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Early-appearing tumour-infiltrating natural killer cells play a crucial role in the generation of anti-tumour T lymphocytes

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SUMMARY

Natural killer (NK) cells that infiltrated into the primary tumour site at an early stage of tumour development, were examined for their participation in the generation of anti-tumour cytotoxic T lymphocytes (CTL). NK cells, which were detected by anti-NK1.1 monoclonal antibody (mAb), increased in the peritoneal exudate cells (PEC) on days 3 and 7 after an intraperitoneal (i.p.) inoculation of syngeneic B16 melanoma cells. These tumour-infiltrating NK cells showed a high level of cytotoxic activity against NK-sensitive YAC-1 cells and an increased expression of interferon-γ (IFN-γ) mRNA and interleukin-2 (IL-2) mRNA. The *in vivo* depletion of NK cells with anti-NK1.1 mAb, prior to i.p. inoculation of B16 melanoma cells, resulted in an increased number of tumour cells in the PEC compared to NK cell non-depleted mice. Interestingly, the differences in tumour cell number between both groups were more prominent on days 7 and 14 than on day 3, which strongly suggested that early-infiltrating NK cells have a large influence on the subsequent anti-tumour response. The *in vivo* depletion of NK cells prior to immunization with melanoma cells abrogated the capacity of the spleen cells to generate $CD8^+$ tumour-specific CTL after *in vitro* restimulation. This inability of generating anti-tumour CTL was partially restored by additional i.p. injections of recombinant IL-2 and/or IFN-γ simultaneously with the immunization of melanoma cells. The *in vitro* depletion of NK cells prior to the *in vitro* restimulation with melanoma cells partially impaired the anti-tumour CTL generation from the spleen cells of the immunized mice. Lastly, the *in vivo* depletion of NK cells prior to immunization with melanoma cells abolished the protective immunity against melanoma cells at the rechallenge. Overall, these results indicate that early-appearing tumour-infiltrating NK cells not only participate in the anti-tumour early defence by themselves, but also play a crucial role in the generation of anti-tumour CTL.

INTRODUCTION

Natural killer (NK) cells are large granular lymphocytes that have the ability to lyse certain tumour cells and virus-infected cells without previous sensitization[1,2] or major histocompatibility complex (MHC) restriction.[3] NK cells are also known to regulate haematopoiesis[4] and the antibody production of B cells.[5] Among the various NK cell functions, much attention has been focused on the lytic activity against tumour cells and many studies have attributed NK cells with significant biological qualities such as immunological surveillance against spontaneously arising tumours and tumour metastases.[6–10]

It has been difficult to address directly the issue that NK cells participate in the anti-tumour early defence because the study of NK cells at an early stage of tumour development has been hampered by the difficulty in obtaining a sufficiently large number of tumour-infiltrating cells for analytical procedures. In this respect, we analysed peritoneal exudate cells (PEC) after the intraperitoneal (i.p.) inoculation of syngeneic tumour cells, because the PEC of i.p. tumour-inoculated mice are regarded as tumour-infiltrating lymphocytes (TIL).[11] Using this experimental model, we have shown previously that early-appearing tumour-infiltrating NK cells show high levels of cytotoxic activity and interferon-γ (IFN-γ) production and therefore prolong the survival of the tumour-bearing mice.[12]

In several kinds of effector cells against syngeneic tumours, the T cell is regarded as the main effector.[13] In particular, anti-tumour specific cytotoxic T lymphocytes (CTL) are considered

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Abbreviations: CTL, cytotoxic T lymphocyte; FCM, flow cytometry; IFN, interferon; IL, interleukin; i.p., intraperitoneal(ly); i.v., intravenous(ly); LAK, lymphokine-activated killer; MMC, mitomycin c; NK, natural killer; PCR, polymerase chain reaction; PEC, peritoneal exudate cells; TIL, tumour-infiltrating lymphocytes.

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to be the final and most important effector against such tumours. On the other hand, recent reports have revealed that the effective induction of an anti-tumour T-cell response requires co-operation between T cells and other lymphoid cells such as B cells and macrophages.[14–16] Nevertheless, the interaction between NK cells and T cells in anti-tumour immunity still remains unclear, although both are regarded as important effectors in the earlier and later stages of tumour development, respectively. In this study, we designed the experiments to investigate the influence of NK cells on the generation of anti-tumour CTL. Our results demonstrate that early-appearing NK cells in the tumour-developing site are functionally activated and also have a strong influence on the subsequent tumour growth. More importantly, such NK cells also play a crucial role in the generation of anti-tumour CTL.

