Induction of Tumor-specific T Cell Immunity by Anti–DR5 Antibody Therapy

Kazuyoshi Takeda,[1] Noriko Yamaguchi,[1] Hisaya Akiba,[1] Yuko Kojima,[2] Yoshihiro Hayakawa,[3] Jane E. Tanner,[3] Thomas J. Sayers,[4] Naoko Seki,[5] Ko Okumura,[1] Hideo Yagita,[1] and Mark J. Smyth[3]

[1]Department of Immunology and [2]Division of Pathology, Central Laboratory of Medical Science, Juntendo University School of Medicine, Tokyo 113-8421, Japan
[3]Cancer Immunology Program, Peter MacCallum Cancer Centre, 8006 Victoria, Australia
[4]Basic Research Program, SAIC-Frederick Inc., and [5]Laboratory of Experimental Immunology, National Cancer Institute Frederick, Frederick, MD 21702

Abstract

Because tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) preferentially induces apoptosis in tumor cells and plays a critical role in tumor surveillance, its receptor is an attractive target for antibody-mediated tumor therapy. Here we report that a monoclonal antibody (mAb) against the mouse TRAIL receptor, DR5, exhibited potent antitumor effects against TRAIL-sensitive tumor cells in vivo by recruiting Fc receptor–expressing innate immune cells, with no apparent systemic toxicity. Administration of the agonistic anti–DR5 mAb also significantly inhibited experimental and spontaneous tumor metastases. Notably, the anti–DR5 mAb-mediated tumor rejection by innate immune cells efficiently evoked tumor-specific T cell immunity that could also eradicate TRAIL-resistant variants. These results suggested that the antibody-based therapy targeting DR5 is an efficient strategy not only to eliminate TRAIL-sensitive tumor cells, but also to induce tumor-specific T cell memory that affords a long-term protection from tumor recurrence.

Key words: apoptosis • macrophage • NK cells • CTL • TRAIL

Introduction

TNF–related apoptosis–inducing ligand (TRAIL) is a type II transmembrane protein belonging to the TNF family, which preferentially induces apoptotic cell death in a wide variety of transformed cells but not in normal cells (1–3). We have recently demonstrated a substantial role for TRAIL in tumor immune surveillance as a natural suppressor of metastasis in the liver and chemically induced or spontaneous tumor development (4–6). Moreover, we also found that IFN-γ–mediated TRAIL induction on NK cells played a critical role in the antimetastatic effects of IL-12 and α-galactosylceramide (7). Thus, endogenously expressed TRAIL appears to be a key effector molecule for the immune surveillance and immunotherapy of tumors (8, 9).

Preclinical studies in mice and nonhuman primates have shown that the administration of recombinant soluble forms of TRAIL suppressed the growth of TRAIL-sensitive human tumor xenografts, with no apparent systemic toxicity (10–12). This supported the potential utility of recombinant TRAIL as a cancer therapeutic. An agonistic mAb specific for death-inducing TRAIL receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5 in humans and mTRAIL-R2/DR5 in mice) might be more effective for tumor eradication because the decoy receptors (TRAIL-R3/DcR1 and TRAIL-R4/DcR2 in humans and mDcTRAILR1 and mDcTRAILR2 in mice) have been proposed as the key modulators of TRAIL-mediated cytotoxicity (13, 14). However, anti–DR4 or anti–DR5 mAbs might be more toxic because the decoy receptors have also been implicated in protection of normal cells from TRAIL. It has recently been reported that agonistic anti–human DR4 or

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DR5 mAbs exhibited potent tumoricidal activities against human tumor xenografts in nude or SCID mice (15, 16). However, these studies could not address the potential toxicity of anti–DR4 and anti–DR5 mAbs in vivo or their effects on host immunity because the mAbs were not reactive with mouse DR5 on normal tissues and the therapy was performed in immunodeficient mice. In this study, we have examined the antitumor effects of an anti–mouse DR5 mAb, MD5-1, against syngeneic tumor growth and metastasis in immunocompetent mice. This mAb not only inhibited the growth of TRAIL-sensitive tumor cells in vivo without toxicity, but also primed tumor-specific T cells that could eradicate TRAIL-resistant variants. The critical contribution of FcR–bearing innate immune cells to these processes was revealed.

