The Journal of Immunology

Absence of the CD1 Molecule Up-Regulates Antitumor Activity Induced by CpG Oligodeoxynucleotides in Mice

Lucia Sfondrini, Dario Besusso, Maria Teresa Zoia, Monica Rodolfo, Anna Maria Invernizzi, Masaru Taniguchi, Toshinori Nakayama, Mario Paolo Colombo, Sylvie Ménard and Andrea Balsari

J Immunol 2002; 169:151-158; ;
doi: 10.4049/jimmunol.169.1.151
http://www.jimmunol.org/content/169/1/151

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Absence of the CD1 Molecule Up-Regulates Antitumor Activity Induced by CpG Oligodeoxynucleotides in Mice[1]

Lucia Sfondrini,* Dario Besusso,* Maria Teresa Zoia,* Monica Rodolfo,† Anna Maria Invernizzi,* Masaru Taniguchi,§ Toshinori Nakayama,§ Mario Paolo Colombo,‡ Sylvie Ménard,* and Andrea Balsari[2]¶

The role of NKT cells on antitumor activity of CpG oligodeoxynucleotides (ODNs) was evaluated by peritumoral injections of CpG-ODNs in s.c. melanoma-bearing mice of strains differing in the number of NKT cells (athymic nude mice, recombination-activating gene$^{-/-}$/transgenic Vα14/Vβ8.2 mice that generate NKT cells; Jα281$^{-/-}$ mice and CD1$^{-/-}$ mice, which both have a strongly reduced number of NKT cells; and C57BL/6 wild-type mice). Tumor growth was significantly inhibited in strains enriched or depleted of NKT cells. The two murine strains having a reduced number of NKT cells differed significantly in the CpG-dependent tumor growth inhibition: in Jα281$^{-/-}$ mice this inhibition was superimposable to that observed in C57BL/6 mice, while in CD1$^{-/-}$ mice the inhibition was dramatic. The increased tumor inhibition in CD1$^{-/-}$ correlated with a significantly higher ratio of IFN-γ-IL-4 production in response to CpG as compared with C57BL/6 and Jα281$^{-/-}$ mice. Experiments in which preparations of APCs and lymphocytes of the three strains were mixed showed that in the presence of APCs not expressing CD1, the production of CpG-ODN-induced type 1 cytokines was higher. Phenotype analysis of IFN-γ- and IL-4-producing cells revealed that the differences between CD1$^{-/-}$ and C57BL/6 in the production of these two cytokines were mainly due to CD3$^+$ T lymphocytes. These data point to a regulatory role for the CD1 molecule in antitumor activity induced by danger signals, independently of Vα14 NKT cells. The identification of a CD1-dependent suppressive subpopulation(s) might have important implications for the study of tolerance in the context of cancer, autoimmunity, and transplantation. ***The Journal of Immunology*****, 2002, 169: 151–158.**

Bacterial DNA can activate the vertebrate immune system through recognition of unmethylated cytosine-guanosine dinucleotides, i.e., CpG motifs, within a specific pattern of flanking bases (1). Synthetic oligodeoxynucleotides (ODNs)[3] containing CpG motifs also activate various immune cell subsets, including macrophages and NK cells, when injected into mice (1, 2), and induce production of a wide variety of Th1-promoting cytokines independently of any known antisense effect (1–5). Recent studies have demonstrated the powerful adjuvant effect of CpG-ODNs, which can be used to trigger protective and curative Th1 responses in vivo (3, 6–8). Indeed, promising results have been reported in infectious diseases such as hepatitis B and *Listeria* (9, 10). ODNs have also been used as adjuvants for immunization against tumor Ags (11, 12). Recently, an antitumor effect after direct injection of ODNs in the area around neuroblastomas in mice has been reported, sometimes leading to complete eradication of the tumors (13). In that model, NK cells appeared to play a critical role in the antitumor activity, consistent with the finding that CpG-ODNs rapidly activate NK cells in vivo to display lytic activity (14). Moreover, in a murine model of experimental metastasis, activation of NK and NK-like NK1.1$^+$ T (NKT) cells was reported to be critical in the CpG-induced antimetastatic effect (15).

In vitro data suggest that NK cell activation is mediated by the large amounts of IL-12 produced by CpG-activated APCs (14). IL-12 also appears to induce lytic activity of NKT cells (16), although it is unclear whether CpG-ODNs activate these cells. The NKT subset, which expresses the NK1.1 Ag and intermediate levels of TCR, represents a distinct and functionally important T cell lineage (17–21). Most NKT cells use a limited TCR repertoire, comprising an invariant Vα14-Jα281 chain paired preferentially to Vβ8.2 in mice (17–23) or the homologous invariant Vα24-JαQ chain paired to Vβ11 in humans (24–27). Most murine NKT cells are restricted by CD1d molecules (27), which resemble MHC class I molecules structurally (28–30) and are expressed mainly on cells of the different hemopoietic lineages (31–33). CD1d molecules are also essential for the development of a major subset of NKT cells, as demonstrated by studies using CD1d-deficient mice (34–36). An important characteristic of NKT cells is their ability to rapidly produce high levels of cytokines, especially IL-4 and IFN-γ (18). Recent studies have revealed an active role for NKT cells in tumor destruction in several tumor models in mice (16, 37), and the antitumor effect of the CD1d-binding ligand α-galactosylceramide (38), which specifically stimulates NKT cells, is being tested in phase I clinical trials (39). In contrast, some studies indicate an immunosuppressive function for NKT cells. This NKT activity has been observed in UV-induced immune suppression (40) and in

*Molecular Targeting Unit, †Melanoma Genetics Unit, and ‡Immunotherapy and Gene Therapy Unit, Department of Experimental Oncology, Istituto Nazionale Tumori, Milan, Italy; §CREST and Department of Molecular Immunology, Graduate School of Medicine, Chiba University, Chiba, Japan; and ¶Institute of Pathology, University of Milan, Milan, Italy

Received for publication March 7, 2002. Accepted for publication May 1, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was partially supported by Associazione Italiana per la Ricerca sul Cancro. L.S. was supported by a fellowship from Fondazione Italiana Ricerca sul Cancro.

[2] Address correspondence and reprint requests to Dr. Andrea Balsari, Chair of Immunology, c/o Molecular Targeting Unit, Department of Experimental Oncology, Istituto Nazionale Tumori, Via Venezian 1; 20133 Milan, Italy. E-mail address: balsari@istitutotumori.mi.it

[3] Abbreviations used in this paper: ODN, oligodeoxynucleotide; RAG, recombination-activating gene; tg, transgenic.

0022-1767/02/$02.00

anterior chamber-associated immune deviation (41). Moreover, in a mouse model in which tumors spontaneously regress after initial growth and then recur, a negative regulation of tumor immunosurveillance by IL-13, possibly triggered by $CD4^+$NKT cells (42), has been demonstrated.

Recent evidence indicates that the tumor surveillance activity of NKT cells differs from that of NK cells (43). In some models, both NK and NKT cells exert protective activity against tumor metastasis or development, whereas in other settings NK cell-mediated tumor rejection clearly does not require NKT cell activity (43).

To further define the role of NKT cells in the antitumor activity induced by CpG motifs, we compared the therapeutic effect of peritumoral injections of CpG-ODNs in athymic mice, wild-type C57BL/6 mice, recombination-activating gene (RAG) knockout/transgenic (tg) Vα14/Vβ8.2 mice, CD1 knockout mice, and Jα281 knockout mice, which differ in the number of NKT cells. CpG-ODN 1668, which has been demonstrated to activate immune cells via Toll-like receptor 9, a family member of the phylogenetically conserved receptors mediating innate immunity (44), has been used in this study.

Materials and Methods

Cells

B16 murine melanoma cells were routinely cultured in DMEM supplemented with 10% FBS, glutamine, and antibiotics.

