repsonsible for IL-12 induced cytotoxicity of hepatic MNC

Target	EL4			3LL	
Treatment of MNC	% cytotoxicity at different E/T ratio			% cytotoxicity	
	50 : 1	25 : 1	12.5 : 1	50 : 1	25 : 1
—	35±2.8	25±0.5	18±0.4	10±0.6	5±0.6
Complement	32±1.6	27±2.1	16±2.1	11±1.0	6±0.2
Anti-NK1+C	3±0.6	2±1.4	1±1.1	1±0.6	<1
Anti-CD3+C	9±1.0	5±1.5	3±0.9	<1	0.5±0.6
Anti-CD4+C	7±1.3	4±1.2	1±1.0	2±1.1	<1
Anti-CD8+C	35±2.1	29±4.4	17±0.7	12±0.7	7±1.0
Unsorted		30±0.2	16±0.5	17±1.2	8±1.2
Sorted $CD3^-NK1^+$		18±1.7	10±0.2	12±0.8	5±0.6
Sorted $CD3^{int}NK1^{high}$		70±2.1	43±4.1	27±2.0	14±1.0
Sorted CD3 bright $NK1^-$		7±1.6	2±1.4	3±0.6	1±0.8

Data show mean (±SD) percent cytotoxicities at different E/T ratios. Twenty-four h after IL-12 administration (0.5 μg/ml), 5 mice were sacrificed and hepatic MNC were obtained. Hepatic MNC were treated with respective Abs and C and cytotoxic assays were carried out. For sorting, 20 IL-12 injected mice were sacrificed and hepatic MNC were obtained. Repeated experiments showed similar results.

Table 2. Inhibitory effects of IL-12 against experimental metastases of a variety of tumor cells in the liver and lung

Mouse		Tumor	Site of metastases tested	Tumor metastases		
				Control	Treated	% inhibition
BALB/c	+/+	RL♂1	liver	216±24	28±2	87%, $p<0.001$
		Colon 26	lung	125±25	16±10	87%, $p<0.001$
DBA/2	+/+	P815	liver	173±12	10±1	94%, $p<0.001$
C57BL/6	+/+	B16	lung	61±16	5±1	91%, $p<0.001$
BALB/c	nu/nu	RL♂1	liver	147±23	10±3	93%, $p<0.001$
		Colon 26	lung	58±11	4±1	93%, $p<0.001$
C57BL/6	bg/bg	EL4	liver	107±17	16±6	85%, $p<0.001$

The mice were inoculated with syngeneic tumors. Data of tumor metastases and % of inhibition are expressed as mean±SD of 8–10 mice per group.

the antimetastatic effector, isolated hepatic MNC were treated respectively with Ab and C before transfer. Depletion of $CD3^+$ cells, $NK1^+$ cells or $CD4^+$ cells greatly inhibited hepatic MNC transfer-induced antimetastases induced in the liver and the lung.[28] Thus, $NK1^+CD3^+CD4^+$ cells, namely $NK1^+$ $\alpha\beta$ T cells, are revealed to be the effectors for antimetastasis. IL-12-activated $NK1^+$ $\alpha\beta$ T cells are CD4 dim positive or double negative.[28] Interestingly, hepatic MNC from IL-12 treated nude mice, when transferred into tumor pre-injected euthymic mice, also inhibited metastases of both the liver and lung, and this effect was abrogated by the depletion of $CD3^+$ cells (Fig. 3b),[28] revealing that extrathymically developed $NK1^+$ $\alpha\beta$ T cells are functionally competent.