Materials and Methods

Mice. BALB/c and CB17-scid (SCID) mice at 6 wk of age were from Charles River Japan Inc. and The Walter and Eliza Hall Institute of Medical Research. IFN-γ–deficient (IFN-γ$^{-/-}$), perforin-deficient (perforin$^{-/-}$), or TRAIL-deficient (TRAIL$^{-/-}$) BALB/c mice and RAG-2–deficient (RAG-2$^{-/-}$) C57BL/6 mice were derived as previously described (4–7). FcR common γ chain–deficient (FcRγ$^{-/-}$) or FcγRIIB-deficient (FcγRIIB$^{-/-}$) BALB/c mice were from Taconic (17, 18). All mice were maintained under specific pathogen-free conditions and used in accordance with the institutional guidelines of Juntendo University and Peter MacCallum Cancer Centre. Animal care was provided in accordance with the procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Antibodies. Anti–mouse CD16/32 (FcR) mAb (2.4G2), anti–mouse CD11b mAb (5C6), control hamster Ig (UC8-1B9), anti–mouse CD4 mAb (GK1.5), anti–mouse CD8 mAb (53-6.7), anti–mouse FasL mAb (MFL3), and anti–mouse TRAIL mAb (N2B2) were prepared in our laboratory as previously described (4, 19). Biotinylated anti–mouse F4/80 mAb (A3-1) was purchased from Caltag, biotinylated anti–mouse CD205 mAb (NLDC-145) was purchased from Cedarlane Laboratories, and biotinylated rat IgG2aκ (R35-95) and rat IgG 2bκ (A95-1) were purchased from BD Biosciences. A hamster IgG2 anti–mouse DR5 mAb (MD5-1) was generated by immunizing an Armenian hamster with mouse DR5-Ig fusion protein (DakoCytomation), fusing the splenocytes with P3U1 mouse myeloma cells, and then screening the reactivity to mouse DR5-transfected BHK (BHK-mDR5) cells by flow cytometry. MD5-1 was purified from ascites using a protein G column. Biotinylation was performed as previously described (20). Requests for MD5-1 should be addressed to H. Yagita (hyagita@med.juntendo.ac.jp).

Tumor Cell Lines. TRAIL-sensitive 4T1 mammary carcinoma, TRAIL-resistant FcγRIIB-expressing A20 B lymphoma and P815 mastocytoma, and TRAIL-resistant L5178Y T lymphoma have been described (4, 6, 18, 21). TRAIL-sensitive R331-mock renal carcinoma and TRAIL-resistant R331–FLICE inhibitory protein (FLIP) transfectants were prepared as previously described (22). Mouse Fas- or mouse Fn14-transfected L5178Y cells were prepared as previously described (23).

Flow Cytometric Analysis. Immunofluorescent staining with biotinylated mAb and flow cytometric analyses were performed as previously described (4).

Cytotoxicity Assay. Cytotoxic activity was tested by a 4-h ^{51}Cr release assay as previously described (4, 5). Susceptibility of tumor cells to TRAIL-mediated cytotoxicity was examined using murine TRAIL-transfected 2PK-3 (mTRAIL/2PK-3) or mock-transfected 2PK-3 (mock/2PK-3) as the effector cells (4, 5). Susceptibility to MD5-1 cytotoxicity was tested in the presence of FcR-expressing P815 cells, NK cells, or macrophages. Splenic NK cells and peritoneal exudate macrophages were prepared from RAG-2$^{-/-}$ or FcRγ$^{-/-}$ mice as previously described (4, 24). In some experiments, the cytotoxicity assay was performed in the presence of 10 μg/ml anti-FcR mAb, 10 μg/ml z-VAD-fmk (Peptide Institute), or 5 nM concanamycin A (CMA; Wako Pure Chemicals). In some experiments, the indicated amount of biotinylated MD5-1 was cross-linked with 5 μg/ml streptavidin (Wako Pure Chemicals) or unlabeled MD5-1 was cross-linked with effector cells fixed with 1% paraformaldehyde (25). Data are represented as the mean ± SE of triplicate samples.