Oligonucleotides

Purified single-stranded phosphorothioated ODN 1668 (5′-TCCATGA CGTTCCTGATGCT-3′) containing a CpG motif, and control ODN AP1 (5′-GCTTGATGACTCAGCCGGAA) lacking a CpG motif (7) were synthesized in endotoxin-free conditions by M-Medical-GENENCO (Firenze, Italy). Phosphorothioate modification was used to reduce the susceptibility of the ODN to DNase digestion and thereby significantly prolong its $t_{1/2}$ in vivo. Toxicity of ODNs on tumor cells was evaluated in sulforhodamine B proliferation assay (45). Briefly, B16 cells were seeded at 1.5×10^3 cells/well in 96-well microplates; ODNs at various concentrations (5×10^{-6}, 5×10^{-7}, or 5×10^{-8} M) were added at day 1 of culture, and tumor cell growth was evaluated at day 3, as described (45). Tests were performed in quadruplicate.

Mice

CD1.1 ($CD1^{-/-}$) mice (36, 46) were kindly provided by G. Eberl (Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, Epalinges, Switzerland); $RAG^{-/-}$ V$\alpha14^{tg}$V$\beta8.2^{tg}$ (NKT) mice (47) and J$\alpha281^{-/-}$ mice (16) were provided by M. Taniguchi (Chiba University, Chiba, Japan); C57BL/6 mice and athymic (nude) mice were purchased from Charles River (Calco, Italy). All mice were bred in the Istituto Nazionale Tumori (INT) animal facilities, and all studies were approved by the INT Institutional Review Board.

In vivo experiments

Mice were inoculated with 8×10^5 B16 melanoma cells in the right flank, and 10 days later, inoculated s.c. in the vicinity of the tumor with ODN 1668 or AP1 (40 μg dissolved in 100 μl saline) or with 100 μl saline daily for 5 days. Two perpendicular diameters of the tumor mass were measured with a caliper every 2 days, and tumor volumes were calculated as: $\pi/6 \times$ length $\times$ width^2. Differences between groups were analyzed using the two-tailed unpaired t test and were considered significant at $p < 0.05$.

ELISA and ELISPOT

Spleen cells were aseptically removed from C57BL/6, J$\alpha281^{-/-}$, and $CD1^{-/-}$ mice. No difference in the total number of splenocyte population was observed among the three murine strains. Spleen cells (10^7 splenocytes) were cultured for 18 h in 24-well plates in 2 ml complete RPMI supplemented with 10% defined FBS ($\leq$10 Eu/ml endotoxin; HyClone, Logan, UT) in the presence of serial dilutions of ODNs. Supernatants were analyzed by ELISA (BD PharMingen, San Diego, CA) for IFN-γ and IL-12 (p40 form) productions. IFN-γ and IL-12 production after in vivo stimulation with ODNs was analyzed as above using spleen cell suspensions prepared from mice injected i.v. with 200 μg ODNs 90 min before and incubated in 24-well plates (10^7 cells/well) for 18 h in complete RPMI supplemented with 10% defined FBS.

IL-4 production after in vitro stimulation with ODNs was measured by ELISPOT assay. Splenocytes (5×10^5 cells/well) were incubated in MAHA nitrocellulose microtiter plates (Millipore, Bedford, MA) precoated with 10 μg/ml anti-mouse IL-4-coating Ab (Endogen, Woburn, MA) in the presence of ODNs. After 24 h, plates were washed with PBS-Tween 0.05%, incubated with 1 μg/ml biotinylated anti-mouse IL-4 Ab (Endogen) for 2 h, washed again, and incubated with alkaline phosphatase-streptavidin diluted 1/1000 (Bio-Rad, Hercules, CA). Spots were developed with 5-bromo-4 chloro-3-indolyl phosphate in AMP substrate buffer (Bio-Rad) and counted by an ELISPOT reader (AID, Gmbh Strassberg, Germany).

Flow cytometry

Liver cells were isolated, as previously described (48). Briefly, total liver cells were resuspended in 40% isotonic Percoll solution (Pharmacia Biotech, Uppsala, Sweden) and underlaid with 80% isotonic Percoll solution. Centrifugation for 20 min at 2000 rpm isolated the mononuclear cells at the 40–80% interface. The cells were washed twice with PBS containing 2% FCS. Spleen or liver cells from C57BL/6, $RAG^{-/-}$ V$\alpha14^{tg}$V$\beta8.2^{tg}$, $CD1^{-/-}$, and J$\alpha281^{-/-}$ mice were analyzed for NKT cell number by double staining with FITC-conjugated anti-CD3 (145-2C11) and PE-conjugated anti-NK1.1 (PK136) Abs or with FITC-conjugated anti-DX5 and biotin-conjugated anti-CD3 (145-2C11) Abs, followed by staining with streptavidin-PE (BD PharMingen). To detect the percentage and phenotype of cytokine-secreting cells after in vitro stimulation with ODNs, cells were labeled for 5 min at a concentration of 10^7 cells/ml in ice-cold medium with mouse IFN-γ or IL-4 catch reagent, an anti-IFN-γ or anti-IL-4 mAb conjugated to leukocyte cell surface-specific mAb (cytokine secretion assays; Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were diluted with medium to a final concentration of 10^5 cells/ml and incubated for 45 min at 37°C. After the cytokine-capturing period, cells were harvested, resuspended at a concentration of 10^7 in PBS containing 0.5% BSA and 2 mM EDTA, and stained for 10 min on ice with PE-conjugated anti-IFN-γ or anti-IL-4 Ab (Miltenyi Biotec) and with FITC-conjugated anti-CD3 (145-2C11), or anti-NK1.1 (PK136), or anti-CD4 (H129.19), or anti-CD8 (53-6.7) (BD PharMingen). All Abs were used at concentrations recommended by the manufacturer. Samples were analyzed by flow cytometry (FACSCalibur flow cytometer) and data analysis performed using CellQuest software (BD Biosciences, Mountain View, CA).

Isolation and stimulation of MHC class II-positive and class II-negative cells

Mouse MHC class II (Ia) microbeads (Miltenyi Biotec) were used for the positive selection or depletion of APCs from spleen cells of C57BL/6 and $CD1^{-/-}$ mice, according to the manufacturer's protocol. Briefly, cells were magnetically labeled with the microbeads for 15 min at 4°C at a concentration of 10^8 cells/ml, washed, resuspended in PBS (pH 7.2) supplemented with 0.5% BSA, and passed through a separation MS^+ column placed in the magnetic field of a MACS separator (Miltenyi Biotec). Labeled and unlabeled fractions, enriched or depleted of MHC class II cells, respectively, were separately recovered and counted, and their purity was evaluated by FACScan analysis after staining with PE-conjugated anti-B220 (RA3-6B2) or anti-Mac1 (M1/70) Ab (BD PharMingen). MHC class II^+ and MHC class II^- cells obtained from C57BL/6, $CD1^{-/-}$, and J$\alpha281^{-/-}$ splenocytes were cultured for 18 h in 96-well plates in 0.15 ml complete RPMI supplemented with 10% defined FBS in the presence of ODNs, mixing (1:1) the two fractions obtained from cells of the three strains in different combinations.

Results

Antitumor effects of CpG-ODNs in $RAG^{-/-}$ V$\alpha14^{tg}$V$\beta8.2^{tg}$ mice and athymic nude mice

