Ultraviolet Radiation-Induced Impairment of Tumor Rejection Is Enhanced in Xeroderma Pigmentosum A Gene-Deficient Mice

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Xeroderma pigmentosum (XP)A gene-deficient mice display dermatologic abnormalities similar to human XP, such as enhanced ultraviolet (UV)-induced acute inflammation and high incidence of UVB-induced skin cancer. We have previously reported that UVB-induced immunosuppression of contact hypersensitivity was greatly enhanced in XPA mice. In the present study, we examined the effects of UVB radiation on tumor rejection in XPA mice. Tumor cells established from UVB-induced squamous cell carcinoma in XPA mice were injected subcutaneously. No difference in the development of tumors was observed between the non-irradiated XPA and wild-type mice. Tumors developed, grew in size, and reached the maximum at 7–10 d after the inoculation. Thereafter, all tumors decreased in size and were completely rejected by 4 wk in both strains of mice. When tumor cells were inoculated into the skin that had been irradiated with 50–150 mJ per cm^2 of UVB, tumor grew in 60% (12 of 20) of the XPA mice, but only in 4% (one of 23) of wild-type mice. Phenotyping of tumor-infiltrating cells revealed that the migration of natural killer cells and CD8(+) T cells was inhibited in UVB-irradiated XPA mice. These data suggest that enhanced UVB-induced impairment of tumor rejection could be partially involved in the cancer development of XP patients.

Key words: immuno-suppression/tumor immunity/UVB/xeroderma pigmentosum/XPA-gene
J Invest Dermatol 124:1313–1317, 2005

The tumor immunity plays an important role in the development, growth, and metastasis of cancer cells and is a major defense strategy against neoplasms. It is now generally accepted that ultraviolet (UV) B radiation has mutagenic properties and also immunosuppressive effects, which may contribute to the development of sunlight-induced skin cancers (Streilein *et al*, 1994; Berneburg and Krutmann, 2000). Underlying mechanisms of UV-induced immunosuppression have been extensively studied in T cell-mediated immune reactions including contact hypersensitivity (CHS) and delayed type of hypersensitivity (DTH) (Noonan *et al*, 1981; Ullrich *et al*, 1986; Miyauchi and Horio, 1995). The UVB irradiation before the sensitizing procedure can induce hapten- or antigen-specific suppressor or regulatory T cells, resulting in tolerance to the sensitizer (Shreedar *et al*, 1998; Moodycliffe *et al*, 2000; Schwarz *et al*, 2000). Therefore, the affected animals cannot be re-sensitized with the same antigen at a later time without further irradiation, but can be sensitized to other unrelated antigens. Depletion or dysfunction of antigen-presenting cells and production of a variety of cytokines or soluble factors with immunomodulatory activity may be involved in the UV-induced immunosuppression.

There is strong evidence that DNA damage in the skin plays a crucial role in the UV-induced immunosuppression of CHS and DTH. The initial studies were performed using the marsupial *Monodelphis domestica*, whose cells have a photolyase DNA repair enzyme that is activated to repair cyclobutane pyrimidine dimers (CPD) by exposure to photoreactivating visible light. Photo-repair of UVB-induced CPD restored suppression of the CHS response in the animal (Applegate *et al*, 1989). Furthermore, topical treatment with liposomes containing the excision repair enzyme T4 endonuclease V prevented UVB-induced suppression of CHS and DTH responses (Kripke *et al*, 1992). We have previously studied the effects of UVB on the immunologic function of xeroderma pigmentosum (XP)A gene-deficient mice that have defects in nucleotide excision repair. Although CHS was induced in non-irradiated XPA mice as well as wild-type mice, UVB-induced local and systemic immunosuppressions were greatly enhanced in XPA mice (Miyauchi-Hashimoto *et al*, 1996). There are some studies that patients with XP have defects in cell-mediated immunity such as impaired cutaneous responses to recall antigens and a contact sensitizer (Morison *et al*, 1985; Wysenbeek *et al*, 1986; Goldstein *et al*, 1990). Our studies using XPA mice suggested that the immunologic dysfunction in XP patients is not innate but acquired possibly after exposures to the sunlight. We have also investigated the natural killer cells, which play a crucial role in antigen non-specific tumor immunity, and found that XPA mice displayed a higher level of spontaneous and polyisosinic:polycytidylic acid-inducible natural killer cell activity than wild-type mice, unless they were exposed to UVB. But, the activity in XPA mice was impaired by UVB irradiation at doses that did not affect the natural killer activity in wild-type mice (Miyauchi-Hashimoto *et al*, 1999). These results strongly suggest that UV-induced