MATERIALS AND METHODS

Antibodies

Anti-NK1.1 antigen monoclonal antibody (mAb) (PK136; mouse IgG2a) was obtained from ATCC (Rockville, MD). Rabbit anti-asialo GM1 serum was obtained from Wako Chemical Industries Ltd (Osaka, Japan). To prepare anti-NK1.1 mAb, PK136 hybridoma cells were grown in a serum-free medium (101; Nissui Pharmaceutical Co. Ltd, Tokyo, Japan), the supernatant collected and the mAb concentrated by 50% ammonium sulphate precipitation. Anti-heat-stable antigen mAb (J11D; rat IgM), anti-CD4 mAb (RL172.4; rat IgM) and anti-CD8 mAb (83.12.5; rat IgM) were obtained from ATCC. Anti-human IgA mAb (mouse IgG2a; Biotest AG, Frankfurt, Germany) was used as an isotype control antibody of anti-NK1.1 mAb. The mAb diluted to 1 mg/ml in phosphate-buffered saline (PBS) were stored at $-70°$ until use. The mAb used in the flow cytometric analysis were: phycoerythrin (PE)-conjugated anti-Thy-1.2 mAb (Caltag, South San Francisco, CA), PE-conjugated anti-CD4 mAb (BRL, Gaithersburg, MD), fluorescein isothiocyanate (FITC)-conjugated anti-NK1.1 mAb (PharMingen, San Diego, CA), FITC-conjugated anti-TCR$\alpha\beta$ mAb (H57-597; the kind gift of Dr R. T. Kubo, Cytel Corp., San Diego, CA), FITC-conjugated anti-TCR$\gamma\delta$ mAb (PharMingen), FITC-conjugated anti-CD3ε mAb (145-2C11; was kindly donated by Dr J. A. Bluestone, University of Chicago, Chicago, IL), FITC-conjugated anti-CD8 mAb (BRL) and FITC-conjugated anti-I-$A^{b/k}$ mAb (Meiji Institute of Health Science, Tokyo, Japan).

Mice

Female C57BL/6J (B6) mice were obtained from Japan SLC (Shizuoka, Japan) and kept in a specific pathogen-free animal facility at our institution. They were used in the experiments at 7–10 weeks of age. In some experiments, the mice were injected intravenously (i.v.) with 200 μg of anti-NK1.1 mAb or with mouse serum on day -4 and -1 before an i.p. inoculation of tumour cells. This treatment of anti-NK1.1 mAb completely depleted the NK1.1$^+$ cells and NK activity of the spleen cells (data not shown).

Tumours

The tumour cells used were B16 melanoma (H-2^b) and EL-4 thymoma (H-2^b) derived from B6 mice, P815 mastocytoma (H-2^d) derived from a DBA/2 mouse and YAC-1 lymphoma (H-2^a) derived from an A/Sn mouse. B16 melanoma cells expressed MHC class I but not class II molecules (data not shown). These tumour cell lines were maintained *in vitro* in a complete culture medium, which consists of RPMI-1640 (Gibco, Grand Island, NY) supplemented with 40 μg/ml gentamicin, 2 mM L-glutamine, 10 mM HEPES buffer and 10% Nu serum (a substitute for fetal calf serum; Collaborative Research Inc., Lexington, MA) at 37° in a humidified atmosphere with 5% CO_2.

Preparation of TIL and tumour-infiltrating NK cells from PEC

One million B16 melanoma cells were inoculated i.p. into the B6 mice. Peritoneal lavages were performed on days 3, 7 or 14 after tumour inoculation with 10 ml of complete culture medium, as previously reported.[12]

To enrich the NK cells from the PEC on day 3 after an i.p. inoculation with B16 melanoma cells, B cells and CD4$^+$ and CD8$^+$ T cells were depleted by treatment with anti-heat-stable antigen mAb (J11D), anti-CD4 mAb (RL172.4) and anti-CD8 mAb (83.12.5) plus Low-Tox-M Rabbit Complement (Cedarlane Laboratories, Ontario, Canada). After treatment, to remove the B cells further, the viable cells were applied onto anti-mouse IgG and IgM antibody (Tago Inc., Burlingame, CA)-coated plates, incubated for 60 min at 4° and then the non-adherent cells collected. To remove the $\gamma\delta$ T cells from the non-adherent cells, the immunomagnetic bead method was used, as follows. The non-adherent cells were resuspended in Hanks' balanced salt solution (HBSS), incubated with biotin-conjugated anti-TCR$\gamma\delta$ mAb (PharMingen) on ice for 30 min and washed three times with HBSS. Then the cells were incubated with streptavidin-coated immunomagnetic beads (Dynal AS, Oslo, Norway) on ice with gentle agitation for 20 min, and the $\gamma\delta$ T cells removed by a magnetic holder (Dynal). The resulting NK-enriched cells consisted of more than 83% of NK1.1$^+$ cells and less than 0·2% of CD3$^+$ cells (data not shown).

Preparation of peritoneal macrophages

B16 melanoma cells were treated with 100 μg/ml mitomycin c (MMC; Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan) for 90 min. The control or mAb-treated mice were inoculated i.p. with one million MMC-treated B16 melanoma cells on day 0. On day 3 or 7, the PEC were obtained and the cells adherent to plastic dishes (Falcon 3001; Becton Dickinson, Oxnard, CA) were collected as peritoneal macrophages. The expression of MHC class II antigen (I-A^b) on the surface of peritoneal macrophages was determined by a flow cytometry (FCM) analysis.

FCM analysis

A FCM analysis was performed on a FACScan flow cytometer (Becton Dickinson). Whole PEC were stained with FITC-conjugated anti-NK1.1 mAb and PE-conjugated anti-Thy1.2 mAb or FITC-conjugated anti-CD8 mAb and PE-conjugated CD4 mAb, and analysed. The gates were set on forward scatter to exclude any dead cells and red blood cells (RBC). The data were displayed by a two-colour contour plot. In some experiments, in order to investigate the expression of MHC class II antigen (I-A^b) on peritoneal macrophages, the peritoneal

adherent cells were stained with FITC-conjugated anti-I-$A^{b/k}$ mAb. Rabbit serum (Cedarlane Laboratories) was used to block the non-specific FC receptor binding.