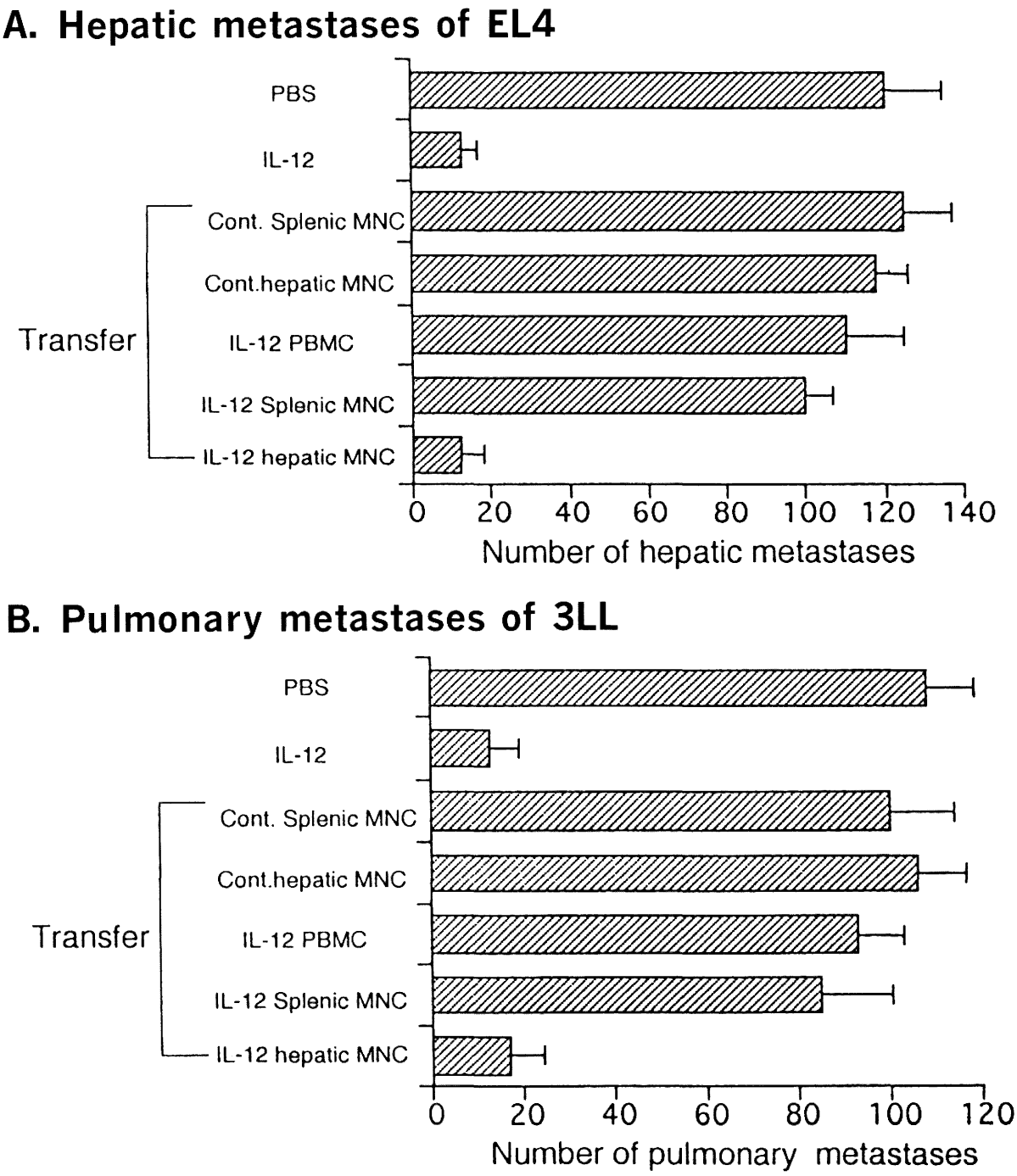

Fig. 2. Adoptive transfer of IL-12 stimulated hepatic MNC inhibit tumor metastases in the liver (**A**) and lung (**B**). 2×10^6 hepatic MNC, 2×10^7 splenic MNC or 2×10^6 blood MNC from IL-12 treated mice (0.5 μg IL-12/mouse was injected 24 h before sacrifice) or untreated mice were intravenously injected into mice which had been pre-injected with 0.5×10^5 liver metastatic EL4 cells or lung metastatic 3LL cells 1 d before transfer. Transfers were carried out 5 times on alternate days, starting from day 1. Control mice received injections of 0.5 μg IL-12/injection or PBS 5 times on the same days as the MNC transfers. After 14 days, the mice were sacrificed and test organs were removed and fixed in Bouin's solution to facilitate visualization of tumor foci prior to counting the number of foci. The data represented are the numbers of tumor foci (mean$\pm$SD) of 5 to 10 mice of each group.

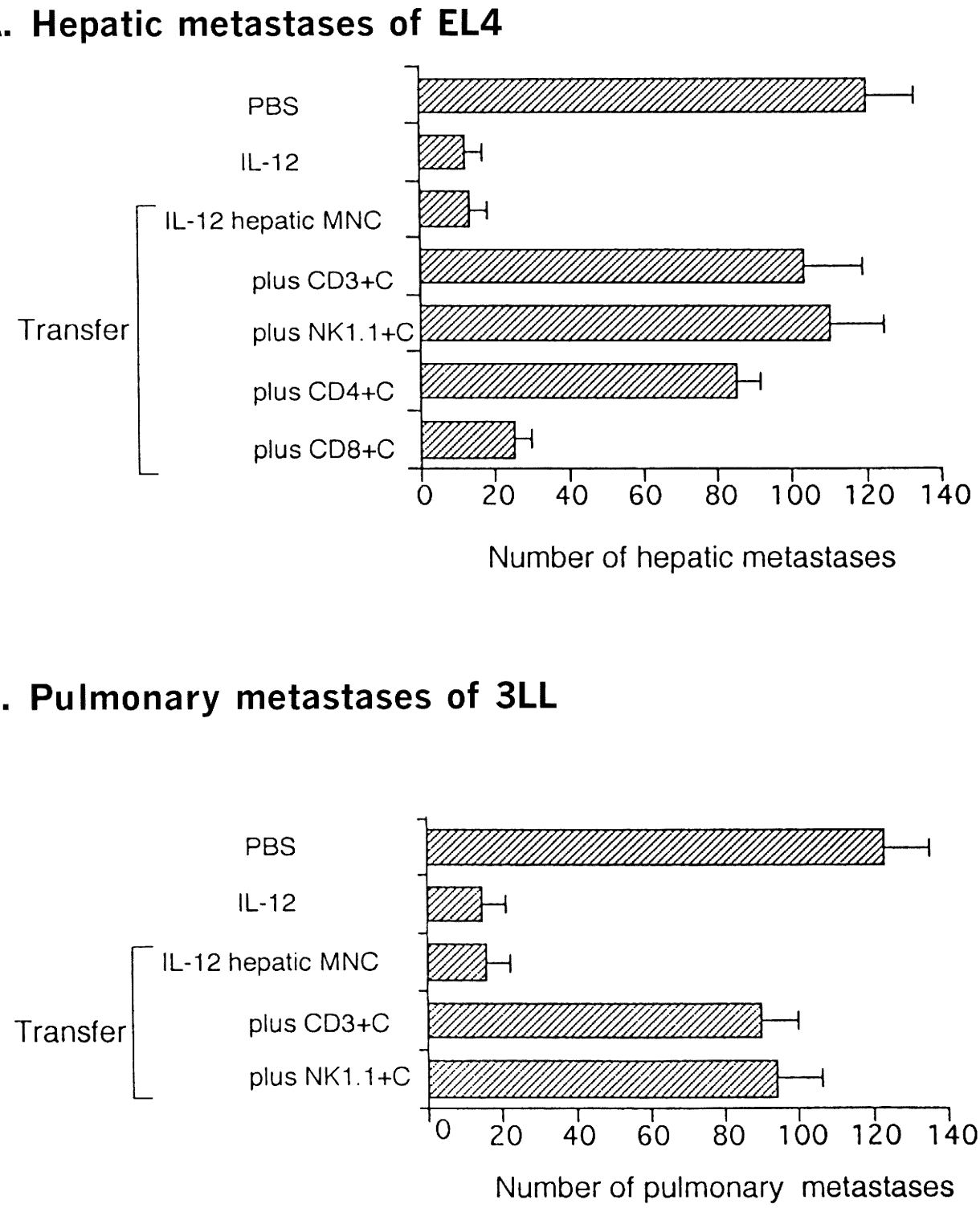

Fig. 3a. $CD4^{low}NK^{high}TCR^{int}$ cells are antimetastatic effectors in the liver and lung. $CD3^+$, $NK1^+$, $CD4^+$ or $CD8^+$ cells were depleted from IL-12 stimulated hepatic MNC by respective Ab and C, after which transfers were again carried out. The data represented are the numbers of tumor foci (mean±SD) of 5 to 10 mice of each group.