Abbreviations: CHS, contact hypersensitivity; CPD, cyclobutane pyrimidine dimer; DTH, delayed type of hypersensitivity; IL, interleukin; PG, prostaglandin; SCC, squamous cell carcinoma; UV, ultraviolet; XP, xeroderma pigmentosum

DNA damage is responsible not only for gene mutations but also for immunologic impairment, and thereby skin cancers developed especially in XP patients. To address this issue, in the present experiments, we examined the effects of UVB radiation on the tumor immunity in XPA mice.

Results

UVB inhibited rejection of transplanted squamous cell carcinoma (SCC) cells from XPA mice First, 3×10^7 of tumor cells were inoculated into the skin of non-irradiated recipient mice (eight wild-type and eight XPA mice) and the time course of the size of tumors was examined. There was no difference in development of tumors between non-irradiated wild-type and XPA mice. In both types of mice, tumors inoculated into non-irradiated skin grew in size, and reached the maximum at 7 or 10 d after the inoculation. Thereafter, all tumors decreased in size and were rejected by 4 wk after the inoculation in both strains of mice (Fig 1).

Next, the effect of UVB irradiation on the ability to reject inoculated tumor cells was examined. One group of mice (four each of wild-type and XPA mice) was irradiated with 25 mJ per cm^2 UVB for 2 d. The second group (six of wild-type and five of XPA mice) received single irradiation with 100 mJ per cm^2 UVB. The third group (13 of wild-type and 11 of XPA mice) was exposed to 75 mJ per cm^2 on 2 consecutive days. Therefore, each group was exposed totally to 50, 100, and 150 mJ per cm^2, respectively. One day after the last irradiation, 3×10^7 of SCC tumor cells were inoculated into the irradiated skin. When exposed to 150 mJ per cm^2-UVB, the tumor volume increased after the inoculation, and reached a maximum at day 25 in XPA mice (Fig 2). The macroscopic features of mice at day 17 after tumor inoculation into 150 mJ per cm^2-UVB irradiated skin are shown in Fig 2*B*. Tumors in wild-type mice had already subsided whereas those in XPA mice were growing with central necrosis. Almost the same results were observed in the experiment in which tumor cells were inoculated into the skin irradiated with 50 and 100 mJ per cm^2-UVB (data not shown). When tumors were engrafted into the skin exposed to 150 mJ per cm^2-UVB, the percentage of tumor-bearing wild-type mice decreased by day 12 after the inoculation, and there are no mice having tumors on day 42. In contrast, 55% of XPA mice had tumors even on day 42. The number of tumor-bearing mice at the sixth wk is summarized in Table I. When mice were exposed to 50, 100, or 150 mJ per cm^2-UVB, 75% (three of four), 60% (three of five), or 55% (six of 11) of XPA mice, respectively, maintained the tumors, but almost all tumors had been rejected by wild-type mice at the sixth wk.

Histological examination showed proliferation of inoculated tumor cells in UVB-irradiated skin of XPA mice Histological study was performed in three mice each

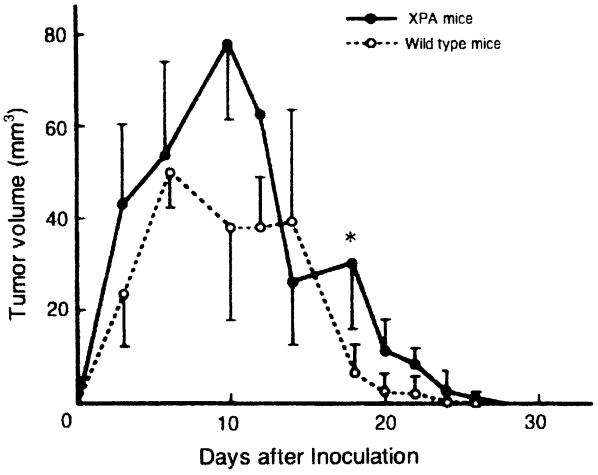

Figure 1
Tumors developed and were rejected from the skin in which cultured tumor cells were inoculated. The cell line was derived from squamous cell carcinoma that had been induced by chronic ultraviolet B irradiation in xeroderma pigmentosum (XP)A mice. A suspension of 3×10^7 tumor cells was injected subcutaneously into non-irradiated XPA and wild-type mice (n = 8 each). Tumor size was measured periodically after inoculation. Tumor volume was calculated as transverse diameter × right-angled diameter × height (mm^3). The results are presented as mean ± SD. *Significant difference with p = 0.02.