Assay of cytotoxic activity

A graded number of either NK cells or $CD8^+$ T cells was incubated with 1×10^4 ^{51}Cr-labelled target cells in 200 μl of the complete culture medium, for 4 hr in a round-bottomed, 96-well microtitre plate (Nunc, Roskilde, Denmark). After incubation, 100 μl of supernatants were harvested. The radioactivity of the supernatants was measured in a gamma-counter and the percentage specific ^{51}Cr release was calculated according to the formula:

$$\frac{(\text{experimental release} - \text{spontaneous release}) \times 100}{\text{maximal release} - \text{spontaneous release}}.$$

Spontaneous release was determined from the sample of target cells incubated without effector cells, while the maximal release was determined from the sample of target cells incubated with 10% Triton-X (Wako Chemical Industries Ltd). The spontaneous release was less than 20% of the maximal release. All samples were assayed in triplicate and the values were shown as the means ± SD.

Polymerase chain reaction (PCR)

The total RNA of NK-enriched cells was isolated by the acid guanidinium thiocyanate–phenol/chloroform extraction method.[17] One microgram of total RNA was reverse-transcribed using RNAase H^- reverse transcriptase (RT; BRL) and a random primer (BRL) according to the manufacturer's instructions. Tenfold serial dilutions of cDNA were prepared and amplified by PCR with 2·5 U Taq DNA polymerase (Promega, Madison, WI) and 40 pM of sense and antisense oligonucleotide primers in a total volume of 100 μl. The reaction buffer consisted of 50 mM KCl, 1·5 mM $MgCl_2$, 10 mM Tris–HCl, pH 9·0, 0·1% Triton X-100 and 0·15 mM dNTP. The PCR cycles were allowed to run for 1 min at 94°, followed by 1 min at 54° and 30 seconds at 72°. Before the first cycle, a denaturation step of 7 min at 94° was included. After 28 cycles, the extension at 72° was prolonged to 4 min. The following primers were used: β-actin sense, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′; β-actin antisense, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′; interleukin-2 (IL-2) sense, 5′-TGATGGACCTACAGGAGCTC-CTGAG-3′; IL-2 antisense, 5′-GAGTCAAATCCAGAACA-TGCCGCAG-3′; IFN-γ sense, 5′-AGCGGCTGACTGAACT-CAGATTGTAG-3′; IFN-γ antisense, 5′-GTCACAGTTTT-CAGCTGTATAGGG-3′.

A 10-μl aliquot of each PCR product was electrophoresed through a 1·8% agarose gel, transferred to a Nytran nylon membrane (Schleicher & Schuell, Dassel, Germany), hybridized with ^{32}P-labelled oligonucleotide probes, and then exposed to X-ray film. The following oligonucleotide probes were used: β-actin, 5′-TTCTGCATCCTGTCAGCAAT-3′; IL-2, 5′-GAGCATCCTGGGGAGTTTCA-3′; IFN-γ, 5′-GG-TCACTGCAGCTCTGAATG-3′.

In vivo *immunization and* in vitro *generation of anti-tumour CTL*

The control of mAb-treated mice were injected i.p. with MMC-treated B16 melanoma cells (1×10^6) on day 0. On day 14, the harvested spleen cells (2×10^7 cells/well) were cultured *in vitro* with MMC-treated B16 melanoma cells (4×10^5 cells/well) in six-well dishes (Coster, Cambridge, MA) in 4 ml complete culture medium supplemented with 10% fetal calf serum (FCS). After a 96-hr culture, the cultured cells were examined for their CTL activities by a 4-hr ^{51}Cr-release assay. In some experiments, the cultured cells were enriched for $CD8^+$ T cells as follows. The cultured spleen cells were treated with anti-CD4 mAb (RL172.4) and anti-asialo GM1 antibody plus Low-Tox-M rabbit complement. After treatment, to remove B cells, the viable cells were applied onto anti-mouse IgG and IgM antibody-coated plates and non-adherent cells were used as $CD8^+$ T cells for the CTL assay. The resulting cells consisted of more than 90% of $CD8^+$ T cells and less than 0·1% of $NK1.1^+$ cells and 1% of $CD4^+$ cells (data not shown). In some experiments, either NK cells or $CD4^+$ cells were removed from the spleen cells, before the *in vitro* restimulation with inactivated tumour cells, by treatment with anti-asialo GM1 antibody or anti-CD4 mAb plus complement, respectively.

In vivo *administration of IL-2 and/or IFN-*γ

In some experiments, the mice were injected i.p. with 500 U of recombinant (r)IL-2 (kindly supplied by Shionogi Pharmaceutical Co. Ltd, Osaka, Japan) and/or 500 U of rIFN-γ (Gibco) twice daily for 3 days.

Counting the number of tumour cells in the peritoneal cavity

The mice were inoculated i.p. with 1×10^6 melanoma cells on day 0. On days 3, 7 and 14, the tumour cells in the peritoneal cavity were harvested by the same method as peritoneal lavage used for PEC collection. Tumour growth was determined by the number of viable tumour cells in the peritoneal cavity per mouse.

Assay of protective immunity

Control of mAb-treated mice were immunized i.p. with 1×10^6 MMC-treated B16 melanoma cells on day 0. On day 14, these immunized mice were inoculated subcutaneously (s.c.) with 2×10^5 B16 melanoma cells in 200 μl HBSS. After tumour inoculation, two tumour diameters at right angles were measured with calipers every 3 days and the product of the two values taken as the tumour size (mm^2).

Statistics

The statistical significance of the data was determined by Student's *t*-test. A *P*-value of less than 0·05 was considered to be significant.