Knockout tg $RAG^{-/-}$ V$\alpha14^{tg}$V$\beta8.2^{tg}$ mice (B6 background) lack the RAG and thus do not develop T or B cells. Moreover, $RAG^{-/-}$ V$\alpha14^{tg}$V$\beta8.2^{tg}$ mice, unlike $RAG^{-/-}$ mice, do not maintain detectable levels of NK cells, probably because Vβ8.2 expression inhibits NK cell development and promotes NKT cell development (47). Thus, $RAG^{-/-}$ V$\alpha14^{tg}$V$\beta8.2^{tg}$ mice are rich only in NKT lymphocytes. We tested the antitumor activity of CpG sequences in these mice and in athymic nude mice (BALB/c background), which lack T cells, but not NK cells. As a tumor model, we used

the poorly immunogenic B16 melanoma, a well-established melanoma model in mice. The antitumor activity of CpG-ODN against this melanoma has been reported to be NK cell dependent in athymic mice (49). A total of 25 RAG$^{-/-}$ Vα14^{tg}Vβ8.2^{tg} mice and 29 nude mice was injected s.c. with 8×10^5 B16 melanoma cells. Tumor growth was slightly faster in the athymic nude mice, and at day 10 tumor nodules were measurable only in nude mice, although tumors were palpable in all mice of both strains. Mice of each strain were randomly divided into three groups and injected at the tumor site on each of 5 days with CpG-ODN 1668, ODN AP1 lacking the CpG motif (40 μg/100 μl saline daily), or with saline. In both strains, treatment with CpG-ODN reduced tumor growth as compared with treatment with control ODN AP1 or saline (Fig. 1). By day 17 after tumor implantation, tumor volumes in CpG-ODN-treated nude mice were 67% smaller than in saline-treated nude mice (mean ± SE: 659 ± 132 mm^3 vs 2025 ± 266 mm^3, $p = 0.0001$) and 49% smaller than in the AP1 ODN-treated group (659 ± 131 mm^3 vs 1296 ± 195 mm^3, $p = 0.01$), while RAG$^{-/-}$ Vα14^{tg}Vβ8.2^{tg} mice treated with CpG-ODN had 82% smaller tumor volumes than their saline-treated counterparts (mean ± SE: 247 ± 67 mm^3 vs 1355 ± 266 mm^3, $p = 0.0001$) and 65% smaller than those that received ODN AP1 (247 ± 67 mm^3 vs 713 ± 197 mm^3, $p = 0.04$). The antitumor activity of CpG-ODNs did not appear to be mediated by a direct toxicity on tumor cells because no growth inhibition was observed when CpG-ODN at various concentrations was added to in vitro cultured melanoma cells (data not shown).

Antitumor effects of CpG-ODN in C57BL/6 mice and in CD1$^{-/-}$ and Jα281 chain$^{-/-}$ knockout mice

Because both athymic and RAG$^{-/-}$ Vα14^{tg}Vβ8.2^{tg} mice lack T cells and thus cannot provide information on a possible role for NKT cells in shaping initial T cell activation in response to CpG ODNs, we evaluated the antitumor growth effects of CpG-ODNs in wild-type C57BL/6 mice as compared with two knockout strains, homogeneous in genetic background and with markedly reduced NKT cell numbers. C57BL/6 mice knocked out for CD1 or Jα281 have reduced numbers of NK1.1$^+$ T cells in the thymus, spleen, bone marrow, and liver, but similar numbers of CD4$^+$, CD8$^+$, and other lymphocytes to those in wild-type mice (34). Consistent with previous data (21, 50), our multiparameter flow cytometry analysis revealed a marked reduction, but not the absence of NKT cells in the spleen of these two knockout strains (data not shown).

Mice of each strain were injected s.c. with B16 melanoma cells (8×10^5/200 μl). Ten days later, when tumor nodules were palpable, mice of each strain were randomly divided into three groups and injected at the tumor site with ODNs (40 μg/100 μl daily for 5 days) containing or lacking the CpG sequence, or with saline. A superimposable growth rate of the tumors injected in the three strains was observed after treatment with saline (Fig. 2). Tumor growth was greatly reduced in all three strains of mice receiving CpG-ODNs (Fig. 2), while slight tumor growth inhibition was observed after treatment with control ODNs. By day 21 after tumor implantation, tumor volumes in C57BL/6 mice injected with CpG-ODNs were 75% smaller than in mice receiving saline (mean ± SE: 533 ± 115 mm^3 vs 2154 ± 458 mm^3, $p = 0.0009$) and 54% smaller than in control ODN-injected mice (533 ± 115 mm^3 vs 1162 ± 319 mm^3, $p = 0.05$); Jα281$^{-/-}$ mice receiving CpG-ODNs had tumor volumes 75% smaller than in the saline-injected group (mean ± SE: 410 ± 116 mm^3 vs 1654 ± 565 mm^3, $p = 0.04$) and 71% smaller than in the control ODN group (410 ± 116 mm^3 vs 1396 ± 422 mm^3, $p = 0.04$). CD1$^{-/-}$ mice injected with CpG-ODNs had tumors that were 98% smaller than the saline group (mean ± SE: 52 ± 23 mm^3 vs 2642 ± 206 mm^3, $p < 0.0001$) and 95% smaller than in the control ODN group (52 ± 23 mm^3 vs 1122 ± 331 mm^3, $p = 0.0046$). The reduction in tumor growth was significantly greater in CD1$^{-/-}$ mice than in the other two strains ($p = 0.01$ vs C57BL/6 and $p = 0.02$ vs Jα281$^{-/-}$).

Cytokine production after CpG-ODN stimulation

The immune response to CpG-containing ODNs is characterized by production of Th1-promoting cytokines, especially IL-12 and IFN-γ (2–4). IL-12 has been reported to induce a temporally selective loss of CD3$^+$NK1.1$^+$ cells (48). We observed in C57BL/6 mice 24 h after injection of CpG-ODNs a strong depletion of liver CD3$^+$NK1.1$^+$ cells, while the percentage of CD3$^+$DX5$^+$ cells resulted in no modification (Fig. 3). To determine whether the different antitumor responses observed in CD1$^{-/-}$, Jα281$^{-/-}$, and C57BL/6 mice might reflect the differential production of these regulatory cytokines in response to CpG-ODN stimulation, IL-12 and IFN-γ production in response to ODNs containing or lacking

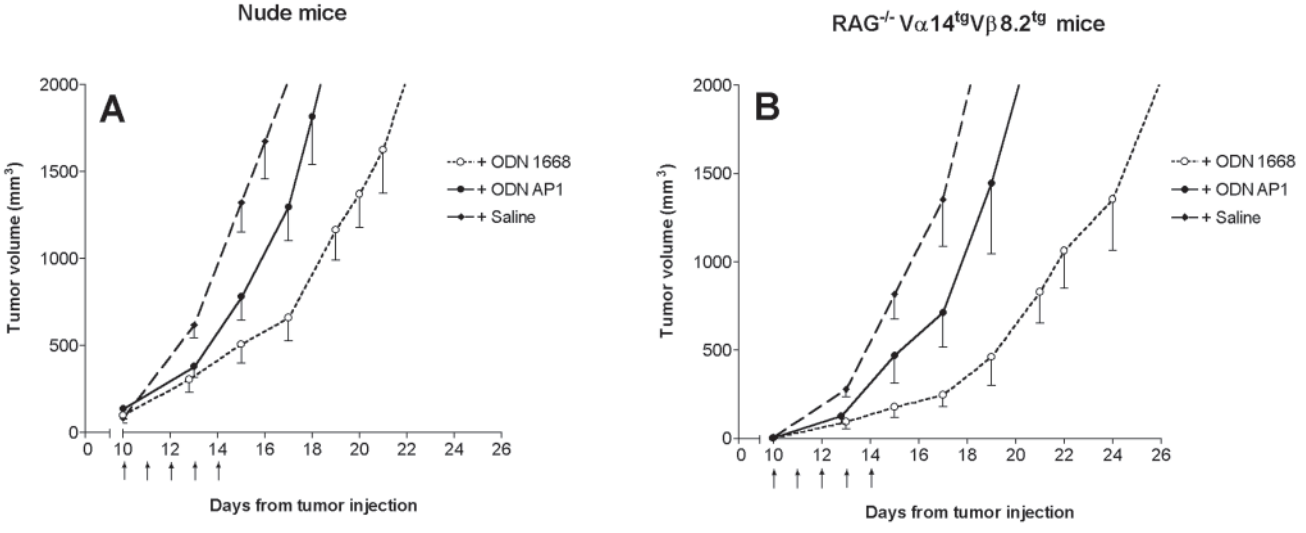

FIGURE 1. Tumor volumes (mean ± SE) in nude mice (*A*) and transgenic (Vα14^{tg}Vβ8.2^{tg}) mice (*B*) treated 10 days after B16 melanoma cell injection with saline (nude mice, $n = 5$; Vα14^{tg}Vβ8.2^{tg} mice, $n = 4$), control ODN AP1 (nude mice, $n = 12$; Vα14^{tg}Vβ8.2^{tg} mice, $n = 11$), or CpG-ODN 1668 (nude mice, $n = 12$; Vα14^{tg}Vβ8.2^{tg} mice, $n = 10$) for 5 days (arrows) at the tumor site.