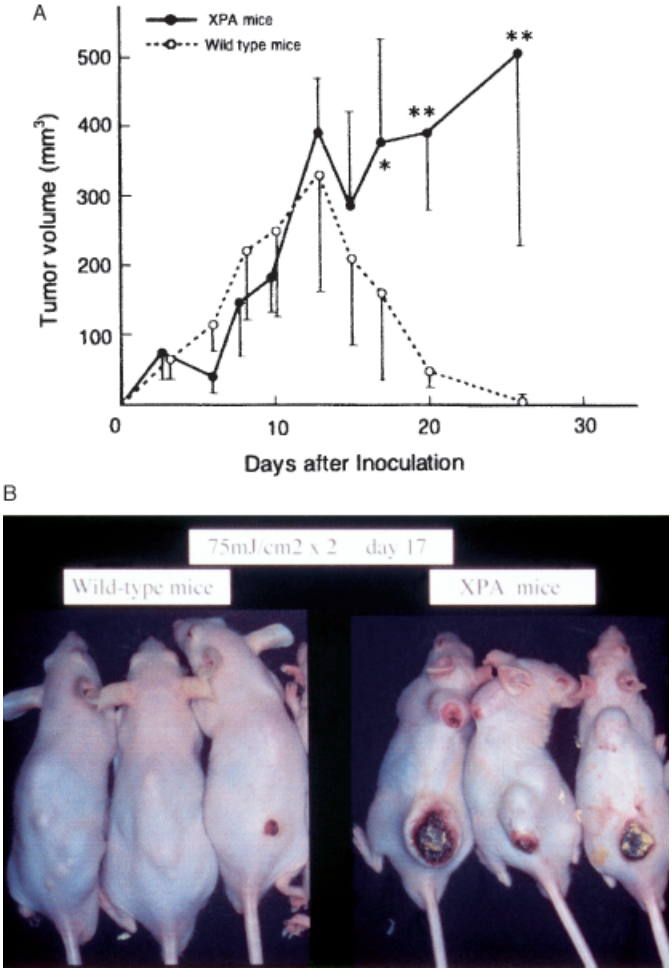

Figure 2
Ultraviolet (UV) B radiation suppressed rejection of transplanted tumor cells from xeroderma pigmentosum (XP)A mice. Mice were exposed to UVB at a dose of 75 mJ per cm^2 for 2 consecutive days. One day after the second exposure, a suspension of 3×10^7 squamous cell carcinoma was injected subcutaneously into UVB-irradiated XPA (n = 11) and wild-type mice (n = 13). Tumor volume was measured after irradiation (*A*). The results are presented as mean ± SD. *, ** Significant differences with p = 0.05 and p < 0.0001, respectively. Seventeen days after tumor cell inoculation, wild-type mice completely rejected tumors, whereas XPA mice had large tumors (*B*).

Table I. UVB effect on the rejection of tumor cells

Group	UVB treatment, total dose (dose × d)	Tumor incidence[a] at 6 wk	
		Wild-type	XPA mice
I	50 mJ per cm^2 (25 mJ per cm^2 × 2)	0/4	3/4
II	100 mJ per cm^2 (100 mJ per cm^2 × 1)	1/6	3/5
III	150 mJ per cm^2 (75 mJ per cm^2 × 2)	0/13	6/11
	Total	1/23	12/20[b]

[a]Number of tumor-bearing mice/number of challenged mice.
[b]Significant difference with $p = 0.002$.
UVB, ultraviolet B, XPA, xeroderma pigmentosum A.

of wild-type and XPA mice at various time points after tumor cell inoculation into the skin irradiated with 150 mJ per cm^2-UVB. In wild-type mice, tumor masses proliferated in the subcutaneous tissue at day 9 after the inoculation. At day 12, marked inflammatory cells including mononuclear cells, neutrophils, and eosinophils infiltrated in and around tumor masses, and tumor masses revealed necrotic change. At day 16, tumor masses were almost replaced with necrotic tissue and keratin materials with calcification, and were surrounded by fibrosis and granulation. In XPA mice, tumor masses revealed necrotic change surrounded by inflammatory cells in subcutaneous tissue, but a small part of tumor masses proliferated up to the dermis breaking down to the muscle at day 9. Remaining tumor cells proliferated at day 12 associated with prominent inflammatory cells infiltrated around tumor masses. At day 16, tumor masses were further proliferating progressively into the upper part of the dermis associated with inflammatory cell infiltration.