RESULTS

Kinetic study of the phenotypes of TIL in the peritoneal cavity after i.p. inoculation of B16 melanoma cells

We have previously reported that NK cells infiltrate and are activated at the early stage of tumour development after an i.p. inoculation of syngeneic 3LL lung carcinoma.[12] In this study, we tried to investigate the participation of NK cells in the generation of anti-tumour CTL, but 3LL was found to be unable to induce any tumour-specific CTL by *in vitro* restimulation (data not shown). Therefore, we changed 3LL for B16 melanoma because B16 is able to induce anti-tumour

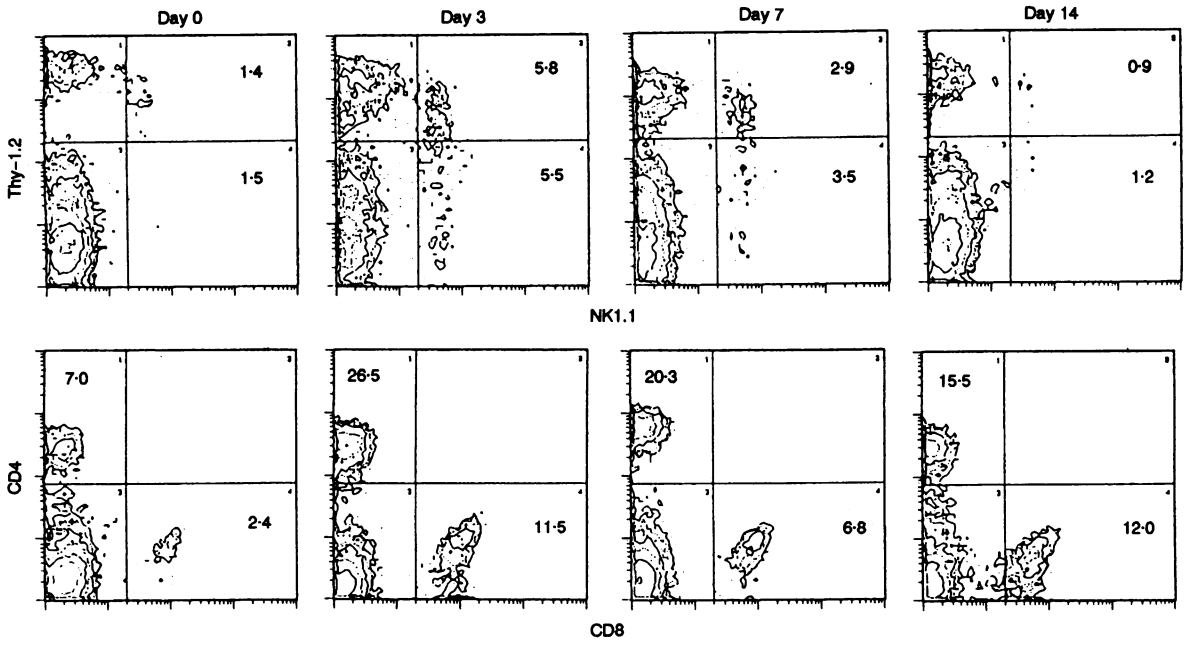

Figure 1. A FCM analysis on phenotypes of whole PEC on days 3, 7 and 14 after i.p. inoculation of B16 melanoma. The cells were stained with FITC-conjugated anti-NK1.1 mAb and PE-conjugated anti-Thy-1.2 mAb or FITC-conjugated anti-CD8 mAb and PE-conjugated anti-CD4 mAb. Rabbit serum was used to block the non-specific binding to Fc receptor. The *x* and *y* axes depict the intensity of fluorescence on a log scale.

CTL by *in vitro* restimulation. At first, we examined the phenotypic profiles of TIL in the PEC after an i.p. inoculation of B16 melanoma cells. Figure 1 shows that $NK1.1^+$ cells increased dramatically at the early stage of tumour development, from 2·9% on day 0 to 11·3% on day 3 and to 6·4% on day 7, similar to the findings for 3LL. $CD4^+$ T cells also increased in the population as early as day 3 after tumour inoculation, and thereafter slightly decreased. $CD8^+$ T cells also increased as early as day 3 and thereafter transiently decreased and then increased again on day 14. We could not detect any $NK1.1^+/CD3^+$ double-positive cells in the PEC after tumour inoculation (data not shown). The experiments were repeated three times and consistent results were obtained.

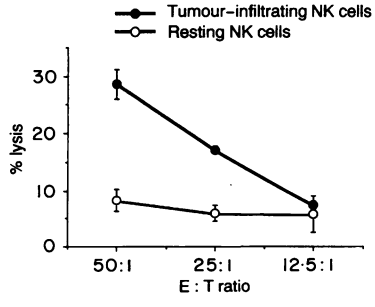

Figure 2. The NK activity of tumour-infiltrating NK cells or resting NK cells. The NK-enriched cells from the PEC on day 3 after i.p. inoculation of B16 melanoma (closed circles) and the NK-enriched cells from naive B6 spleen cells (open circles) were examined for their NK activity by a 4-hr ^{51}Cr-release assay. The procedure of enriching NK cells was as described in the Materials and Methods.