Complement-mediated Lysis. Complement-mediated lysis with rabbit serum (Low-Tox M; Cedarlane Laboratories) or mice serum was performed as previously described (26). As a positive control, freshly isolated splenocytes were lysed after incubation with anti-Thy1.2 mAb (J1j).

Histological Examination. Hematoxylin/eosin (H/E) staining of paraffin-embedded liver and thymus sections was performed as previously described (27). For immunohistochemistry, 3-μm cryostat sections were air dried and fixed with acetone for 10 min at 4°C. Endogenous biotin was blocked by Avidin/Biotin Blocking Kit (Vector Laboratories). The sections were then incubated with an appropriate concentration of primary antibody followed by detection using the avidin-biotinylated peroxidase complex method (ABC; Vector Laboratories). CD205 (DEC205) was detected by further amplification with biotinylated tyramide (PerkinElmer) according to the manufacturer's instructions.

Serum Transaminases. Naive BALB/c mice were i.p. injected with MD5-1 or control Ig (300 μg/mouse) three times at 3-d intervals, and then the sera were collected from individual mice 2, 8, 20, 48, and 72 h after the last injection. Serum transaminase (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) activities were measured by the standard photometric method using Hitachi type 7350 automatic analyzer.

Subcutaneous Tumor Growth. Mice were inoculated with the indicated number of 4T1 cells or R331-mock cells s.c. in the hind frank, and then administered i.p. with 200 μg MD5-1 or control Ig on days 0, 4, and 8. Some groups of mice were also treated i.p. with 300 μg anti-CD11b mAb and/or 50 μg anti-asialo GM1 (ASGM1) Ab (Wako Pure Chemicals) on days −1, 4, 9, and 13. In some experiments, mice were inoculated with 2 × 10⁴ 4T1 cells, and then 200 μg MD5-1 were i.p. injected five times at 3-d intervals starting at day 15 or 25. Tumor size was measured periodically as previously described (5). Data are represented as the mean ± SE of five mice in each group.

Assessment of Tumor-specific T Cell Immunity. Wild-type BALB/c or SCID mice were inoculated with the indicated number of 4T1 cells in the mammary gland and administered i.p. with 200 μg MD5-1 or control Ig on days 0, 4, and 8. The MD5-1–treated tumor-free mice were then rechallenged with 5 × 10³ 4T1 or R331-mock cells s.c. in the opposite flank on day 42. Some groups of these mice were administered i.p. with 100 μg anti-CD4 and/or anti-CD8 mAb on 0, 4, 7, and 11 d after the rechallenge. Effective depletion of CD4$^+$ and/or CD8$^+$ T cells was verified by flow cytometry. As a control, naive mice were challenged with the same number of 4T1 or R331-mock cells. As

another control, naive mice were inoculated s.c. with 5×10^5 irradiated (20,000 rad) 4T1 cells resuspended in serum-free RPMI 1640 medium after preincubation with 10 µg/ml MD5-1 or control Ig and rechallenged with 5×10^3 or 2×10^5 live 4T1 cells on day 42. In adoptive transfer experiments, 5×10^7 nylon wool–purified T cells from the spleens of naive mice or the mice that had rejected 4T1 after MD5-1 treatment were i.v. injected into naive SCID mice 2 d after 4T1 (5×10^3) inoculation into mammary fat pad. Data are represented as the mean $\pm$ SE of five to seven mice in each group.