Flow cytometric analysis of tumor-infiltrating cells Tumors were removed at various time points after inoculation of 3×10^7 SCC cells in the skin exposed to 150 mJ (75 mJ × 2) per cm^2. The number of tumor-infiltrating cells, separated from tumor suspensions by centrifugation with Histopaque (Sigma, St Louis, Missouri), was counted, and the cells were phenotyped by flow cytometry. In wild-type mice, the size of tumors peaked on day 12 after tumor cell inoculation and decreased thereafter. But, the total number of tumor-infiltrating cells was maintained at the peak even after the tumor size had begun to decrease (Fig 3). On the other hand, the peak of the number of tumor-infiltrating cells in XPA mice was delayed, reached at day 21, and thereafter decreased rapidly although tumor size was increasing. The time course of infiltration of NK cells in wild-type mice was similar to that of whole infiltrating cells, reaching the maximum at day 12 and decreasing thereafter. But, only a few NK cells infiltrated into the tumors in XPA mice throughout the period of observation. In wild-type mice, infiltration of CD3(+) lymphocytes into the tumors increased until day 19, 1 wk after the peak of infiltration of NK cells. A few days after the peak of tumor size, the amount of infiltrating CD3(+) cells decreased. The timing and the level of peak of infiltrating CD8(+) T cells in wild-type mice were almost

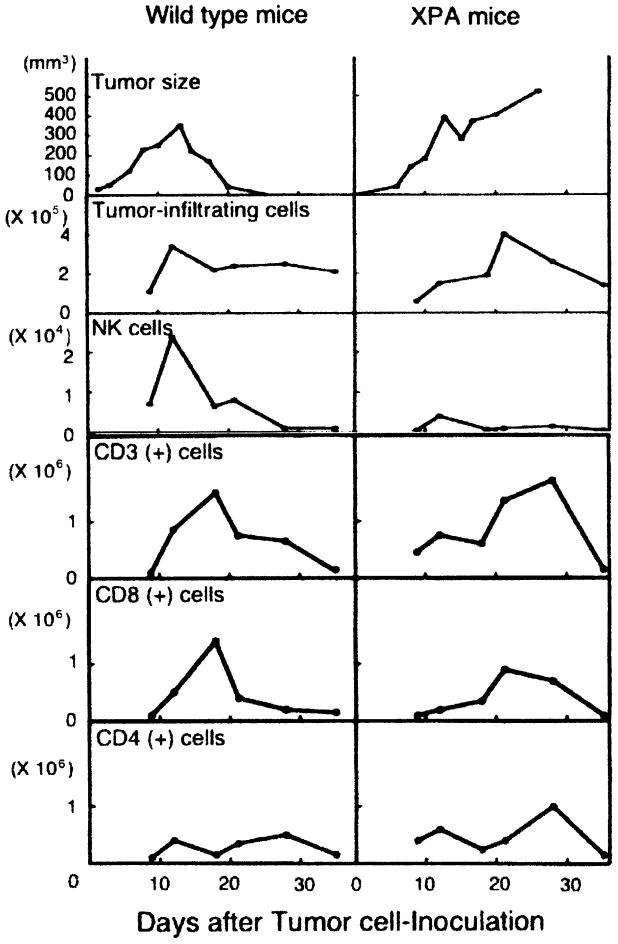

Figure 3
Flow cytometric analysis of tumor-infiltrating cells. Tissues for analysis were obtained from tumors that developed in wild-type and xeroderma pigmentosum A (XP)A mice as described in Fig 2. Tumor-infiltrating cells were prepared as described in Materials and Methods. Number and phenotypes of tumor infiltrating cells were analyzed by flow cytometry. Statistical analysis and error bars are not provided, because the experiment was performed only once using four tumors (one per mouse).

identical to those of CD3(+) cells, suggesting that a large part of tumor-infiltrating lymphocytes was CD8(+) T cells with possible cytotoxicity to tumor cells. The infiltration of CD4(+) T cells delayed and was at a low level. In XPA mice, infiltration of CD3(+), especially CD8(+) cells, was slight in early phase of the tumor growth. The infiltrating CD3(+) and CD4(+) cells increased and reached maximum in the later phase.