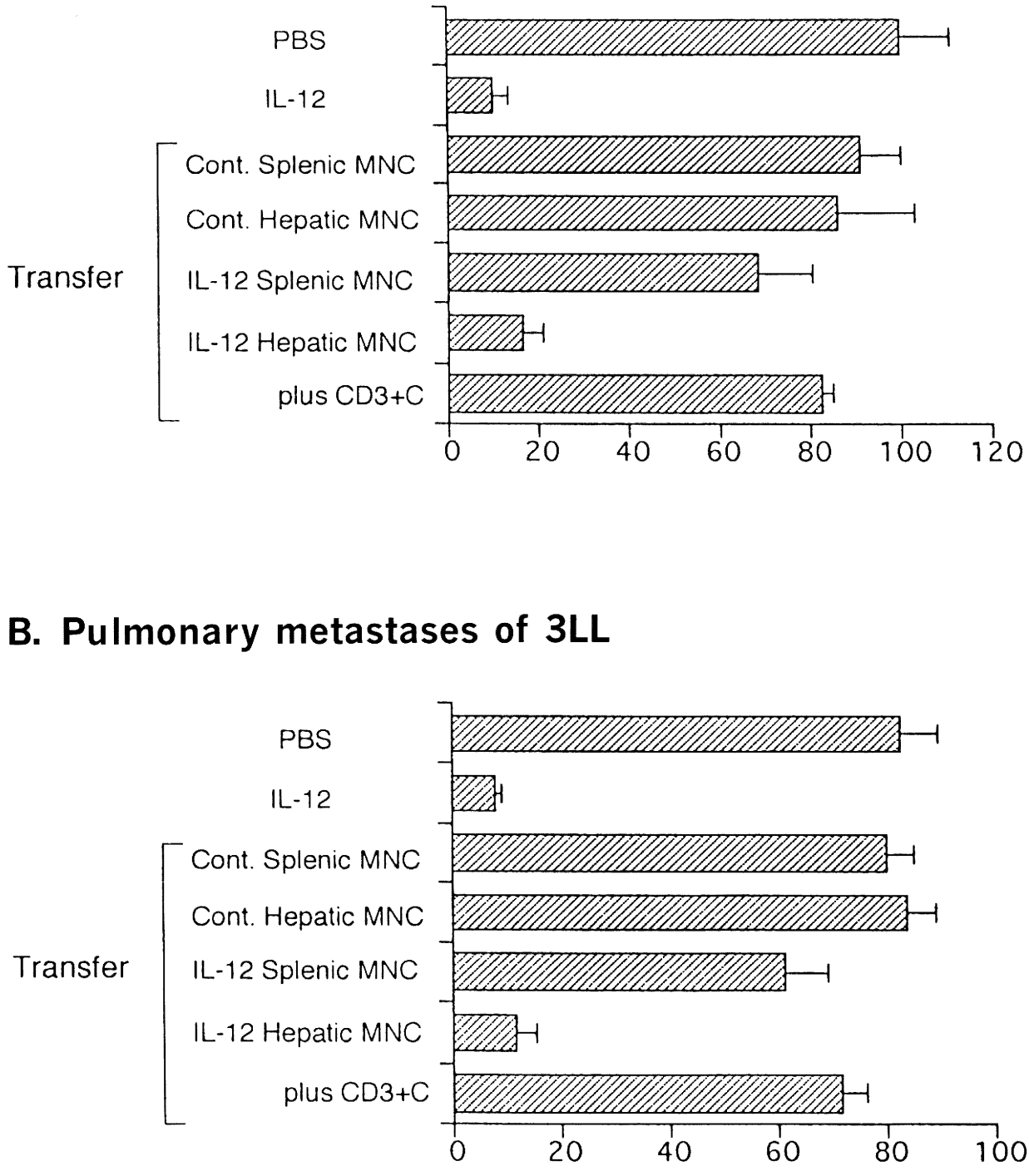

Fig. 3b. $NK^{high}TCR^{int}$ cells in the liver of nude mice also have an antimetastatic effect. Twenty-four h after IL-12 (0.5 μg/mouse, i.p.) administration into C57BL/6 nu/nu mice, hepatic and splenic MNC were obtained and transferred into EL4 (**A**) or 3LL (**B**) pre-injected +/+ mice. The data represented are from 5 mice in each group.

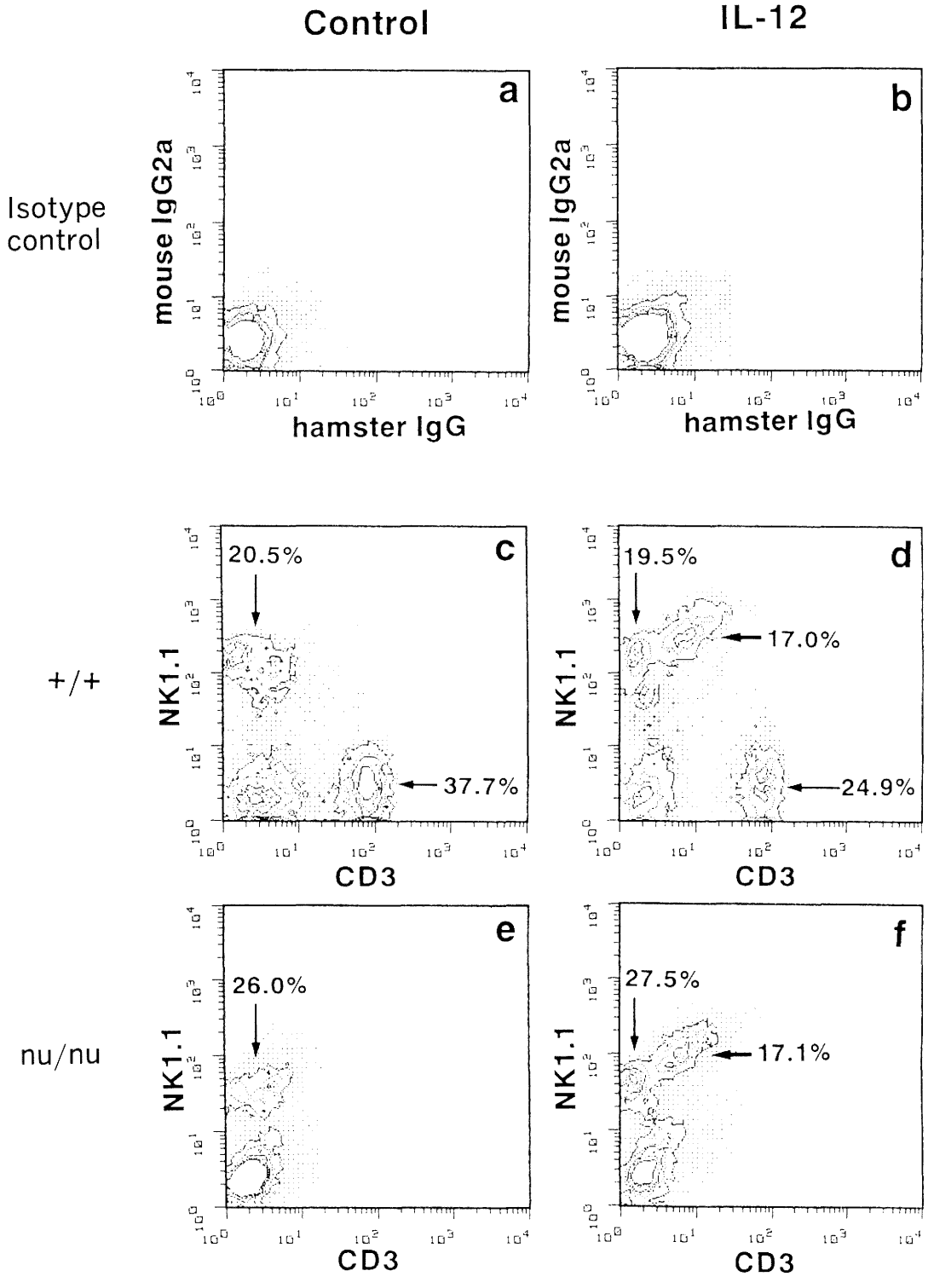

Fig. 4. IL-12 induces NK1$^+$ $\alpha\beta$ T cells in the lungs of both +/+ and athymic nu/nu mice. Twenty-four h after IL-12 i.p. administration, lungs were removed and pulmonary MNC were examined. Fig. 4a and b show isotype controls for anti-CD3 Ab and anti-NK1 Ab.