Cytotoxic activity of early-appearing tumour-infiltrating NK cells

We next examined the cytotoxic activity of enriched NK cells from the PEC on day 3 after i.p. inoculation of B16 melanoma. The NK-enriched PEC contained more than 83% of $NK1.1^+$ cells and less than 0·2% of $CD3^+$ cells, and neither $NK1.1^+$ $CD8^+$ nor $NK1.1^+$ $CD4^+$ cells were detected (data not shown). Figure 2 shows that the NK-enriched PEC showed a high level of cytotoxicity against NK-sensitive YAC-1. These cells showed 10% cytotolytic activity against NK-resistant and lymphokine-activated killer (LAK)-sensitive P815 at an effector : target ratio of 50 : 1 (data not shown). On the other hand, the NK-enriched cells obtained from spleen cells of untreated B6 mice showed a very low level of NK activity.

The increased expression of IFN-γ mRNA and IL-2 mRNA in tumour-infiltrating NK cells

The NK cell is reported to be a potent producer of lymphokines such as IFN-γ and IL-2,[18–20] and we have also previously reported that tumour-infiltrating NK cells produce a high level of IFN-γ.[12] We therefore tried to determine whether or not tumour-infiltrating NK cells produce IL-2, with a bioassay, but without success, probably because of contamination of the tumour cells (data not shown). Therefore, using the RT-PCR technique, we examined the expression of IL-2 mRNA in the enriched NK cells obtained from PEC on day 3 after an i.p. inoculation of B16 melanoma. Figure 3 shows that the expression of IL-2 mRNA was detected appreciably in the tumour-infiltrating early-appearing NK cells, but not in the NK cells of normal spleen cells. We also examined the expression of IFN-γ mRNA in the tumour-infiltrating NK

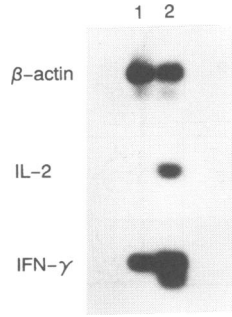

Figure 3. The expression of cytokine mRNA in either tumour-infiltrating NK cells or resting NK cells. Total RNA was prepared from enriched NK cells obtained from naive B6 spleen cells (lane 1) or from PEC on day 3 after i.p. inoculation of B16 melanoma (lane 2), and was analysed as described in the Materials and Methods.

cells and found that such NK cells also increased their expression of IFN-γ mRNA. These findings indirectly suggest that early-infiltrating NK cells in the tumour-developing site have the potential to produce both IFN-γ and IL-2.

It has been reported that the IFN-γ produced by NK cells activates macrophages and up-regulates the expression of MHC class II macrophages.[21] Therefore, we examined the possibility that IFN-γ produced by tumour-infiltrating NK cells increases the frequency of MHC class II-positive macrophages. As a result, the *in vivo* depletion of NK cells prior to i.p. injection of melanoma cells caused a slight but significant reduction in the frequency of I-A^b-positive peritoneal macrophages on day 7 (data not shown).

Kinetic analysis of the tumour cell number in NK cell-depleted or NK cell non-depleted mice

To investigate the influence of NK cells on tumour growth, we examined the kinetic changes of the number of tumour cells in either NK cell-depleted or NK cell non-depleted mice. To deplete NK cells *in vivo*, anti-NK1.1 mAb was injected as described in the Materials and Methods. Figure 4 shows that the number of B16 melanoma cells in the peritoneal cavity was significantly higher in the NK cell-depleted mice than in the NK cell non-depleted mice on day 7 (twofold) and day 14 (fourfold). Interestingly, the differences in the number of tumour cells between NK cell-depleted and NK cell non-depleted mice were more prominent in the later stages (days 7 and 14) than in the earlier stages (day 3). The number of tumour-infiltrating $CD4^+$ or $CD8^+$ T cells in the PEC after the i.p. inoculation of melanoma cells showed no significant difference between both groups of mice at any stage of tumour development (data not shown).

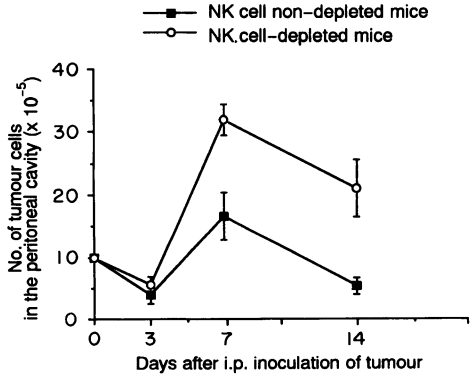

Figure 4. The kinetic changes of i.p. injected B16 melanoma cells in either NK cell-depleted and NK cell non-depleted mice. The number of B16 melanoma cells in the peritoneal cavity after i.p. inoculation of 1×10^6 B16 melanoma was examined. The mice were treated i.v. with 200 μg of anti-NK1.1 mAb (open circles) or with mouse serum (closed squares) on days −4 and −1 before i.p. injection of B16 melanoma cells. The values are the means ± SD for five mice. The differences in the number of tumour cells on days 7 and 14 were significant ($P < 0.05$ by Student's *t*-test).