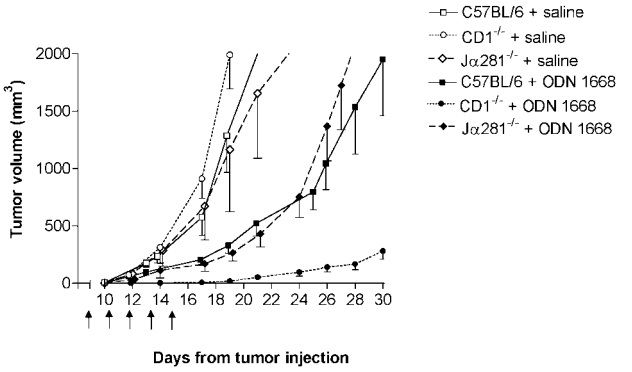

FIGURE 2. Tumor volumes (mean ± SE) in C57BL/6, Jα281$^{-/-}$, and CD1$^{-/-}$ mice treated 10 days after B16 melanoma cell injection with saline (C57BL/6 mice, $n = 10$; Jα281$^{-/-}$ mice, $n = 11$; and CD1$^{-/-}$ mice, $n = 5$), or CpG-ODN 1668 (C57BL/6 mice, $n = 13$; Jα281$^{-/-}$ mice, $n = 11$; and CD1$^{-/-}$ mice, $n = 10$) for 5 days (arrows) at the tumor site.

a CpG sequence was evaluated by ELISA in the supernatant of spleen cells from mice of the three strains incubated with different doses of ODNs for 18 h. CpG-ODNs induced high level production of IL-12 or IFN-γ in all three mouse strains, while no production of either cytokine was detected in response to stimulation with the control ODN, even at the highest concentration tested. IL-12 levels were not significantly different in the three strains (mean ± SE: C57BL/6, 2291 ± 149; CD1$^{-/-}$, 1965 ± 217; Jα281$^{-/-}$, 1540 ± 338, at 1 μg/ml ODN 1668), whereas CD1 knockout mice produced a significantly higher level of IFN-γ than did the other two strains (Fig. 4*A*). Analysis of IL-12 and IFN-γ production by splenocytes obtained 90 min after i.v. injection of ODN (200 μg) revealed production of these cytokines only with ODN containing CpG sequences. These ex vivo data are consistent

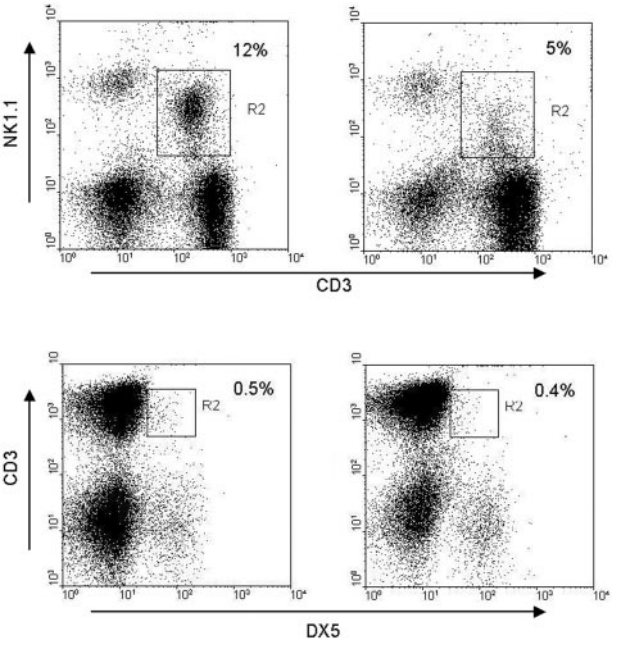

FIGURE 3. FACS analysis of liver NKT cell population from C57BL/6 mice before (*left*) and 24 h after i.v. injection of 200 μg ODN 1668 (*right*). Liver cells were stained with FITC-conjugated anti-CD3 (*x*-axis) and PE-conjugated anti-NK1.1 (*y*-axis) Abs or with FITC-conjugated anti-DX5 (*x*-axis) and biotin-conjugated anti-CD3 Abs plus streptavidin-PE (*y*-axis). For analysis, live lymphocytes were gated according to light scatter properties and propidium iodide exclusion. One representative experiment of three is shown.

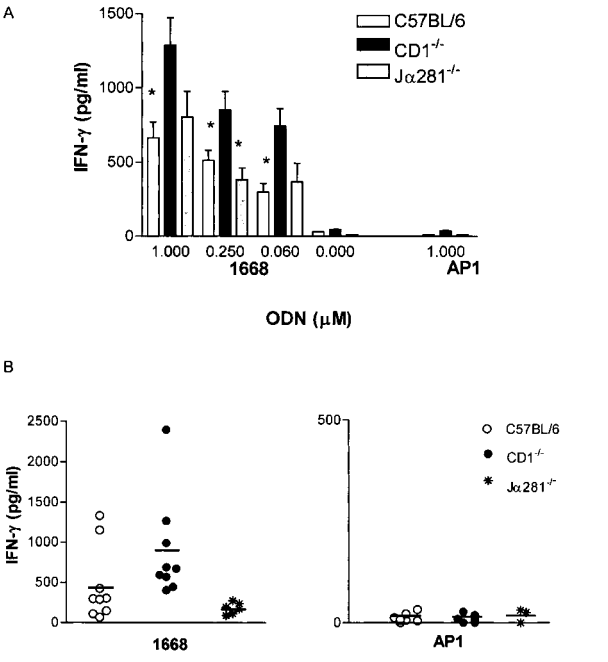

FIGURE 4. IFN-γ production in C57BL/6, Jα281$^{-/-}$, and CD1$^{-/-}$ mice after stimulation with CpG-ODN 1668 or control ODN AP1. *A*, Single cell suspensions were prepared from spleens, placed in culture, and stimulated with ODNs or not stimulated. Culture supernatants were harvested after 18 h, and IFN-γ levels were determined by ELISA. Data (mean ± SE) were pooled from two independent experiments, each performed with four to five mice per group. *, $p < 0.05$ vs CD1$^{-/-}$ mice by two-tailed unpaired *t* test. *B*, Single cell suspensions were prepared from spleens of mice 90 min after i.v. injection of 200 μg ODNs. Culture supernatants were harvested after 18 h, and IFN-γ levels were determined by ELISA. Symbols represent the IFN-γ production by each single mouse; horizontal lines represent the mean value in each group.

with in vitro results, because despite the high variability among individual mice, the CD1 knockout strain produced higher IFN-γ levels compared with the other two strains (Fig. 4*B*), while a similar production of IL-12 was observed (mean ± SE: C57BL/6, 1876.8 ± 245.1; CD1$^{-/-}$, 1634.1 ± 185.1; seven mice/group; Jα281$^{-/-}$ not evaluated). ELISA evaluation revealed no detectable IL-4 production in response to stimulation with ODNs in the three strains after stimulation with CpG-ODN or control ODN. However, in CD1$^{-/-}$ splenocytes stimulated in vitro with CpG ODN, the more sensitive ELISPOT assay revealed a significantly

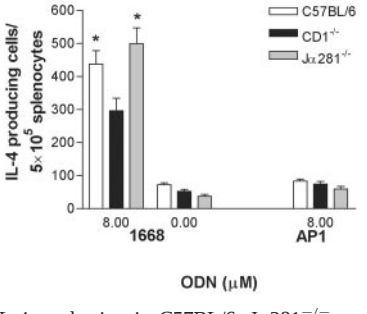

FIGURE 5. IL-4 production in C57BL/6, Jα281$^{-/-}$, and CD1$^{-/-}$ mice after stimulation with CpG-ODN 1668 or control ODN AP1. Single cell suspensions were prepared from mouse spleens and cultured in nitrocellulose plates coated with anti-IL-4 Ab in the presence of ODNs. Spot number was determined after 24 h of incubation. Data (mean ± SE) were pooled from two independent experiments, each performed with four to five mice per group. *, $p < 0.05$ vs CD1$^{-/-}$ mice group by two-tailed unpaired *t* test.