VI. *In vivo* depletion of NK1$^+$ $\alpha\beta$ T cells abolishes IL-12-induces the cytotoxicity of hepatic MNC and lung MNC and antimetastasis in both organs

Although mouse lungs normally have NK cells but not NK1$^+$ $\alpha\beta$ T cells (Fig. 4c, e), IL-12 induced NK1$^+$ $\alpha\beta$ T cells (Fig. 4d, f) with potent cytotoxicity in the lung. This phenomenon is also apparent in hepatic vein blood.[28] To further confirm that NK1$^+$ $\alpha\beta$ T cells are antimetastatic effectors, mice were treated with anti-asialo GM1 (AGM1) Ab or anti-NK1 Ab *in vivo* before IL-12 administration. Anti-AGM1 Ab depleted NK cells in the liver and other organs but did not affect NK1$^+$ $\alpha\beta$ T cells (Fig. 5), while anti-NK1 Ab depleted both populations (Fig. 5). Intriguingly, although NK cell depletion significantly reduced cytotoxicity of the liver and lung MNC of IL-12 untreated mice, IL-12 administration could induce strong cytotoxicity of liver and lung MNC in NK cell depleted mice, nearly comparable to that of mice without NK cell depletion, while IL-12 could not induce any cytotoxicity of liver and lung MNC which were depleted of both NK cells and NK1$^+$ $\alpha\beta$ T cells (Table 3). Moreover, NK cell depletion did not lead to an increase in metastases in the livers and lungs of IL-12 untreated mice, and IL-12 could inhibit metastases in both organs, whereas depletion of both populations resulted in marked increases in the numbers of metastases with or without IL-12 treatment (Table 4). Thus, NK1$^+$ $\alpha\beta$ T cells are more potent antimetastatic effectors than NK cells in livers and lungs with or without IL-12 stimulation. Although it was generally suggested that NK cells are responsible for the antimetastases in either organ, this is not the case; instead, NK1$^+$ $\alpha\beta$ T cells appear to be a novel antitumor population in these organs. It should be emphasized that anti-NK1 Ab alone can not discriminate NK1$^+$ $\alpha\beta$ T cells from NK cells. We believe that our finding is important in the field of tumor immunology, because humans also have a lymphocyte population similar to NK1$^+$ $\alpha\beta$ T cells of mice (as described below), and liver and lung metastases of tumors are lethal outcomes in many cancer patients. Without consideration and investigation of

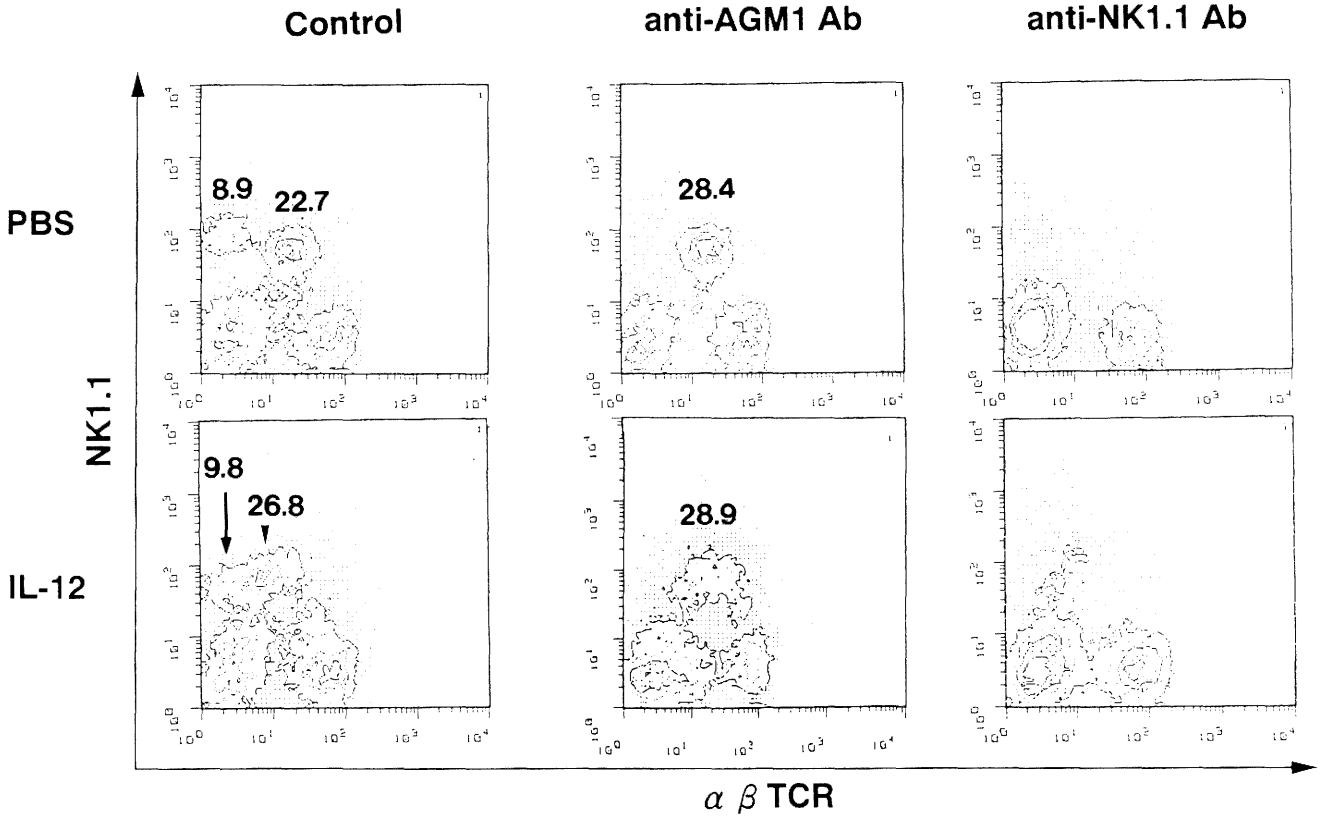

Fig. 5. αNK1 Ab but not αAGM1 Ab treatment *in vivo* depletes NK1$^+$ int cells. Four days before sacrifice, mice were injected (i.p.) with respective Abs or rabbit serum (control). One day before sacrifice, mice were injected (i.p.) with 0.5 μg of IL-12 or PBS.