In another set of experiments, wild-type or perforin$^{-/-}$ BALB/c mice were s.c. inoculated with 10^4 R331-mock or R331-FLIP cells and i.p. administered with 200 µg MD5-1 or control Ig on days 0, 4, and 8. As a control, naive mice were inoculated with 5×10^5 irradiated (20,000 rad) R331-mock cells and rechallenged with 10^4 live R331-mock cells on day 42. The mice that had rejected R331-mock cells after MD5-1 treatment were then rechallenged with 10^4 R331-mock or R331-FLIP cells s.c. in the opposite flank on day 35. Some groups were depleted of CD8$^+$ and/or CD4$^+$ T cells as described above or i.p. administered with 300 µg anti-FasL mAb and/or anti-TRAIL mAb on 0, 1, and 7 d after the rechallenge. To determine the kinetics of T cell priming, mice were inoculated with 10^4 R331-mock cells followed by 200 µg MD5-1 on days 0, 4, and 8, and then 10^4 R331-FLIP cells were inoculated into the opposite flank on days 0, 7, 14, or 21. To determine the strength of T cell–mediated rejection, the indicated numbers of R331-mock or R331-FLIP cells were inoculated into mice 48 d after they had rejected R331-mock cells after MD5-1 treatment. Naive or irradiated R331-mock–inoculated mice were similarly challenged as the controls.

CTL Assay. BALB/c mice were immunized with 10^5 irradiated (20,000 rad) 4T1 cells preincubated with 10 µg/ml control Ig or MD5-1, or by rejecting 5×10^3 live 4T1 cells after treatment with MD5-1. 8 wk after immunization, the mice were challenged with 5×10^3 4T1 cells and then 6 d later splenocytes were prepared from the immunized and naive mice. These splenocytes were cultured with irradiated 4T1 cells and 5 U/ml of human IL-2 (BD Biosciences) for 7 d. CTL activity was analyzed by a 4-h ^{51}Cr release assay against 4T1 or R331-mock cells.

*Statistical Analysis.* Statistical analysis was performed by two-sample t test for the cytotoxicity, metastasis, and tumor growth data. Significant differences in tumor incidence at one time point were determined by the Fisher's exact test. P-values <0.05 were considered significant.

Online Supplemental Material. Fig. S1 shows the antimetastatic effect of MD5-1 against spontaneous metastasis of 4T1 cells and experimental metastasis of R331 cells. Metastasis experiments were performed as previously described (6, 22, 28) and procedures are detailed in the Supplemental Materials and Methods. Supplemental Materials and Methods and Fig. S1 are available at http://www.jem.org/cgi/content/full/jem.20031457/DC1.

Results

Cytotoxic Activity of MD5-1 In Vitro. We generated an anti–mouse DR5 mAb, MD5-1, by immunizing a hamster with mouse DR5-Ig fusion protein and screening mAb that bound to mouse DR5-transfected BHK cells, but not to mock, mouse Fas, or mouse Fn14 transfectants (Fig. 1 A). Among over 30 different types of mouse tumor cell lines, all TRAIL-sensitive tumor cells were reactive with MD5-1, as represented by 4T1 mammary carcinoma and R331 renal carcinoma (Fig. 1 A). Conversely, all MD5-1$^-$ tumor cells, such as L5178Y (Fig. 1 A), were TRAIL resistant. MD5-1 specifically precipitated a 44-kD protein from TRAIL-sensitive tumor cell lines, which was also detected by commercially available polyclonal Ab against mouse DR5 (unpublished data). These results indicated that MD5-1 was specific for mouse DR5.