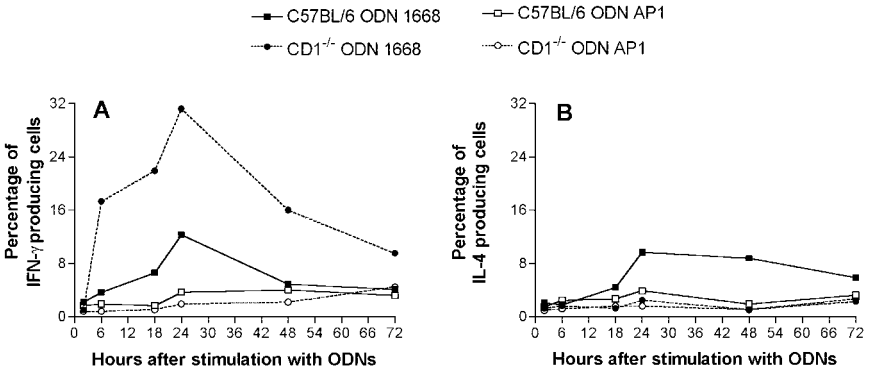

FIGURE 6. Kinetics of activation of IFN-γ (*A*)- and IL-4-producing (*B*) cells by ODN 1668 and control ODN AP1 (1 μM for IFN-γ, 8 μM for IL-4). Single cell suspensions were prepared from spleens, placed in culture, and stimulated with ODNs. At the time points indicated, cells were labeled with IFN-γ or IL-4 catch reagent, allowed to secrete cytokines for 45 min at 37°C, and stained with PE-conjugated anti-IFN-γ or anti-IL-4 Ab. For FACScan analysis, live lymphocytes were gated according to light scatter properties (forward and side scatter) and propidium iodide exclusion.

lower number of cells ($p < 0.05$) producing IL-4 as compared with the other two strains (Fig. 5).

The kinetics and magnitude of IFN-γ and IL-4 response in C57BL/6 and CD1$^{-/-}$ mice were evaluated using a new sorting technique based on the surface capture of secreted cytokines by bispecific Ab-Ab conjugates, which bind to IL-4 or IFN-γ and to murine CD45, a highly abundant leukocyte cell surface protein (51). FACScan analysis of C57BL/6 and CD1$^{-/-}$ spleen cells incubated with CpG-ODN or control ODN for different times revealed similar kinetics of IFN-γ release induced by CpG-ODN in the two strains, but a significantly higher number of cells secreting IFN-γ in CD1$^{-/-}$ than in C57BL/6 mice ($p = 0.04$) (Fig. 6). In contrast, the number of cells producing IL-4 was significantly higher in C57BL/6 mice ($p = 0.02$) (Fig. 6).

Role of APC on CpG-ODN-induced IFN-γ production

The regulatory role of APC expressing or not expressing CD1 on the CpG-ODN-induced IFN-γ production was then evaluated. Spleen cells from the three strains (C57BL/6, Jα281$^{-/-}$, and CD1$^{-/-}$) were separated into MHC class II$^+$ and MHC class II$^-$ fractions using MHC class II microbeads, and the positive fractions containing APCs were mixed with the negative fractions containing the lymphocytes. After stimulation with CpG-ODNs, the production of IFN-γ and IL-4 was evaluated by ELISA. No detectable levels of IL-4 were revealed in supernatants of any of the fractions obtained from the three strains (not shown).

Increased production of IFN-γ by cells present in the MHC class II$^-$ fractions from C57BL/6 or Jα281$^{-/-}$ mice in response to CpG-ODN was observed when syngeneic APC were replaced with APC-containing fractions obtained from CD1$^{-/-}$ mice ($p < 0.05$ vs each syngeneic combination) (Fig. 7). In contrast, IFN-γ production by the lymphocyte-containing fraction from CD1$^{-/-}$ mice was not significantly modified when syngeneic CD1$^{-/-}$ APC were replaced with APCs from C57BL/6 or Jα281$^{-/-}$ mice.

In agreement with Warren et al. (52), who found no direct effect of CpG-ODNs on purified T cells in vitro, no IFN-γ production was observed by purified MHC class II-negative fractions (Fig. 7).

Phenotype of cells secreting IFN-γ and IL-4 in response to CpG ODNs in C57BL/6 and CD1$^{-/-}$ mice

To characterize the phenotype of spleen cells secreting IFN-γ and IL-4 in response to CpG-ODN in C57BL/6 and CD1$^{-/-}$ mice, a multiparametric analysis using the capturing cytokine-bispecific Ab and specific mAbs directed against CD3 and NK1.1 molecules was performed. CpG-ODN stimulation induced IFN-γ secretion from both CD3$^+$ cells and NK1.1$^+$ cells (Fig. 8). A significantly higher number of CD3$^+$ T lymphocytes, secreting IFN-γ in response to CpG-ODN, was detectable in CD1$^{-/-}$ mice as compared with C57BL/6 mice (49.4 vs 12.2%, $p = 0.006$). Both CD8$^+$ and CD4$^+$ populations were found to contribute to the increased production of IFN-γ observed in CD1$^{-/-}$ mice. No significant differences in the number of NK1.1$^+$ cells secreting IFN-γ were detectable between the two strains (1.9% in CD1$^{-/-}$ vs 1.3% in C57BL/6, $p = 0.55$).

The CD3$^+$ T lymphocytes were found to be the major source of the increased production of IL-4 in C57BL/6 mice as compared with CD1$^{-/-}$ mice (8.1 vs 2.2%, $p = 0.04$) (Fig. 8). Very few NK1.1$^+$ cells secreting IL-4 were observed in both strains after CpG-ODN stimulation (0.05% in C57BL/6 and 0.09% in CD1$^{-/-}$). Therefore, the phenotype analysis of IFN-γ- and IL-4-producing cells revealed that the differences between CD1$^{-/-}$ and C57BL/6 in the production of these two cytokines were mainly due to CD3$^+$ T lymphocytes.

Discussion

Our data indicate that direct injection of CpG-ODNs in the peritumoral area represents a simple means of achieving immunotherapeutic effects against B16 melanoma without the need for selection and purification of tumor Ags. It is noteworthy that the CpG-ODN effect

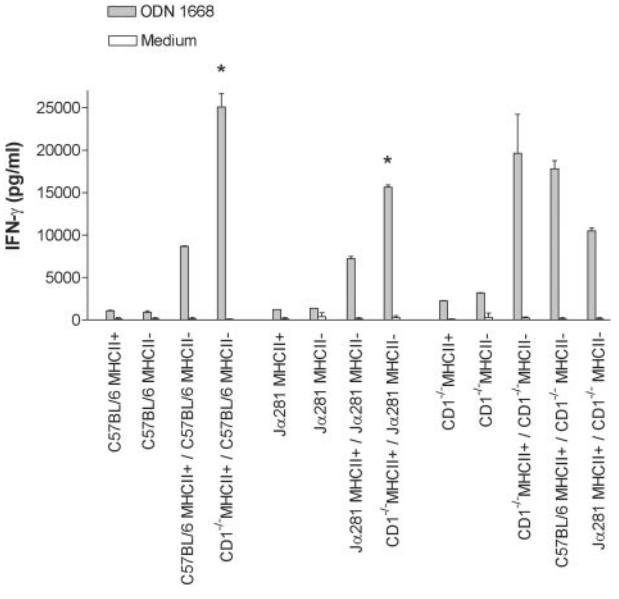

FIGURE 7. IFN-γ production after culture for 18 h in the presence of CpG-ODN 1668 (1 μM) by the APC-containing MHCII$^+$ fraction or lymphocyte-containing MHCII$^-$ fraction obtained from C57BL/6, Jα281$^{-/-}$, and CD1$^{-/-}$ mice, or after coculture of the two fractions (1:1) in the different combinations from the three strains. The experiment reported is representative of four independent experiments. *, $p < 0.05$ vs each syngeneic combination by two-tailed unpaired *t* test.