Table 3. NK1$^+$int T cells are responsible for IL-12 induced antitumor cytotoxicity of hepatic and lung MNC

Organ of MNC	Treatment	Target	% cytotoxicity at different E/T ratio			
			100 : 1	50 : 1	25 : 1	12.5 : 1
	PBS		21±3	13±1	10+1	6±1
	R.Serum		20±3	12±2	10±1	5±1
	αAGM1 Ab		5±2	3±1	3±1	2±1
Liver	αNK1 Ab	Yac-1	1±1	1±0	<1	<1
	IL-12		57±3	47±3	40±2	26±3
	R. Serum+IL-12		58±3	48±4	42±3	25±4
	αAGM1+IL-12		45±3	37±1	26±2	19±2
	αNK1+IL-12		2±1	1±1	<1	<1
	PBS		9±3	3±1	2±1	<1
	R. Serum		10±2	4±1	<1	<1
	αAGM1 Ab		2±1	1±0	<1	<1
Liver	αNK1 Ab	P815	0	0	0	0
	IL-12		20±4	13±1	8±1	2±1
	R. Serum+IL-12		21±3	14±2	8±1	3±1
	αAGM1+IL-12		16±2	10±2	7±1	3±1
	αNK1+IL-12		1±0	<1	0	0
	PBS		5±1	3±1	<1	<1
Lung	IL-12	P815	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

Table 4. Anti-NK1 Ab treatment *in vivo* increases liver and lung metastases

Treatment	EL4 liver metastases	3LL lung metastases
R. Serum	96±18	118±23
αAGM1 Ab	86±15	102±22
αNK1 Ab	156±26	188±25
IL-12	15± 4	21± 5
αAGM1+IL-12	20± 5	24± 6
αNK1+IL-12	125±19	146±28

Data represented are mean±SD from 6 mice of each group. αNK1 Ab treatment significantly ($p<0.001$) increased metastases both in the liver and lung of IL-12 untreated mice. IL-12 decreased metastases in both organs of mice with or without αAGM1 treatment ($p<0.0005$).

these cells, the mechanism of tumor metastases, defense mechanism of hosts against metastasis and its therapeutic strategy can not be fully researched. Our findings also strongly suggest that these cells in the liver can move to other organs and suppress tumor metastases.

VII. LPS induces cytotoxic NK1$^+$ αβ T cells via production of IL-12 from Kupffer cells: a speculation as to why NK1$^+$ αβ T cells are abundant in the liver

Since heat killed bacteria activate hepatic αβ T cells with intermediate TCR (presumably NK1$^+$),[29] and LPS (endotoxin, a cell wall component of gram negative bacteria) reportedly induces IL-12 production from macrophages or monocytes,[30,31] we sought to determine whether or not LPS activates Kupffer cells and NK1$^+$ αβ T cells. LPS and a synthetic analog of lipid A (the biologically active part of LPS), ONO 4007, but not detoxified LPS nor other cytokines, endowed a strong cytotoxicity to hepatic MNC (Table 5).[32] LPS also enhanced NK1 expression of NK1$^+$ αβ T cells, which are responsible for LPS-induced cytotoxicity of hepatic MNC.[32] We then examined IL-12 mRNA expression of hepatic MNC by RT-PCR. Unstimulated plastic-adherent Kupffer

cells expressed mRNA of the IL-12 p35 chain alone, but Kupffer cells stimulated by LPS *in vivo* expressed mRNA of both the p35 and p40 chains of IL-12. In contrast, nonadherent hepatic MNC did not express mRNA for p40 chain of IL-12 with or without LPS stimulation (Fig. 6).[32] These suggest that the LPS-induced activation of NK1$^+$ $\alpha\beta$ T cells is a result of IL-12 production by Kupffer cells.

To confirm that IL-12 is involved in the LPS-induced effect, anti-IL-12 Ab or Abs to other cytokines were injected simultaneously with LPS and the cytotoxicity of hepatic MNC was examined. The results revealed that the LPS-induced cytotoxicity of hepatic MNC was decreased only by the neutralizing anti-IL-12 Ab (C17.8) and anti-IFN-γ Ab (Table 6).[32] However, anti-IFN-γ Ab exerted less of an inhibitory effect than did the neutralizing anti-IL-12 Ab. Namely, IL-1 and TNF are not involved in the enhancement of the cytotoxicity of hepatic MNC, and IFN-γ alone can not explain the effect of IL-12.[32] It is suggested that IL-12 itself or other factors are essential for the IL-12 induced effect. Most of LPS and bacteria that enter the blood stream reportedly accumulate in the liver and are removed therefrom by Kupffer cells or hepatocytes. In addition, LPS and peptidoglycan polysacharride (a component of cell walls of both gram positive and negative bacteria) are continuously brought (sometime at high doses) to the liver from the intestine via the portal vein.[33-36] In fact, i.v. injection of heat killed bacteria (both gram positive and negative) into mice induces IL-12 p40 mRNA of Kupffer cells (our unpublished observation). NK1$^+$ $\alpha\beta$ T cells gradually emerge after birth and are abundant in the liver by adulthood[10]; this phenomenon is accelerated in mice housed and fed in conventional non-specific pathogen free conditions more than in specific pathogen free conditions.[10]

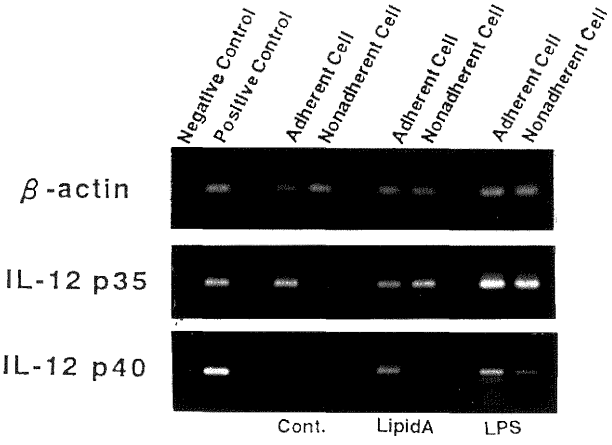

Fig. 6. LPS and lipid A induces mRNA for a p40 heavy chain of IL-12 in Kupffer cells. Liver plastic adherent cells and nonadherent MNC were collected 6 h after LPS or lipid A administration, and p35 and p40 mRNA of IL-12 were detected by PCR. Positive control is from Con A stimulated splenocytes.