Biotinylated MD5-1 exhibited a substantial cytotoxic activity against 4T1 cells only when cross-linked by streptavidin (Fig. 1 B) or when MD5-1 was immobilized or cross-linked by anti–hamster Ig mAb (unpublished data). MD5-1 also exhibited a high cytotoxic activity against 4T1 cells when cross-linked by FcR on P815 cells, which was abolished by anti-FcR mAb (2.4G2; Fig. 1 C). By contrast, MD5-1 did not inhibit the cytotoxic activity of mouse TRAIL (Fig. 1 D). These results indicated that MD5-1 acted as a death-inducing agonist when it was cross-linked.
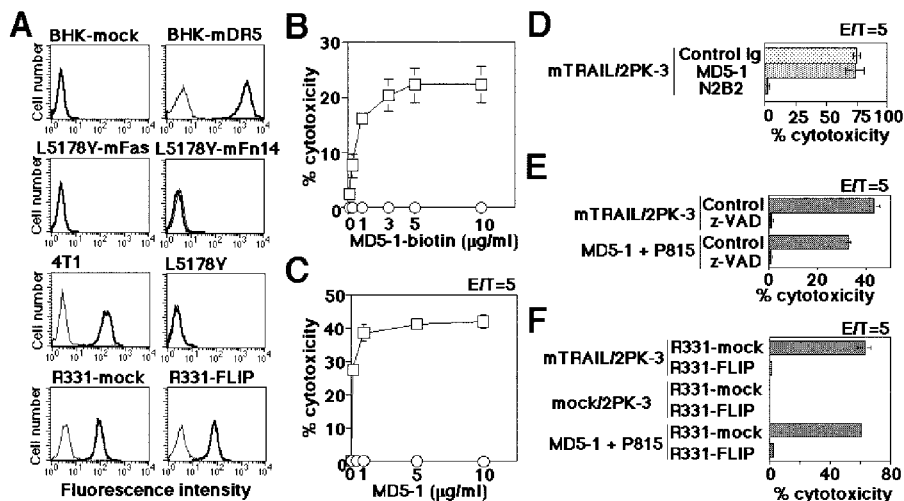
Figure 1. Characterization of MD5-1 in vitro. (A) Reactivity with mouse DR5, Fas, and Fn14 transfectants and mouse tumor cell lines. The thick histograms represent MD5-1 staining and the thin histograms represent control Ig staining. (B) Cytotoxic activity of biotinylated MD5-1 against 4T1 cells in the presence (□) or absence (○) of streptavidin. (C) Cytotoxic activity of MD5-1 against 4T1 cells in the presence of P815 cells with (○) or without (□) anti-FcR mAb (2.4G2). (D) Cytotoxic activity of mTRAIL/2PK-3 against 4T1 cells in the presence of MD5-1 or anti-TRAIL mAb (N2B2). (E) Effect of z-VAD-fmk on MD5-1–mediated cytotoxicity against 4T1 tumor cells. (F) Cytotoxic activity of MD5-1 and mTRAIL/2PK3 against R331-mock and R331-FLIP cells. Similar results were obtained in two or three independent experiments.
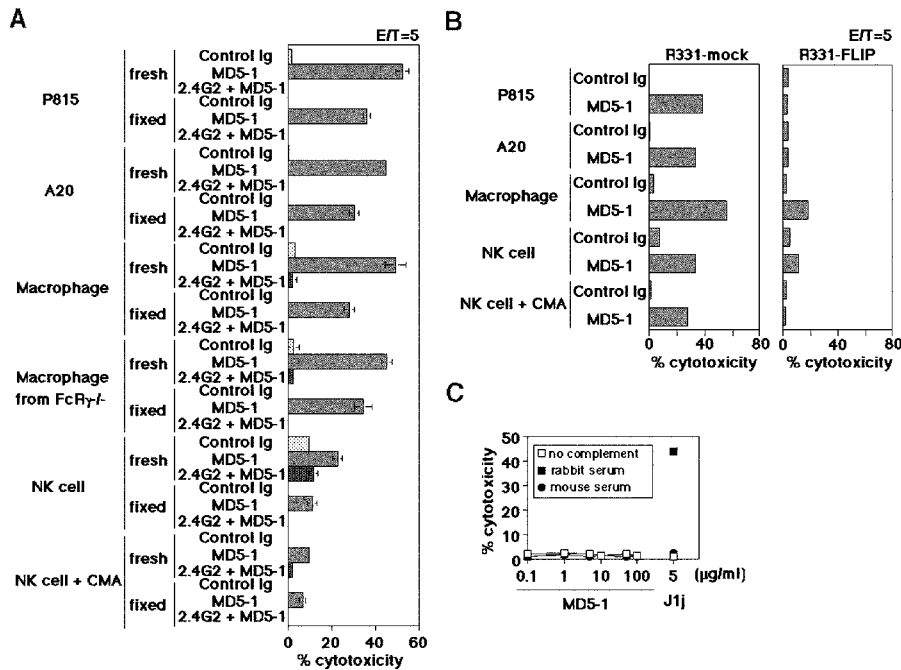
Figure 2. Cytotoxic activity of MD5-1 in the presence of FcR–bearing cells. (A) FcR–mediated cytotoxic activity of MD5-1 against 4T1 tumor cells in the presence of fresh or fixed effector cells. CMA was added to inhibit perforin-dependent cytotoxicity and 2.4G2 was used to block FcγRII and FcγRIII. (B) Cytotoxic activity of MD5-1 against R331-mock and R331-FLIP tumor cells in the presence of various effector cells. (C) Cytotoxic activity of MD5-1 against 4T1 tumor cells in the presence of rabbit or mouse complement.