The crucial role of tumour-infiltrating NK cells in the generation of anti-tumour CTL

Up to now, we have demonstrated that early-appearing tumour-infiltrating NK cells are functionally activated and have a strong influence on the subsequent anti-tumour immunity. Taking into account the fact that T cells, in particular $CD8^+$ T cells, are the main and final effectors against melanoma,[22] we examined further the possibility that NK cells play a role in the subsequent generation of anti-tumour CTL, as shown in Fig. 5. NK cell-depleted or NK cell non-depleted mice were immunized with MMC-treated B16 melanoma cells and, on day 14, the harvested spleen cells were restimulated *in vitro* with MMC-treated B16 melanoma cells without any cytokine. Finally, the $CD8^+$ T cells, which were enriched from the cultured cells, were examined for their CTL activities. The results were that $CD8^+$ T cells derived from the spleen cells of NK cell non-depleted and immunized mice showed a high level of cytolytic activity against B16 melanoma but a low level of cytotolytic activity against P815, as a LAK target, and EL-4 target, as a control syngeneic tumour cell line (data not shown). Although tumour-specific antigens of B16 melanoma have not been defined, the CTL activity against B16 melanoma was blocked by the presence of anti-H-$2K^bD^b$ mAb (data not shown). In contrast, $CD8^+$ T cells derived from the spleen cells of the NK cell-depleted and immunized mice did not show any cytotoxicity against B16 melanoma. These results indicate that tumour-infiltrating NK cells are essential for the subsequent generation of anti-tumour CTL after *in vitro* restimulation.

To address the possibility that cytokines derived from tumour-infiltrating NK cells initiated the generation of anti-tumour CTL, we determined whether or not additional i.p. injections of IL-2 and/or IFN-γ could restore the capacity of the spleen cells from NK cell-depleted and immunized mice to generate anti-tumour CTL after *in vitro* restimulation. Table 1 shows that a partial restoration of the anti-tumour CTL activity in the spleen cells from the NK cell-depleted and immunized mice was induced by i.p. injections of 500 U IL-2 and/or 500 U IFN-γ twice daily for 3 days, which were started on the next day of tumour immunization. The effect of IL-2 to restore the CTL activity was stronger than that of IFN-γ. These results indirectly suggest that the inability to generate anti-tumour CTL after *in vitro* restimulation in NK cell-depleted mice is, at least in part, due to the deficiency of the cytokines derived from tumour-infiltrating NK cells.

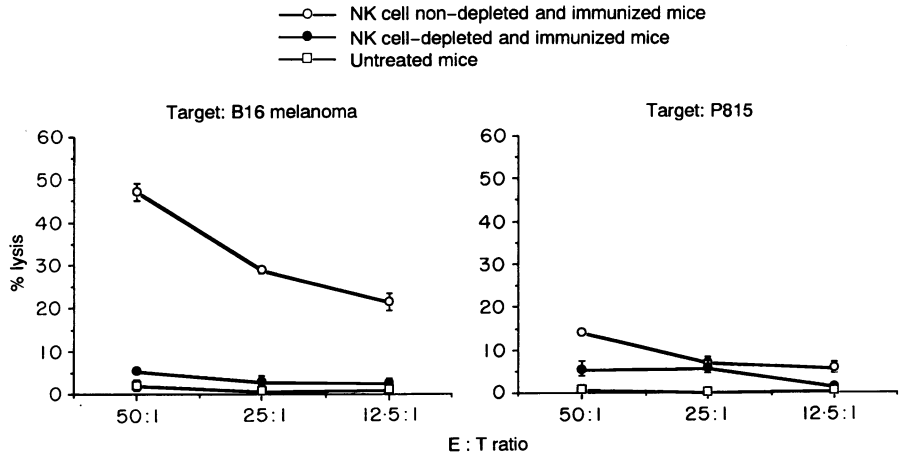

Figure 5. The effect of *in vivo* depletion of NK cells prior to immunization with tumour cells on *in vitro* generation of anti-tumour CTL. The mice were injected i.v. with 200 μg of anti-NK1.1 mAb (closed circles) or with isotype control antibody (open circles) on days −4 and −1 before immunization with i.p. injection of 1×10^6 MMC-treated B16 melanoma cells. On day 14, the spleen cells were harvested and restimulated with MMC-treated B16 melanoma cells for 96 hr. Enriched $CD8^+$ T cells, as described in the Materials and Methods, were examined for cytotoxicity by a 4-hr ^{51}Cr-release assay. Enriched $CD8^+$ T cells from the cultured spleen cells of non-immunized mice (open squares) were used as a control.

The participation of NK cells in the *in vitro* generation of anti-tumour CTL

Next, in order to investigate the participation of NK cells in the *in vitro* generation of anti-tumour CTL, we determined whether or not the *in vitro* depletion of NK cells prior to *in vitro* restimulation has any influence on the generation of anti-tumour CTL. Figure 6 shows that the removal of the NK cells from the spleen cells of the immunized mice before an *in vitro* culture with MMC-treated melanoma cells resulted in a lower level of $CD8^+$ CTL activity against B16 melanoma compared to NK cell non-depleted immune spleen cells. The removal of $CD4^+$ T cells from the responder cells prior to *in vitro* restimulation abrogated anti-tumour $CD8^+$ CTL activity. These results suggest that NK cells are partially required for *in vitro* $CD8^+$ CTL generation but that the *in vitro* generation of anti-tumour CTL is mainly dependent on $CD4^+$ T cells.

A decrease in the protective immunity against melanoma cells at the rechallenge in NK cell-depleted and immunized mice

Finally, we also determined whether or not tumour-infiltrating NK cells have any influence on the protective immunity against

Table 1. Effect of *in vivo* administration of rIL-2 and/or rIFN-γ on the *in vitro* induction of anti-tumour CTL

Group	*In vivo* depletion of NK cells* prior to immunization	Intraperitoneal injection of cytokines† after immunization	CTL activity against B16 melanoma‡ (E : T = 50 : 1) (%)
1	−	−	27·8 ± 1·7
2	+	−	5.8 ± 1·0§
3	+	rIFN-γ	9.9 ± 3.2§
4	+	rIL-2	16·9 ± 3·6¶
5	+	rIFN-γ + rIL-2	12·8 ± 2.6§

* Either NK cell-depleted mice treated with 200 μg of anti-NK1.1 mAb or NK cell non-depleted mice treated with isotype control antibody on days −4 and −1 were immunized i.p. with 1 million MMC-treated B16 melanoma cells on day 0.