Table 5. LPS and ONO-4007 induce cytotoxicity of hepatic MNC comparable to that induced by IL-12

Organ	Treatment	% cytotoxicity at different E/T ratio			
		50 : 1	25 : 1	12.5 : 1	6.25 : 1
Liver	—	6.2±1.8	4.0±0.2	1.9±0.9	0.8±0.3
	LPS	25.4±3.6	17.8±2.9	11.2±1.3	9.2±0.9
	ONO-4007	29.8±5.2	21.3±2.3	16.6±1.4	9.6±1.2
	LPS-D	5.0±1.5	2.1±0.8	0.4±0.4	0
	IL-12	22.7±0.8	16.3±0.7	9.6±1.5	6.3±1.8
	IL-1	7.7±0.6	3.0±1.2	1.6±1.0	0.4±0.4
	TNFα	5.3±1.4	1.3±1.8	1.1±0.5	0.5±0.4
Spleen	—	2.4±1.5	0.7±0.4	0.1±0.2	0.1±0.1
	LPS	4.6±1.5	2.6±0.8	0.4±0.4	0.2±0.2
	ONO-4007	4.3±4.9	0.2±0.2	0	0
	LPS-D	0.2±0.1	0	0.1±0.1	0
	IL-12	5.2±1.3	3.2±0.3	2.1±0.3	0.8±0.2
	IL-1	1.1±0.3	0.6±0.01	0.4±0.2	0
	TNFα	3.6±0.7	1.8±1.7	1.6±0.4	0.5±0.4

Table 6. The cytotoxicity of hepatic MNC induced by LPS was diminished by the anti IL-12 antibody

Treatment	Target	50 : 1	25 : 1	12.5 : 1
—	P815	3.9±2.7	1.7±0.2	1.8±0.1
LPS		22.4±0.3	17.0±2.0	11.4±1.7
LPS+αIL-1α, β		18.3±3.2	14.7±1.8	9.7±1.6
LPS+αTNFα		20.4±1.1	17.6±1.7	9.3±0.7
LPS+αIL-1α, β+αTNFα		21.7±2.5	15.7±2.5	9.2±0.9
LPS+αIL-12 (C17.8)		4.1±0.8	3.0±0.7	1.8±0.3
LPS+αIFNγ		6.8±0.5	5.4±0.5	3.8±1.1
LPS+αIL-12 (C15.1)		23.2±1.9	20.5±0.3	16.2±3.0
—	YAC-1	5.3±0.8	3.9±0.8	3.7±0.6
LPS		38.3±3.3	34.4±0.8	30.3±2.6
LPS+αIL-1α, β		31.1±2.8	29.7±0.3	28.4±2.1
LPS+αTNFα		37.5±7.1	32.4±1.6	30.0±1.6
LPS+αIL-1α, β+αTNFα		34.3±1.4	30.3±3.3	27.8±4.4
LPS+αIL-12 (C17.8)		12.1±2.1	10.7±0.9	8.8±1.8
LPS+αIFNγ		21.2±1.2	18.4±2.1	16.3±1.3
LPS+αIL-12 (C15.1)		40.5±0.8	33.5±1.2	30.6±1.0

Accordingly, it is speculated that these cells originate and expand by way of a symbiotic relationship between the hosts and bacteria in the intestine and other sites (e.g., tonsil and skin).

VIII. Origin and differentiation pathway of NK1$^+$ αβ T cells

Researchers found that DN T (int TCR) cells in the thymus and int T cells (including DN cells) in the liver have Vβ8 T cell predominance and contain self-Mls specific Vβ T cells which are normally deleted in conventional T cell development. However, several years passed before it was recognized that these cells are NK1 positive and are composed of both DN and CD4$^+$ cells,[17-19] partly because only certain strains of mice (e.g., B6 and B10) express NK1. Conventional T cell development takes place in the thymus, in which CD3$^-$4$^-$8$^-$ precursor cells develop into CD4$^+$ or CD8$^+$ single positive cells via an immature CD4$^+$8$^+$ double positive stage. Autoreactive T cells, including self-Mls specific Vβ T cells, are deleted at this double positive stage (so called negative selection). Since NK1$^+$ αβ T cells contain self-Mls specific and thereby potentially autoreactive Vβ T cells, it can be speculated that the differentiation pathway of these cells is different from that of conventional T cells. Although it was proposed that these cells can develop extrathymically because these cells are also present in the liver of nude mice,[9,10] it was some time before this proposition was generally accepted. However, it has recently been found that β2 microglobulin-disrupted (β2m-/-) mice lack most NK1$^+$ αβ T cells in the thymus and liver,[7,15-18] suggesting that, despite the presence of CD4$^+$ T cells, these cells are selected by MHC class I molecules or related molecules which are co-expressed with β2m. Moreover, these cells are selected by bone marrow derived class I bearing cells (including macrophages) but not thymic epithelial cells. This is in marked contrast to conventional thymus derived cells, which need class I molecules (CD8$^+$ cells) or class II molecules (CD4$^+$ cells) expressed on thymic epithelial cells for their differentiation. This finding indicates that NK1$^+$ αβ T cells do not necessarily differentiate in the thymus. Moreover, Sato et al., using bone marrow transplantation into thymectomized mice, clearly demonstrated in a subsequent work that these cells can develop extrathymically. Thus, in the absence of the thymus, int T cells (both NK1$^+$ and NK1$^-$) emerge in the liver and other organs. Therefore, it is concluded that these cells develop extrathymically. Although we do not deny that the thymus produces some of these cells, the pathway should be different from the conventional thymic pathway and should be termed "an alternative thymic pathway".

IX. Human CD56$^+$ $\alpha\beta$ T cells are probably a functional counterpart of NK1$^+$ $\alpha\beta$ T cells in mice

Although only a small percentage (approximately 5%) of CD3$^+$ cells with NK cell marker, CD56, are present in human PBL, human livers contain a much larger population of these cells (Fig. 7).[37] Further, when monocyte depleted human PBL are cultured with 20 U of IL-12 and 100 U of IL-2, either CD56$^+$ $\gamma\delta$ or CD56$^+$ $\alpha\beta$ T cells are selectively expanded (Fig. 8).[37] Which type of TCR cells, $\gamma\delta$ T cells or $\alpha\beta$ T cells, expand is dependent upon individuals. $\gamma\delta$ T cells are CD8$^+$ and DN, while $\alpha\beta$ T cells are CD4$^+$ (data not shown), similar to IL-12 activated murine NK1$^+$ $\alpha\beta$ T cells in the liver. IL-2 alone induces expansion of CD56$^-$ $\alpha\beta$ T cells, and CD56$^+$ T cells induced by IL 12 and IL-2 have a much more potent cytotoxicity than IL-2 activated CD56 $\alpha\beta$ T cells.[37] Furthermore, monocyte-depleted human PBL are cultured with IL-2, IL-12 and immobilized anti-CD3 Ab exclusively expand CD8$^+$CD56$^+$ $\alpha\beta$ T cells with potent cytotoxicity (Fig. 9).[37] It is therefore suggested that these CD56$^+$ T cells are functionally similar populations regardless of their $\alpha\beta$ or $\gamma\delta$ and CD4 or CD8 phenotypes. We believe that human CD56$^+$ $\alpha\beta$ T cells are a counterpart of NK1$^+$ $\alpha\beta$ T cells in mice and can develop extrathymically. NK1$^+$ $\alpha\beta$ T cells in the murine liver greatly decrease by liver fibrosis induced by CCl_4 and lose the cytotoxicity of hepatic MNC against hepatoma MH134 cells,[38] which might help explain the fact that cirrhosis of the liver in humans is frequently accompanied by hepatoma.