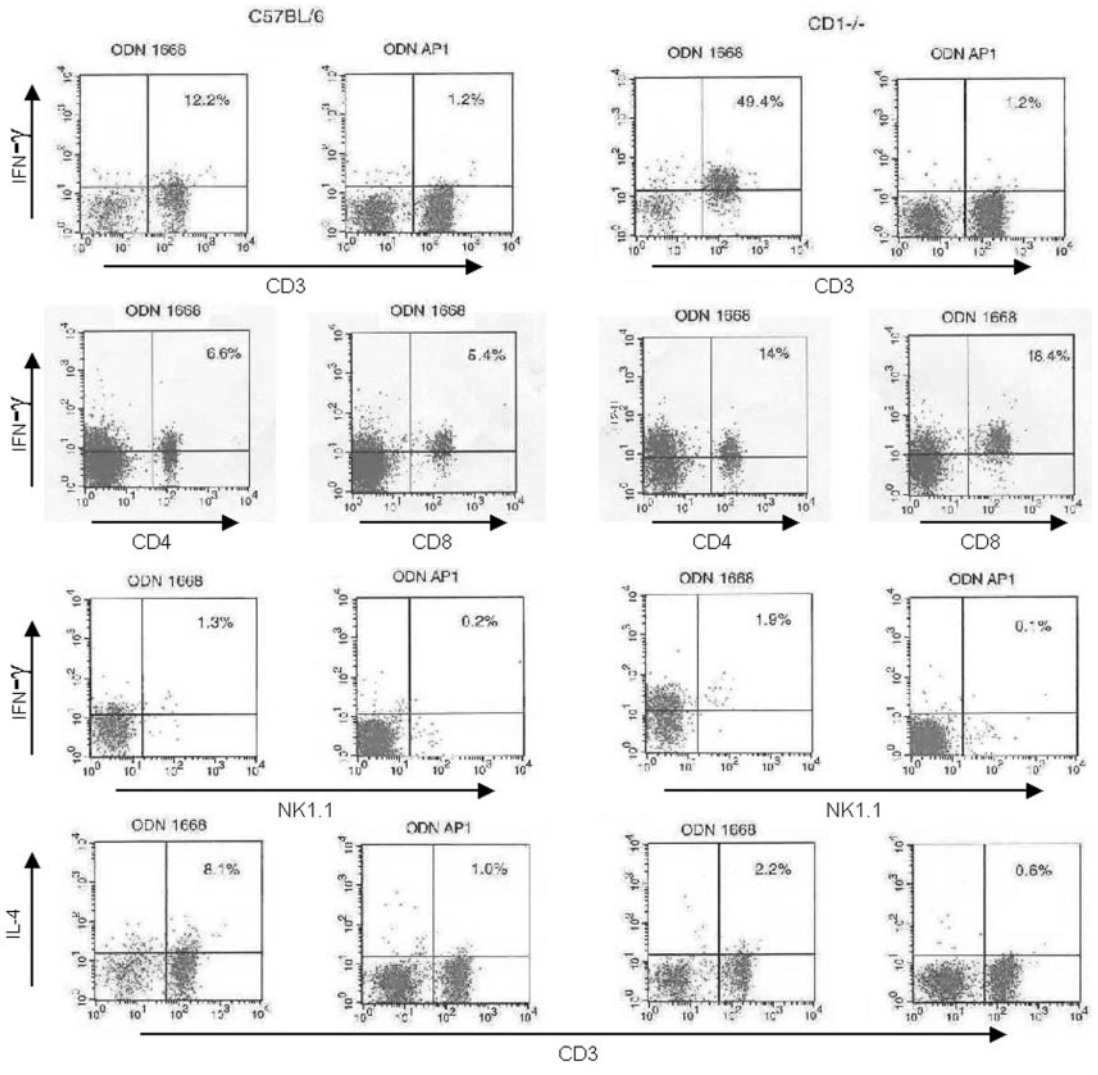

FIGURE 8. Phenotypic analysis of IFN-γ- and IL-4-producing cells after 24 h of incubation with ODN 1668 or control ODN AP1 (1 μM for IFN-γ, 8 μM for IL-4). Cells were labeled with IFN-γ or IL-4 catch reagent, allowed to secrete cytokines for 45 min at 37°C, and stained with PE-conjugated Abs against IFN-γ or IL-4 (*y*-axis) and with FITC-conjugated Abs against CD3 or CD4 or CD8 or NK1.1 (*x*-axis). For analysis, live lymphocytes were gated according to light scatter properties and propidium iodide exclusion. One representative experiment of three is shown.

was observed in nonpathogen-free mice, and the tumor growth rate in all B6 background strains (RAG$^{-/-}$ Vα14^{tg}Vβ8.2^{tg}, Jα281$^{-/-}$, CD1$^{-/-}$, and C57BL/6) treated with saline was superimposable. The slight inhibition of tumor growth in all strains by the control ODN most likely rests in the presence of the phosphorothioate modification (14, 53, 54).

The antitumor responses observed in immunologically different strains might reflect the ability of CpG-ODNs to activate various immune cell subsets. RAG$^{-/-}$ Vα14^{tg}Vβ8.2^{tg} mice, in which NK cells are undetectable, but which are rich in NKT cells, respond similarly to athymic mice in which the anti-B16 cell activity of CpG-ODN is reportedly NK cell dependent (49). These results suggest that NKT cells, like the NK cells in nude mice, in an immune context expressing high level of these cells, can mediate the antitumor activity induced by CpG-ODNs. Accordingly, treatment of C57BL/6 with anti-NK1.1 Abs abrogated by 61% the antitumor activity of CpG-ODNs (data not shown). However, NKT cells may be sufficient, but not necessary, on CpG-induced antitumor activity because superimposable antitumor responses were observed in C57BL/6 and Jα281$^{-/-}$ mice, which partially lack NKT cells. Moreover, it has been suggested that NKT cells may represent a regulatory arm of the T cell response (21, 40, 55); the similar inhibition of tumor growth observed in wild-type C57BL/6 mice and Jα281$^{-/-}$ mice provides evidence against a regulatory role for Vα14 NKT cells in shaping the CpG-induced antitumor immune response.

The stronger antitumor response and the increased ratio of IFN-γ-IL-4 in CD1$^{-/-}$ mice as compared with C57BL/6 wild-type mice are consistent with the data of Terabe et al. (42), obtained using a mouse model in which the tumor shows a growth regression-recurrence pattern. In that study, increased resistance to tumor recurrence was detected in CD1$^{-/-}$ mice, with decreased IL-4 and IL-13 production and increased IFN-γ production. The authors hypothesized that because NKT cells represent a source of IL-4 and IL-13, loss of this subpopulation, which might play a critical role in down-regulation of tumor immunosurveillance, might account for the resistance to tumor recurrence in CD1$^{-/-}$ mice. Data on Jα281$^{-/-}$ mice indicate that, in our model, the greater tumor-protective response in CD1$^{-/-}$ mice is not directly dependent on the loss of Vα14 NKT cells. Moreover, in agreement with in vivo antitumor response, we observed no increase of IFN-γ production in response to CpG-ODNs from C57BL/6 spleen cells depleted of

NKT cells ($CD3^+DX5^+$) using anti-DX5-coated microbeads (data not shown).

Recently, Dao et al. (56) reported that the impairment of peripheral deletion of T cells induced by anti-CD3 Ab in $CD1^{-/-}$ mice (on the B6 × 129 background) is independent of Vα14 NKT cells, because neither β_2-microglobulin-deficient mice nor Jα281-deficient mice reproduce the abnormality found in $CD1^{-/-}$ mice. As proposed by Dao et al. (56), it is possible to hypothesize the presence of T cells that, interacting with CD1 expressed on APC, negatively regulate the immune response. This hypothesis explains the increased IFN-γ production by lymphocytes from C57BL/6 and $J\alpha281^{-/-}$ mice in response to CpG-ODNs observed when syngeneic APCs were replaced with $CD1^{-/-}$ APCs. The development of these suppressive T cells might be CD1 dependent because the IFN-γ production by lymphocytes from $CD1^{-/-}$ mice in the presence of C57BL/6 and $J\alpha281^{-/-}$ APCs was not significantly different from that observed in the presence of syngeneic APCs. As alternative hypothesis, as suggested by data of Chang et al. (57), CD1 might negatively regulate immune response through interaction with a CD1-specific inhibitory receptor(s) that could have a broader cellular distribution that includes T cells. At the present time, we do not know the CD1-reactive cell subset(s) involved in the down-regulation of CpG-induced immune response because besides Vα14 NKT cells, non-Vα14 NKT cells (55) and $NK1.1^-$ T cells also were reported to be dependent on CD1 for function and/or development (30, 58–60).