Discussion

We previously demonstrated that UVB-induced local and systemic immunosuppression of CHS to hapten was enhanced in XPA mice that have a defect in nucleotide excision repair of DNA lesions (Miyauchi-Hashimoto *et al*, 1996). Moreover, UVB-induced suppression of NK cell activity was also enhanced in XPA mice (Miyauchi-Hashimoto *et al*, 1999). These findings suggested that UVB-induced DNA damage is one of the primary molecular mechanisms that leads to the suppression of cell-mediated immunolog-

ical responses. It is well established that the tumor immunity plays an extremely important role in regulating the growth and metastasis of tumors. Especially, cell-mediated immunological reactions play a crucial role in tumor immunity. Thus, to examine directly whether UVB-induced DNA damage is involved in the impairment of immunological reactions against tumors, the effects of UVB radiation on tumor rejection were compared between XPA (−/−) and wild-type mice.

In this study, there was no difference in the ability to reject transplanted or inoculated tumors between wild-type and XPA mice unless they were exposed to UVB. Kripke *et al* demonstrated that UV-induced skin tumors that were transplanted onto normal syngeneic mice revealed an immunological reaction leading to inhibition of tumor growth and to tumor rejection, because such tumors have high antigenicity, and that the tumor rejection was impaired by the previous exposure of recipient mice (Kripke *et al*, 1977, 1994). In this study, however, tumors engrafted into the UVB-irradiated skin of wild-type mice were rejected, similarly to those engrafted to non-irradiated skin at least at the UV doses examined. In contrast, rejection of tumors from UVB-irradiated skin was significantly delayed and impaired in XPA mice. The immunosuppression is UV concentration-dependent. Therefore, wild-type mice would show the same result as XPA mice at a higher dose of UVB. These findings were compatible to our previous report about CHS to hapten in XPA mice (Miyauchi-Hashimoto *et al*, 1996). The CHS response to haptens applied topically was suppressed by UV irradiation of the skin not only at the application site but also distant from the sensitization. This systemic immune suppression was not confirmed in the present UVB-irradiated recipient skin.

Histological examinations in this study suggested that inflammatory cells in the UVB-irradiated XPA mice do not have potent effects to suppress tumor growth. We have previously reported that the number of circulating NK cells in the peripheral blood was decreased and the cytotoxic activity of splenic NK cells was suppressed after exposures to UVB at a dose of 500 mJ per cm^2 for 3 consecutive days in XPA (−/−) mice, but not in XPA (+/+) mice (Miyauchi-Hashimoto *et al*, 1999). In this study, only 150 mJ per cm^2-UVB was sufficient to suppress the infiltration of NK cells to the tumor tissue in the skin.

A number of cytokines and chemical mediators are produced and released from the skin after UVB exposure. We have shown that the production of prostaglandin (PG)E_2, interleukin (IL)-10, and TNF-α was greatly increased in XPA mice after UVB exposure (Kuwamoto *et al*, 2000). These immunosuppressive factors have antagonistic properties against IFN-γ, IL-2, and IL-12, which have an important role in the proliferation and activation of NK cells (Ortaldo *et al*, 1981; Ortaldo and Longo, 1988). Actually, PGE_2 and IL-10 suppress NK activity through the suppression of IFN-γ and IL-2 induction by Th1 cells, macrophages, and NK cells (Koren and Leung, 1982; Lin *et al*, 1994).

In wild-type mice, whether or not exposed to UVB, CD3(+) T cells infiltrated in tumors after the peak of NK cell infiltration associated with the tumor growth. Majority of the accumulated CD3(+) T cells was CD8(+) T cells possibly with cytotoxic activity. In contrast, the infiltration of CD8(+) T cells was suppressed in irradiated XPA mice at the time. Following the decrease in the CD8(+) T cells, the infiltration of CD4(+) T cells predominated in the growing tumor, suggesting that the later T cells might have suppressor or regulatory activity but not cytotoxic activity.

Seo *et al* (1998) analyzed the population of tumor-infiltrating cells in implanted B16 melanoma cells, and showed that NK, NKT, $\alpha\beta$T, and $\gamma\delta$T cells transiently accumulated soon after tumor cell implantation. They also demonstrated that the NK and NKT cells that appeared at an early stage exhibited cytotoxicity to tumor cells, whereas $\alpha\beta$T and $\gamma\delta$T cell populations inhibited the cytotoxic activity of NK and NKT cells. Following the accumulation of these cells, CD4(+) Th1 and CD8(+) cytotoxic T cells infiltrated into tumors. These CD4(+) and CD8(+) T cells were demonstrated to have cytotoxicity against tumor cells in an antigen-specific manner.