X. What is the role of IL-12 activated conventional CD8$^+$ cytotoxic T cells?

As mentioned before, IL-12 was originally described as a new cytokine that activates NK cells and pre-activated T cells. Brunda et al. reported[25] that IL-12-activated CD8$^+$ T cells in mice are antitumor effectors against s.c. inoculated tumors, because *in vivo* depletion of CD8$^+$ cells significantly reduces the inhibition of tumor growth and IL-12 exerts less of an effect in nude mice. Subsequently, Nastala et al. reported[26] that CD8$^+$ cells alone can not induce an inhibition of growth of subcutaneously inoculated tumors because *in vivo* depletion of CD4$^+$ cells abrogates IL-12 induced tumor inhibitory effect. They speculated that CD8$^+$ T cells exert an antitumor effect with the help of CD4$^+$ T cells (probably by cytokines produced by CD4$^+$ cells). Therefore, it is suggested that thymus-derived T cells are important effectors against s.c. inoculated tumors. It is conceivable that the most effective population induced by IL-12 could differ among the tissues in which tumors settle. NK1$^+$ $\alpha\beta$ T cells are important for the surveillance of intra- and perivasculare areas such as the hepatic sinusoid and pulmonary microvenules, while CD8$^+$ T cells are the main antitumor effectors for solid tumors. Nevertheless, it is notable that a significant number of NK1$^+$ $\alpha\beta$ T cells are found among tumor infiltrating lymphocytes.[39] Since NK1$^+$ $\alpha\beta$ T cells are new antitumor effector populations, researchers thus far have not taken account of these cells in tumor experiments in mice. We propose the possibility that CD8$^+$ cytotoxic T cells, NK1$^+$ $\alpha\beta$ T

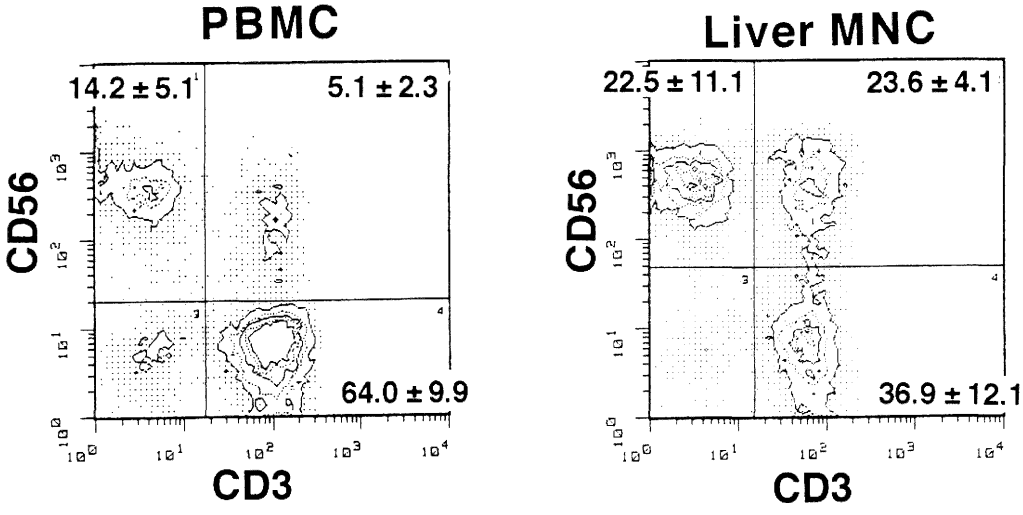

Fig. 7. CD3 and CD56 expression of unstimulated PBL and hepatic MNC of humans.