† The mice were immunized i.p. with MMC-treated B16 melanoma cells on day 0 and then were injected i.p. with or without 500 U rIL-2 and/or 500 U rIFN-γ twice daily on days 1, 2 and 3.

‡ Two weeks after immunization, the spleen cells were cultured with MMC-treated B16 melanoma cells for 4 days and then CTL activity of enriched $CD8^+$ T cells against B16 melanoma was examined by a 4-hr ^{51}Cr-release assay. The data present the means ± SD.

§ The data were significantly lower than that of group 1 ($P < 0.01$).

¶ The data were significantly lower than that of group 1 and significantly higher than that of group 2 or group 3 ($P < 0.01$).

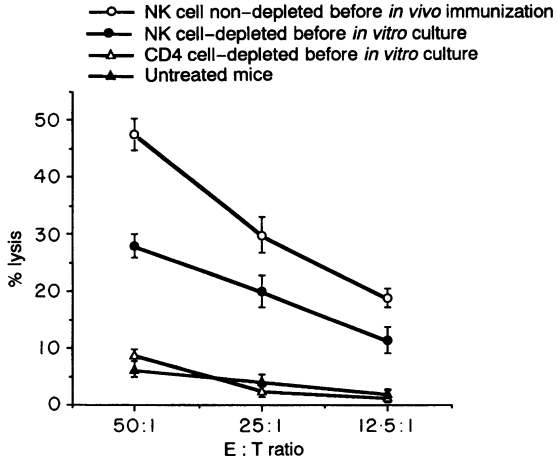

Figure 6. The effect of *in vitro* depletion of NK cells or $CD4^+$ T cells on the *in vitro* induction of anti-tumour CTL. MMC-treated B16 melanoma cells were cultured with either non-depleted (open circles), NK cell-depleted (closed circles) or $CD4^+$ T-cell-depleted (open triangles) spleen cells from mice immunized with MMC-treated B16 melanoma. As a control, spleen cells from naive mice (closed triangles) were cultured with MMC-treated B16 melanoma cells. After a 4-day culture, the $CD8^+$ T cells were enriched from the cultured spleen cells, as described in the Materials and Methods, and examined for cytotoxicity against B16 melanoma by a 4-hr ^{51}Cr-release assay.

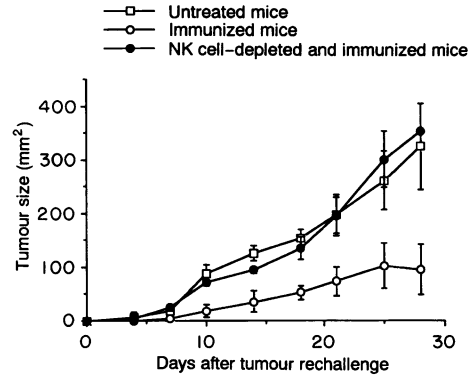

Figure 7. The decreased protective immunity of NK cell-depleted mice against B16 melanoma cells at rechallenge. Either NK cell-depleted mice (closed circles) or NK cell non-depleted mice (open circles) were injected i.p. with 1×10^6 MMC-treated B16 melanoma cells. Two weeks after immunization, these immunized mice or non-immunized mice (open squares) were rechallenged with 2×10^5 B16 melanoma cells via intradermal injection in a midline ventral position. The values are the means ± SD for seven mice.

tumour cells at rechallenge. As shown in Fig. 7, we examined the tumour growth of B16 melanoma after the rechallenge in either NK cell-depleted or NK cell non-depleted mice. As a result, the protective immunity against B16 melanoma significantly decreased in the NK cell-depleted and immunized mice. The NK cell-depleted mice also showed a similar pattern of tumour growth to that of the non-immunized mice. These findings thus suggest that the tumour-infiltrating NK cells play a crucial role in *in vivo* immunization.

DISCUSSION

NK cells are thought to be one of the main effectors responsible for the anti-tumour early defence.[4] However, it has yet to be elucidated whether or not NK cells infiltrate into the tumour developing site and what role NK cells play in the early stage of tumour development. In this respect, with the 3LL lung carcinoma–C57BL/6 system, we have recently reported that early-infiltrating NK cells produce a high level of IFN-γ and show an enhanced cytotoxicity and, as a result, prolong the survival of 3LL-bearing mice.[12] In this study, we reconfirmed that early-infiltrating NK cells in the PEC after i.p. inoculation of B16 melanoma cells enhanced their cytotoxicity (Fig. 2), and show increased expression of IFN-γ mRNA and IL-2 mRNA (Fig. 3). Since we also obtained similar results in the case of a MCA fibrosarcoma–C57BL/6 system (data not shown), we thus consider that NK cells infiltrate into the tumour lesion at an early stage of tumour development and play an important role in the anti-tumour immune response. In addition, we also believe that this phenomenon could possibly be applied to other syngeneic tumour systems.