Attempts to demonstrate a direct role for CD1 on APCs using a mAb against CD1 both in vitro and in vivo (anti-CD1 mAb injected into the tumor site for 5 days, starting 6 h before CpG ODN injection) were not successful (not shown). However, considering that blocking of CD1 activity with the Ab has been proven only in vitro (61), these data cannot be considered conclusive.

Together, our data point to a regulatory role for CD1 in the antitumor response induced by CpG danger signals independently of Vα14 NKT cell activity. The increased antitumor activity in response to CpG-ODN treatment in $CD1^{-/-}$ mice compared with C57BL/6 and $J\alpha281^{-/-}$ mice appears to be related to the enhanced IFN-γ-IL-4 ratio produced in these mice, which affects the Th1/Th2 differentiation process, and in turn might reflect the regulatory role of CD1-restricted lymphocyte subset. Further analyses will provide information about the immunological functions and the ligand specificity of these CD1-dependent subpopulations. A number of recently described regulatory or suppressor T cell subsets represent potentially formidable barriers to successful antitumor immune responses (62–64). It may be that successful immunotherapies will require inclusion of strategies to interfere with the action of suppressor or regulatory cells, possibly by transiently depleting these cells or inhibiting their effector molecules. This approach may be a key requirement for promoting tumor rejection.

Acknowledgments

We thank Drs. M. Bellone and G. Parmiani for helpful suggestions, and L. Mameli for manuscript preparation.

References

1. Krieg, A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature 374:546.*
2. Chace, J. H., N. A. Hooker, K. L. Mildenstein, A. M. Krieg, and J. S. Cowdery. 1997. Bacterial DNA-induced NK cell IFN-γ production is dependent on macrophage secretion of IL-12. *Clin. Immunol. Immunopathol. 84:185.*
3. Chu, R. S., O. S. Targoni, A. M. Krieg, P. V. Lehmann, and C. V. Harding. 1997. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J. Exp. Med. 186:1623.*
4. Roman, M., E. Martin-Orozco, J. S. Goodman, M. D. Nguyen, Y. Sato, A. Ronaghy, R. S. Kornbluth, D. D. Richman, D. A. Carson, and E. Raz. 1997. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med. 3:849.*
5. Yamamoto, S., T. Yamamoto, T. Kataoka, E. Kuramoto, O. Yano, and T. Tokunaga. 1992. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. *J. Immunol. 148:4072.*
6. Lipford, G. B., M. Bauer, C. Blank, R. Reiter, H. Wagner, and K. Heeg. 1997. CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. *Eur. J. Immunol. 27:2340.*
7. Lipford, G. B., T. Sparwasser, M. Bauer, S. Zimmermann, E. S. Koch, K. Heeg, and H. Wagner. 1997. Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. *Eur. J. Immunol. 27:3420.*
8. Zimmermann, S., O. Egeter, S. Hausmann, G. B. Lipford, M. Rocken, H. Wagner, and K. Heeg. 1998. CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. *J. Immunol. 160:3627.*
9. Davis, H. L., R. Weeratna, T. J. Waldschmidt, L. Tygrett, J. Schorr, and A. M. Krieg. 1998. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. [Published erratum appears in *1999 J. Immunol. 162:3103.*] *J. Immunol. 160:870.*
10. Krieg, A. M., L. Love-Homan, A. K. Yi, and J. T. Harty. 1998. CpG DNA induces sustained IL-12 expression in vivo and resistance to *Listeria monocytogenes* challenge. *J. Immunol. 161:2428.*
11. Weiner, G. J., H. M. Liu, J. E. Wooldridge, C. E. Dahle, and A. M. Krieg. 1997. Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. *Proc. Natl. Acad. Sci. USA 94:10833.*
12. Wooldridge, J. E., Z. Ballas, A. M. Krieg, and G. J. Weiner. 1997. Immunostimulatory oligodeoxynucleotides containing CpG motifs enhance the efficacy of monoclonal antibody therapy of lymphoma. *Blood 89:2994.*
13. Carpentier, A. F., L. Chen, F. Maltonti, and J. Y. Delattre. 1999. Oligodeoxynucleotides containing CpG motifs can induce rejection of a neuroblastoma in mice. *Cancer Res. 59:5429.*
14. Wagner, H. 1999. Bacterial CpG DNA activates immune cells to signal infectious danger. *Adv. Immunol. 73:329.*
15. Hafner, M., R. Zawatzky, C. Hirtreiter, W. A. Buurman, B. Echtenacher, T. Hehlgans, and D. N. Mannel. 2001. Antimetastatic effect of CpG DNA mediated by type I IFN. *Cancer Res. 61:5523.*
16. Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, Koseki, M. Kanno, and M. Taniguchi. 1997. Requirement for Vα14 NKT cells in IL-12-mediated rejection of tumors. *Science 278:1623.*
17. Bendelac, A. 1995. Mouse $NK1^+$ T cells. *Curr. Opin. Immunol. 7:367.*
18. Bendelac, A., M. N. Rivera, S. H. Park, and J. H. Roark. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu. Rev. Immunol. 15:535.*
19. Bix, M., and R. M. Locksley. 1995. Natural T cells: cells that co-express NKRP-1 and TCR. *J. Immunol. 155:1020.*
20. MacDonald, H. R. 1995. $NK1.1^+$ T cell receptor-α/β^+ cells: new clues to their origin, specificity, and function. *J. Exp. Med. 182:633.*
21. Vicari, A. P., and A. Zlotnik. 1996. Mouse $NK1.1^+$ T cells: a new family of T cells. *Immunol. Today 17:71.*
22. MacDonald, H. R. 2000. CD1d-glycolipid tetramers: a new tool to monitor natural killer T cells in health and disease. *J. Exp. Med. 192:F15.*
23. Lantz, O., and A. Bendelac. 1994. An invariant T cell receptor α chain is used by a unique subset of major histocompatibility complex class I-specific $CD4^+$ and $CD4^-8^-$ T cells in mice and humans. *J. Exp. Med. 180:1097.*
24. Porcelli, S., C. E. Yockey, M. B. Brenner, and S. P. Balk. 1993. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood $CD4^-8^-$ α/β T cells demonstrates preferential use of several Vβ genes and an invariant TCRα chain. *J. Exp. Med. 178:1.*
25. Dellabona, P., E. Padovan, G. Casorati, M. Brockhaus, and A. Lanzavecchia. 1994. An invariant Vα24-JαQ/Vβ11 T cell receptor is expressed in all individuals by clonally expanded $CD4^-8^-$ T cells. *J. Exp. Med. 180:1171.*
26. Dellabona, P., G. Casorati, B. Friedli, L. Angman, F. Sallusto, A. Tunnacliffe, E. Roosneek, and A. Lanzavecchia. 1993. In vivo persistence of expanded clones specific for bacterial antigens within the human T cell receptor α/β $CD4^-8^-$ subset. *J. Exp. Med. 177:1763.*
27. Exley, M., J. Garcia, S. P. Balk, and S. Porcelli. 1997. Requirements for CD1d recognition by human invariant $V\alpha24^+$ $CD4^-CD8^-$ T cells. *J. Exp. Med. 186:109.*
28. Bendelac, A. 1995. Positive selection of mouse $NK1^+$ T cells by CD1-expressing cortical thymocytes. *J. Exp. Med. 182:2091.*
29. Bendelac, A., O. Lantz, M. E. Quimby, J. W. Yewdell, J. R. Bennink, and R. R. Brutkiewicz. 1995. CD1 recognition by mouse $NK1^+$ T lymphocytes. *Science 268:863.*
30. Cardell, S., S. Tangri, S. Chan, M. Kronenberg, C. Benoist, and D. Mathis. 1995. CD1-restricted $CD4^+$ T cells in major histocompatibility complex class II-deficient mice. *J. Exp. Med. 182:993.*
31. Brossay, L., D. Jullien, S. Cardell, B. C. Sydora, N. Burdin, R. L. Modlin, and M. Kronenberg. 1997. Mouse CD1 is mainly expressed on hemopoietic-derived cells. *J. Immunol. 159:1216.*
32. Park, S. H., J. H. Roark, and A. Bendelac. 1998. Tissue-specific recognition of mouse CD1 molecules. *J. Immunol. 160:3128.*
33. Mandal, M., X. R. Chen, M. L. Alegre, N. M. Chiu, Y. H. Chen, A. R. Castano, and C. R. Wang. 1998. Tissue distribution, regulation and intracellular localization of murine CD1 molecules. *Mol. Immunol. 35:525.*