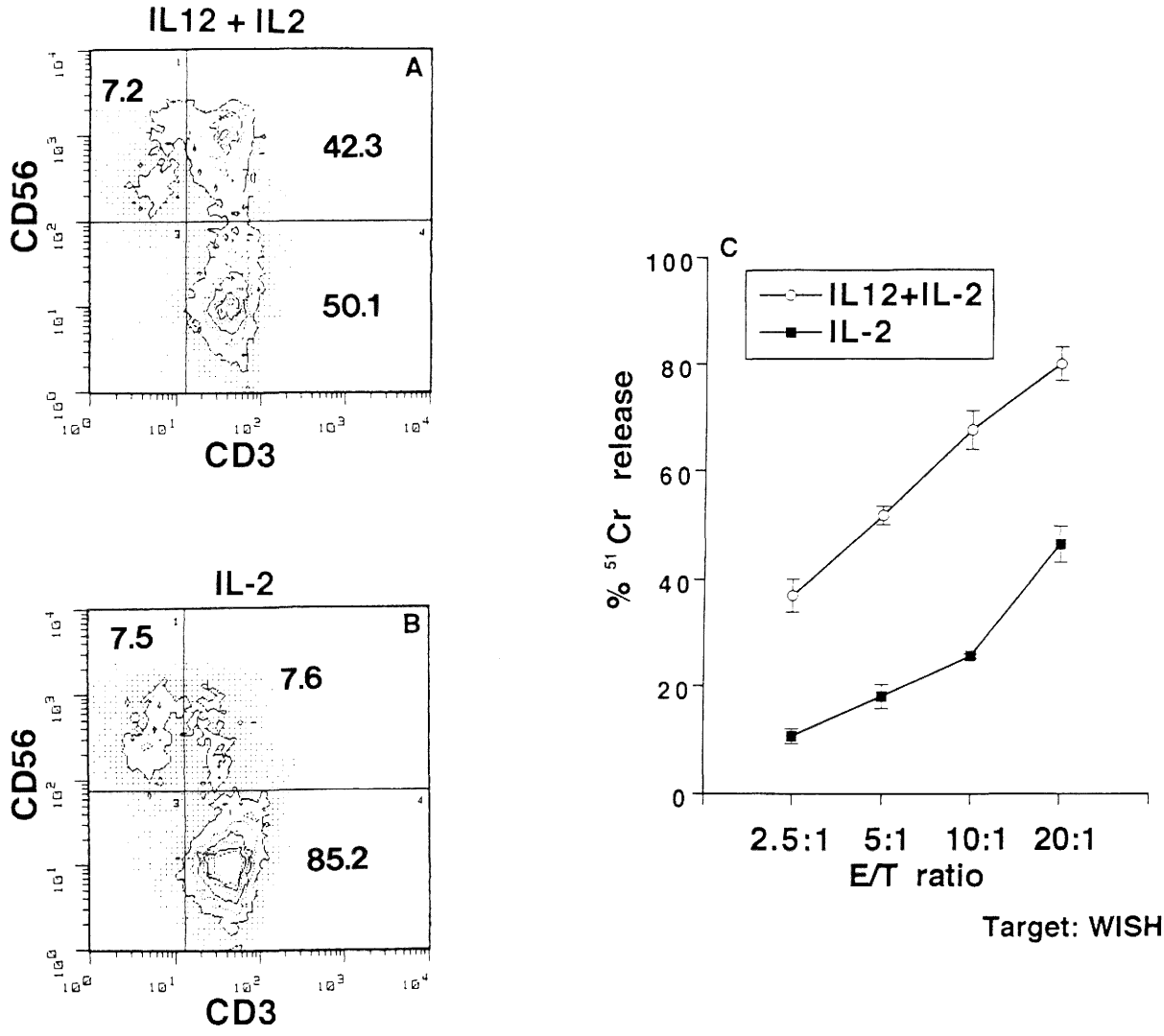

Fig. 8. The effect of a combination of IL-12 and IL-2 or IL-2 alone on the phenotypes and cytotoxic activities of PBL. **A.** Flowcytometric analysis of PBL cultured with both cytokines for 2 weeks. **B.** Flowcytometric analysis of PBL cultured with IL-2 alone for 2 weeks. **C.** Cytotoxic activities of PBL stimulated with both cytokines or IL-2 alone against NK-resistant WISH cells.

cells, NK cells and $\gamma\delta$ T cells probably all share the role of an antitumor barrier induced by IL-12, and may cooperate in the different tissues, areas, and stages of tumor growth.

XI. NK1$^+$ $\alpha\beta$ T cells are a pivotal population that govern Th1 or Th2 type immune response

Although NK1$^+$ $\alpha\beta$ T cells reportedly produce IL-4, and it has been proposed that they induce the differentiation of T helper type 2 reaction, one of the Th1 type cytokine, IL-12, could activate these cells as demonstrated in this study. This effect is at least partly mediated by another Th1 type cytokine, IFN-γ. It is suggested that the type of Th immune reaction induced by NK1$^+$ $\alpha\beta$ T cells may depend upon specific Ags or the environment. In fact, not only IL-12 but also IL-4 can endow cytotoxicity to these cells.[40] We speculate that stimuli such as bacteria, tumors and viruses activate these cells to induce a Th1 type response through IFN-γ and IL-12, and that some parasites and allergic substances may instead induce a Th2 type response by activation of NK1$^+$ $\alpha\beta$ T cells, probably through IL-4 production.

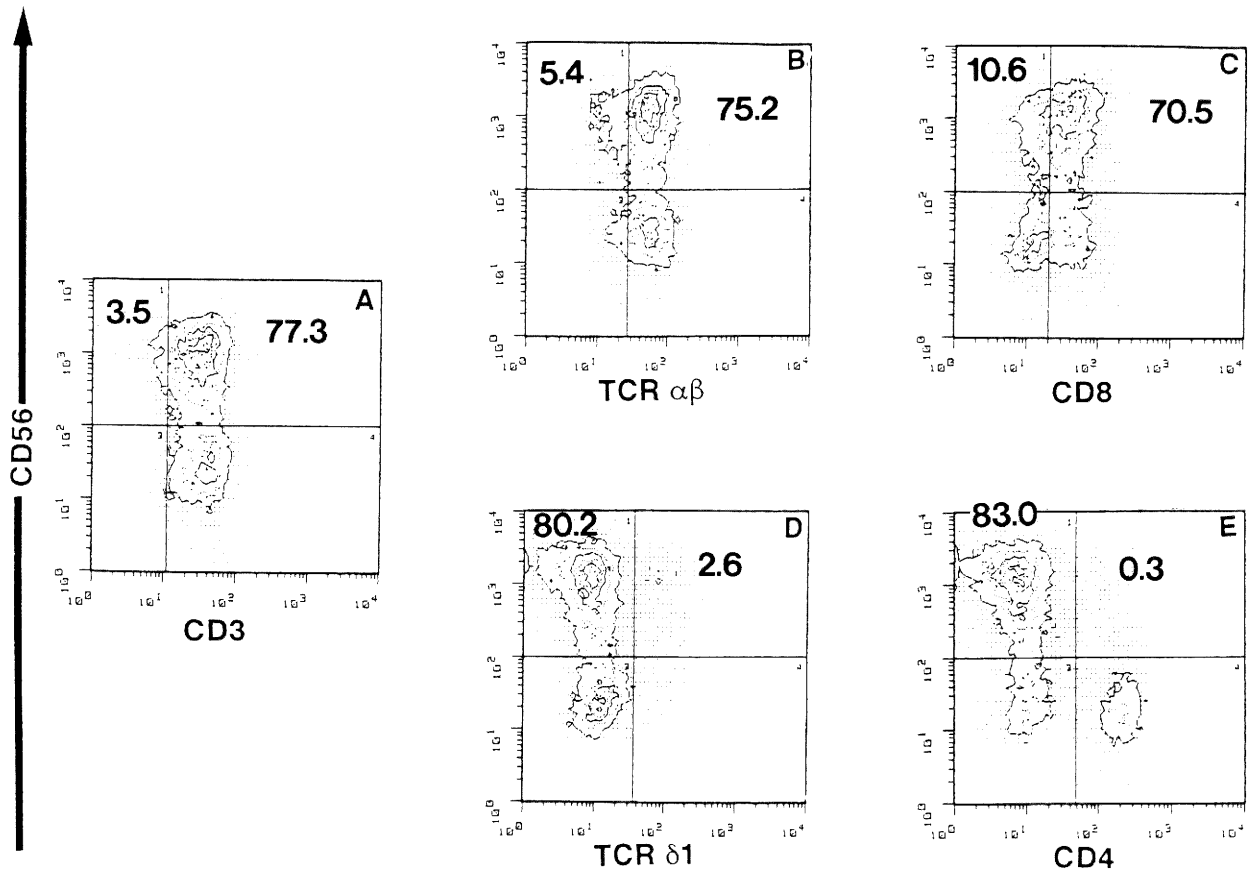

Fig. 9. $CD3^+8^+56^+\alpha\beta$ T cells are induced by cytokines and CD3 costimulation. PBL were cultured with IL-12 and IL-2 in the presence of immobilized anti-CD3 Ab for 2 weeks.

XII. Concluding remarks

In the present report, we demonstrate that T cells with an NK cell marker are preferentially present in the mouse liver and act as one of the major antitumor and antimetastatic populations, not only in the liver but also in the lung. A T cell population with an NK cell marker is also present in the human liver and has similar functional properties. These cells acquire a more potent antitumor cytotoxicity than NK cells and conventional T cells in certain conditions. Thus, these cells should be always considered when one deals with antitumor effectors. Since liver and lung metastases are among the most life threatening courses of cancer patients, we believe that these findings will provide further insights in the field of tumor immunology.

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