We also examined the caspase dependence of MD5-1–mediated cytotoxicity. The cytotoxic activity of MD5-1 induced by FcR–expressing P815 cells against 4T1 cells was completely abrogated by the pan-caspase inhibitor z-VAD-fmk, as was the TRAIL-mediated cytotoxicity (Fig. 1 E). Moreover, R331 cells transfected with FLIP (R331-FIP), which inhibits caspase 8–mediated death receptor signaling (29, 30), were resistant to both TRAIL- and MD5-1–mediated cytotoxicity (Fig. 1 F), despite a similar level of DR5 expression to mock-transfected R331 (R331-mock) cells (Fig. 1 A). Likewise, amongst 30 tumor cell lines tested in vitro, susceptibility to MD5-1 cytotoxicity was strictly correlated with TRAIL sensitivity (unpublished data).

Next, we examined the ability of FcR–expressing effector cells including fresh macrophages and NK cells to mediate MD5-1 cytotoxicity in vitro because these innate immune cells are well-known mediators of antibody-dependent cellular cytotoxicity (ADCC) in vivo (31, 32). Macrophages induced MD5-1 cytotoxicity against 4T1 cells more effectively than NK cells, and both were inhibited by anti-FcR mAb (Fig. 2 A). MD5-1 cytotoxicity was also induced by NK cells in the presence or absence of a perforin inhibitor, CMA, suggesting that MD5-1 did not trigger the perforin-dependent ADCC by NK cells in vitro (Fig. 2 A). Consistent with this notion, macrophages and NK cells from perforin$^{-/-}$ mice or FasL mutant *gld* mice induced MD5-1 cytotoxicity against 4T1 cells at a comparable level to that induced by wild-type cells (unpublished data). Importantly, A20 cells that only express FcγRII, macrophages isolated from FcRγ$^{-/-}$ mice, and paraformaldehyde-fixed FcR$^+$ cells, all triggered MD5-1–mediated cytotoxicity, confirming that cross-linking was sufficient to induce DR5- and caspase-mediated cell death (Fig. 2, A and B). However, live (fresh) effector cells al-

ways induced higher levels of MD5-1 cytotoxicity than those observed with fixed effector cells, suggesting some contribution of ADCC, especially by macrophages (Fig. 2 A). All effector cells also triggered cytotoxicity against R331-mock cells in the presence of MD5-1, however, R331-FLIP cells were rather refractory (Fig. 2 B). Some residual MD5-1 cytotoxicity against R331-FLIP cells in the presence of macrophages or NK cells suggested that these effector cells were capable of mediating a small part of MD5-1–dependent cell death independently of caspase activation. Collectively, these results indicated that MD5-1 was capable of triggering FcR-dependent cytolysis of tumor cells in vitro by cross-linking DR5 and FcR (even FcγRII), inducing both DR5-mediated caspase-dependent cell death and caspase-independent ADCC. Notably, MD5-1 did not kill tumor cells in the presence of rabbit or mouse complement (Fig. 2 C). The fact that macrophages and NK cells effectively mediated MD5-1 cytotoxicity via their FcR implied a possible cytotoxic effect of MD5-1 in vivo.