In conclusion, this study indicated that the defect in excision repair of DNA damage is involved in the enhancement of UVB-induced suppression of tumor immunity against skin cancers in XPA mice. This work confirmed that UV-induced DNA damage is not just an initiator but also a tumor promotor. It is possible that hyperproduction of immunosuppressive factors in the skin by UVB radiation is responsible for the impairment of anti-tumor activity of infiltrated cells. The high incidence of skin cancers in XP patients may be mainly because of frequent mutations of tumor suppressor genes and oncogenes after UV irradiation. In addition, UV-induced impairment of tumor immunity could be partially involved in the cancer development.

Materials and Methods

Animals XPA gene-deficient mice with CBA, C57BL/6, and CD-1 chimeric genetic background (Nakane *et al*, 1995) were backcrossed with hairless albino mice of the inbred strain Hos/HR-1, which were supplied by Hoshino Experimental Animal Farm (Saitama, Japan), and the resultant hairless XPA (−/−) and XPA (+/+) mice were used in this study. All mice were female and 8–10 wk of age at the beginning of each experiment, but within a single experiment, all mice were age-matched. Each experimental panel consisted of 4–18 mice. Animal experiments were approved by the Kansai Medical University Subcommittee on Research Animal Care.

UV-irradiation The UVB source was a bank of six fluorescent sunlamps (FL.20SE.30; Toshiba Medical Supply, Tokyo, Japan) with an emission spectrum of 275–375 nm, mainly UVB range, peaking at 305 nm. The irradiance of UVB was measured by a radiometer (UVR-305/365(II); Toshiba Medical Supply).

Tumor cell line The UVB-induced SCC cell line (SCC 18) was derived from tumor that was induced in XPA mice by chronic UVB irradiation. Cells were maintained in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO_2/air humidified atmosphere.

Inoculation of cultured tumor cells After establishment of tumor cell line, 3×10^7 cells were suspended in 0.2 mL of phosphate-buffered saline (PBS) and injected into a recipient mouse subcutaneously in each side of the upper back a day after the last UVB-irradiation.

Tumor measurement Tumor incidence was defined as the number of palpable tumors greater than 2 mm in diameter. Tumor

size was calculated as transverse diameter × right-angled diameter × height (mm^3).

Histologic examination Biopsy specimens were taken 6, 9, 12, and 16 d after inoculation of tumor cells and were stained with hematoxylin and eosin.

Preparation of tumor-infiltrating cells and flow cytometric analysis SCC 18 cells (3×10^7) were inoculated subcutaneously into recipient mice. Developed tumors were removed, cut into small pieces, crushed gently, and then suspended in 10% PBS through nylon mesh. Ten milliliters of the cell suspension applied on 5 mL of Histopaque 1083 (Sigma) were subjected to centrifugation at 1000 × *g* for 30 min at 20°C. The cells at the interface were collected, washed three times with PBS containing 2% FCS, and used as tumor-infiltrating cells.

Tumor-infiltrating cells obtained from four tumors (one tumor per mouse) were stained with FITC-and/or PE-conjugated monoclonal antibodies for 30 min at 4°C, washed three times, and then analyzed with a flow cytometer (FACScan, Becton Dickinson, Oxnard, California). The mononuclear cell fraction was gated to exclude contaminating tumor cells, and data were displayed on two-color plots or a histogram by FACScan programs. The fluorescence intensity was visualized on a histogram by flow cytometric analysis.

Monoclonal Abs The following monoclonal Abs were purchased from PharMingen (San Diego, California): PE-conjugated anti-NK1.1 (PK136), FITC-conjugated anti-mouse gd TCR (GL3), anti-mouse ab TCR (H57-597), anti-CD3, anti-CD4, and anti-CD8.

Statistical analysis Data were expressed as the mean ± SEM. Statistical significances were assessed by Student's *t* test, and $p < 0.05$ was taken as the level of significance.

This research was partially supported by Grant no. 14570830 and no.14570831 from the Ministry of Education, Science and Culture of Japan.

DOI: 10.1111/j.0022-202X.2005.23717.x

Manuscript received August 24, 2004; revised January 6, 2005; accepted for publication January 12, 2005

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