A notable finding in the present study was that early-appearing tumour-infiltrating NK cells had a strong influence on the subsequent anti-tumour immune response. As shown in Fig. 4, NK cell-depleted mice showed an increased number of B16 melanoma cells in the PEC on days 7 and 14 after tumour inoculation compared to NK cell non-depleted mice. On the other hand, the percentage of infiltrating NK cells in the PEC on day 14 had already returned to the level of that of normal mice. This evidence strongly suggests that the anti-tumour defence mechanism at the later stage of tumour development is not dependent on the NK cells themselves. Furthermore, considering the fact that T cells are a main effector against syngeneic tumours,[13] we hypothesize that the higher number of tumour cells in the NK cell-depleted mice at the later stage of tumour development may be attributed to the impaired induction of the anti-tumour T-cell response. This assumption led us to design an experimental study to determine whether or not tumour-infiltrating NK cells, which are present immediately after tumour immunization, have any influence on the subsequent generation of anti-tumour CTL.

Concerning the specific mechanisms by which NK cells participate in the generation of anti-tumour CTL, one possibility is that NK cells have an influence on anti-tumour CTL generation through production of IFN-γ. NK cells are known to be potent producers of IFN-γ at an early stage of NK cell activation.[18,19] We also demonstrated that tumour-infiltrating NK cells produce a high level of IFN-γ[12] and express an increased level of IFN-γ mRNA (Fig. 3). Considering that the cytokine environment is critical to the development of antigen-specific $CD4^+$ and $CD8^+$ T cells,[23–25] IFN-γ produced by NK cells may help the anti-tumour T-cell response. Furthermore, IFN-γ is well known to up-regulate the expression of MHC class I and class II.[21,26] Accordingly, the up-regulation of MHC class II on macrophages and MHC class I on the tumour cells would allow tumour-specific $CD4^+$ and $CD8^+$ T cells to recognize the tumour-specific antigens presented by macrophages and tumour cells, respectively. Indeed, we observed that the *in vivo* depletion of NK cells resulted in a significant reduction of the frequency of $I\text{-}A^b$-positive peritoneal macrophages on day 7 after an i.p. inoculation of B16 melanoma. We also propose the possibility that IFN-γ

produced by tumour-infiltrating NK cells causes the cytostatic activity in macrophages, because IFN-γ is a key cytokine for macrophages to show cytostatic activity.[27,28]

A second possibility is that NK cells help to generate anti-tumour CTL through IL-2 production. Previous reports revealed that IL-2 is crucial for the generation of anti-tumour cytotoxic effectors such as CTL[23,24] and LAK.[29] In addition, under typical *in vitro* and *in vivo* conditions, an efficient induction of anti-tumour $CD8^+$ T cells is dependent on lymphokines, mainly IL-2, provided by concurrent $CD4^+$ helper T cells. Therefore, it is reasonable to think that, in addition to the IL-2 produced by $CD4^+$ T cells, IL-2 which is locally produced by tumour-infiltrating NK cells in the tumour developing site might activate and augment the anti-tumour T-cell response. In this study, we demonstrated that early-appearing tumour-infiltrating NK cells express IL-2 mRNA (Fig. 3). In addition, the *in vivo* depletion of NK cells prior to immunization with tumour cells almost completely abolished the anti-tumour CTL activity (Fig. 5) and the subsequent additional i.p. injections of IL-2 after tumour immunization partially restored the CTL activity (Table 1). $CD4^+$ T cells are more important than NK cells as cells responsible for the *in vitro* generation of anti-tumour CTL (Fig. 6). These lines of evidence thus suggest that NK cells are critically required for *in vivo* priming of anti-tumour CTL or their precursors, and that the anti-tumour CTL generation after an *in vitro* restimulation with tumour cells is mainly mediated by $CD4^+$ T cells, although NK cells are needed for optical CTL generation.

Another possibility is that the destruction of tumour cells by the activated NK cells may 'help' the processing and presentation of tumour-derived antigens by macrophages. In addition, we cannot rule out the possibility that the presence of tumour-infiltrating NK cells at the time of tumour vaccination deviated the $CD4^+$ helper T cells to the T-helper type-1 (Th1) type and thus resulted in effective immunization, because the Th1-type cytokines are thought to be efficient for anti-tumour T-cell immunity.[11,28] The possibility may also be supported by the report that the presence of NK cells, at the initial stage of parasite infection, results in the immune deviation of T-helper cells preferentially in the Th1 type.[30] Further experiments are now underway to elucidate the precise mechanism.

One of the most practical strategies of cancer immunotherapy is anti-tumour vaccination.[31] The findings presented here provide a rationale for the view that anti-tumour vaccination should be targeted not only to T cells but also to NK cells. The augmentation of T-cell functions by NK cell activation with their cytotoxicity and cytokine production at the time of tumour immunization would result in the subsequent promotion of anti-tumour T-cell immunity. In fact, the adjuvant properties of several various biological response modifiers are mediated in part by their ability to activate NK cells prior to the induction of the T-cell response.[32] From this point of view, we needed to determine whether or not more efficient anti-tumour immunity could be induced in mice that had had their NK activity augmented with OK432, a streptococcal preparation. We found that such an agent has an enhancing effect on anti-tumour vaccination (S. Kurosawa *et al.*, manuscript in preparation).

In conclusion, we have demonstrated that early-appearing NK cells during tumour development have a strong influence on anti-tumour CTL generation. These findings indicate that NK cells play an important role not only in anti-tumour early defence but also in the induction of anti-tumour T-cell immunity. Therefore, early-appearing NK cells should be considered as a significant initiation factor for optimal anti-tumour T-cell immunity.

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