No Apparent Toxicity of MD5-1 In Vivo. Previous studies have suggested a hepatotoxicity of some forms of TRAIL in vitro and in vivo (16, 33). However, significantly elevated serum AST or ALT levels were not observed in BALB/c mice after repeated MD5-1 administration (Fig. 3 A), nor was there obvious histological evidence of tissue pathology in the liver and thymus (Fig. 3 B) as well as other organs including the kidney, brain, lungs, intestine, and skin. Moreover, the MD5-1–treated mice did not show any sign of systemic toxicity as estimated by body weight, gross appearance, or behavior (unpublished data).

Antimetastatic Effect of MD5-1. Spontaneous metastasis of 4T1 mouse mammary carcinoma inoculated into the mammary gland is the most clinically relevant model for
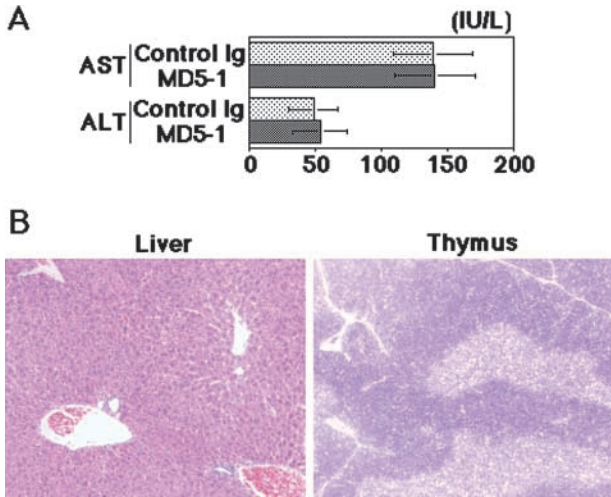
Figure 3. No apparent toxicity of MD5-1 in vivo. (A) Serum AST and ALT levels 20 h after MD5-1 injection. Data are represented as the mean ± SE of 10 mice. Similar results were obtained in two independent experiments at several time points (not depicted). (B) Histological examination (H/E) of liver (×10) and thymus (×5) from MD5-1–treated mice.

evaluating antimetastatic immunotherapies (28). After resection of the primary tumor, mice were i.p. administered with MD5-1 to estimate the antimetastatic effect. Administration of MD5-1 markedly inhibited spontaneous lung and liver metastases as compared with control Ig, and thereby significantly prolonged the survival (Fig. S1, A and B, available at http://www.jem.org/cgi/content/full/jem.20031457/DC1). It should be noted that at the time of primary tumor resection mice harbored a massive 4T1

metastatic burden in the lungs (>50,000 nodules) and livers (>2,000 nodules). A comparable effect was also observed in TRAIL$^{-/-}$ and perforin$^{-/-}$ mice, indicating that the antimetastatic effect of MD5-1 was independent of endogenous TRAIL- or perforin-mediated cytotoxicity.

We also examined the effect of MD5-1 on the experimental metastasis of R331-mock and R331-FLIP tumors. As we recently reported, R331-FLIP exhibited an enhanced liver metastasis in the control Ig–treated mice compared with R331-mock (22). MD5-1 administration efficiently inhibited both lung and liver metastases of R331-mock, but not R331-FLIP (Fig. S1 C, available at http://www.jem.org/cgi/content/full/jem.20031457/DC1). These results were consistent with the susceptibility of R331-mock, but not R331-FLIP, to MD5-1 cytotoxicity in vitro (Fig. 1 F), suggesting that the antimetastatic effect of MD5-1 was primarily mediated by caspase-dependent death signaling via DR5, rather than classical ADCC or complement-mediated lysis.