34. Chen, Y. H., N. M. Chiu, M. Mandal, N. Wang, and C. R. Wang. 1997. Impaired $NK1^+$ T cell development and early IL-4 production in CD1-deficient mice. *Immunity 6:459.*
35. Mendiratta, S. K., W. D. Martin, S. Hong, A. Boesteanu, S. Joyce, and L. Van Kaer. 1997. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity 6:469.*
36. Smiley, S. T., M. H. Kaplan, and M. J. Grusby. 1997. Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. *Science 275:977.*
37. Hashimoto, W., K. Takeda, R. Anzai, K. Ogasawara, H. Sakihara, K. Sugiura, S. Seki, and K. Kumagai. 1995. Cytotoxic NK1.1 Ag^+ α-β T cells with intermediate TCR induced in the liver of mice by IL-12. *J. Immunol. 154:4333.*
38. Kobayashi, E., K. Motoki, T. Uchida, H. Fukushima, and Y. Koezuka. 1995. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol. Res. 7:529.*
39. Yokoyama, T., E. Kobayashi, and Y. Koezuka. 1999. Effect of KRN7000 (α-galactosylceramide) on CTL induction. *Workshop on NKT Cells and CD1-Mediated Antigen Presentation.* San Diego, p. 32.
40. Moodycliffe, A. M., D. Nghiem, G. Clydesdale, and S. E. Ullrich. 2000. Immune suppression and skin cancer development: regulation by NKT cells. *Nat. Immun. 1:521.*
41. Wang, Y., I. Goldschneider, D. Foss, D. Y. Wu, J. O'Rourke, and R. E. Cone. 1997. Direct thymic involvement in anterior chamber-associated immune deviation: evidence for a nondeletional mechanism of centrally induced tolerance to extrathymic antigens in adult mice. *J. Immunol. 158:2150.*
42. Terabe, M., S. Matsui, N. Noben-Trauth, H. Chen, C. Watson, D. D. Donaldson, D. P. Carbone, W. E. Paul, and J. A. Berzofsky. 2000. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat. Immun. 1:515.*
43. Takeda, K., Y. Hayakawa, M. Atsuta, S. Hong, L. Van Kaer, K. Kobayashi, M. Ito, H. Yagita, and K. Okumura. 2000. Relative contribution of NK and NKT cells to the anti-metastatic activities of IL-12. *Int. Immunol. 12:909.*
44. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature 408:740.*
45. Sfondrini, L., M. Rodolfo, M. Singh, M. P. Colombo, M. I. Colnaghi, S. Ménard, and A. Balsari. 2000. Cooperative effects of *Mycobacterium tuberculosis* Ag38 gene transduction and IL-12 in vaccination against spontaneous tumor development in proto-neu-transgenic mice. *Cancer Res. 60:3777.*
46. Eberl, G., R. Lees, S. T. Smiley, M. Taniguchi, M. J. Grusby, and H. R. MacDonald. 1999. Tissue-specific segregation of CD1d-dependent and CD1d-independent NK T cells. *J. Immunol. 162:6410.*
47. Taniguchi, M., H. Koseki, T. Tokuhisa, K. Masuda, H. Sato, E. Kondo, T. Kawano, J. Cui, A. Perkes, S. Koyasu, and Y. Makino. 1996. Essential requirement of an invariant Vα14 T cell antigen receptor expression in the development of natural killer T cells. *Proc. Natl. Acad. Sci. USA 93:11025.*
48. Eberl, G., and H. R. MacDonald. 1998. Rapid death and regeneration of NKT cells in anti-CD3ϵ- or IL-12-treated mice: a major role for bone marrow in NKT cell homeostasis. *Immunity 9:345.*
49. Krieg, A. M. 2000. Immunotherapy with CpG motifs from bacterial DNA. In *Conference on Cancer Vaccines*. Cancer Research Institute, New York, p. S07.
50. Yoshimoto, T., and W. E. Paul. 1994. CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. *J. Exp. Med. 179:1285.*
51. Ouyang, W., M. Lohning, Z. Gao, M. Assenmacher, S. Ranganath, A. Radbruch, and K. M. Murphy. 2000. Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. *Immunity 12:27.*
52. Warren, T. L., S. K. Bhatia, A. M. Acosta, C. E. Dahle, T. L. Ratliff, A. M. Krieg, and G. J. Weiner. 2000. APC stimulated by CpG oligodeoxynucleotide enhance activation of MHC class I-restricted T cells. *J. Immunol. 165:6244.*
53. Davis, H. L. 2000. Use of CpG DNA for enhancing specific immune responses. *Curr. Top. Microbiol. Immunol. 247:171.*
54. Monteith, D. K., M. J. Horner, N. A. Gillett, M. Butler, R. Geary, T. Burckin, T. Ushiro-Watanabe, and A. A. Levin. 1999. Evaluation of the renal effects of an antisense phosphorothioate oligodeoxynucleotide in monkeys. *Toxicol. Pathol. 27:307.*
55. Godfrey, D. I., K. J. Hammond, L. D. Poulton, M. J. Smyth, and A. G. Baxter. 2000. NKT cells: facts, functions and fallacies. *Immunol. Today 21:573.*
56. Dao, T., M. Exley, W. Z. Mehal, S. M. Tahir, S. Snapper, M. Taniguchi, S. P. Balk, and I. N. Crispe. 2001. Involvement of CD1 in peripheral deletion of T lymphocytes is independent of NK T cells. *J. Immunol. 166:3090.*
57. Chang, C. S., L. Brossay, M. Kronenberg, and K. P. Kane. 1999. The murine nonclassical class I major histocompatibility complex-like CD1.1 molecule protects target cells from lymphokine-activated killer cell cytolysis. *J. Exp. Med. 189:483.*
58. Wang, B., T. Chun, and C.-R. Wang. 2000. Comparative contribution of CD1 on the development of $CD4^+$ and $CD8^+$ T cell compartments. *J. Immunol. 164:739.*
59. Lee, D. J., A. Abeyratne, D. A. Carson, and M. Corr. 1998. Induction of an antigen-specific, CD1-restricted cytotoxic T lymphocyte response in vivo. *J. Exp. Med. 187:433.*
60. Behar, S. M., T. A. Podrebarac, C. J. Roy, C. R. Wang, and M. B. Brenner. 1999. Diverse TCRs recognize murine CD1. *J. Immunol. 162:161.*
61. Roark, J. H., S. H. Park, J. Jayawardena, U. Kavita, M. Shannon, and A. Bendelac. 1998. CD1.1 expression by mouse antigen-presenting cells and marginal zone B cells. *J. Immunol. 160:3121.*
62. Seddon, B., and D. Mason. 1999. Peripheral autoantigen induces regulatory T cells that prevent autoimmunity. *J. Exp. Med. 189:877.*
63. Roncarolo, M. G., and M. K. Levings. 2000. The role of different subsets of T regulatory cells in controlling autoimmunity. *Curr. Opin. Immunol. 12:676.*
64. Sakaguchi, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell 101:455.*