Contribution of Macrophages and NK Cells to the Tumoricidal Effects of MD5-1. Administration of MD5-1 substantially inhibited s.c. growth of 4T1 tumors in both SCID (Fig. 4 A) and wild-type BALB/c (Fig. 4 B) mice. Depletion of NK cells by the anti-ASGM1 Ab treatment, but not administration of anti-CD11b mAb (5C6) that inhibits the recruitment of macrophages (34), significantly enhanced 4T1 tumor growth in the control Ig–treated mice, indicating a substantial contribution of NK cells, but not macrophages, to the natural suppression of 4T1 tumor growth. In contrast, the tumoricidal effect of MD5-1 was mostly abrogated by anti-CD11b treatment, but only weakly by NK cell depletion. In the case of R331-mock cells, NK cell de-
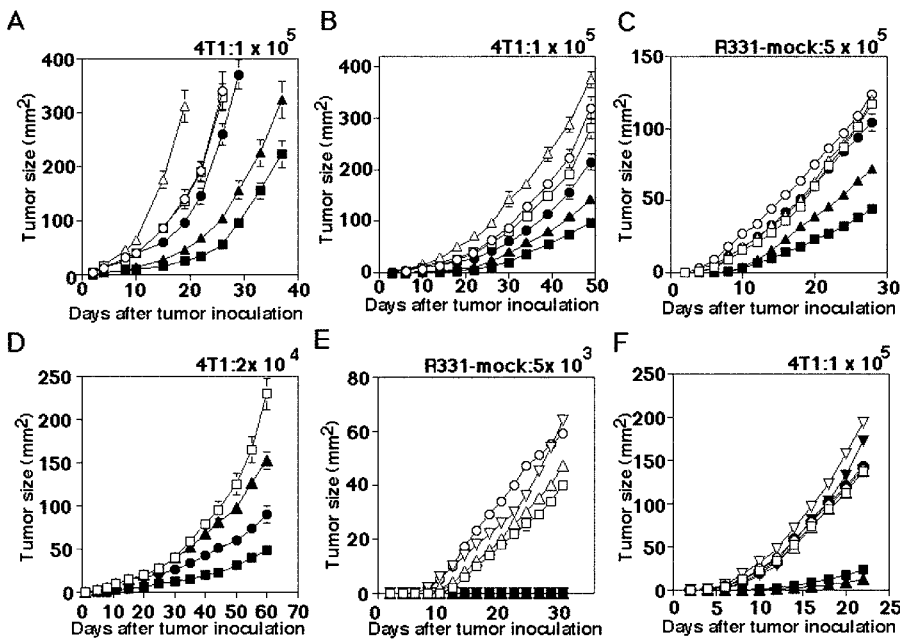
Figure 4. Contribution of macrophages and NK cells to tumoricidal effect of MD5-1. SCID mice (A) or wild-type BALB/c mice (B) were s.c. inoculated with 4T1 cells, and wild-type BALB/c mice (C) were s.c. inoculated with R331-mock cells. The mice were then treated with MD5-1 (solid symbols) or control Ig (open symbols). Some groups of mice were treated with anti-ASGM1 Ab (triangles), anti-CD11b mAb (circles), or control Igs (squares). (D) Wild-type BALB/c mice were s.c. inoculated with 4T1 cells. The mice were then treated with MD5-1 from day 0 (■), 15 (●), or 25 (▲). One group of mice was treated with control Ig from day 0 (□) as the control. (E) Wild-type (squares), TRAIL$^{-/-}$ (circles), perforin$^{-/-}$ (triangles), and IFN-γ$^{-/-}$ (upside down triangles) BALB/c mice were s.c. inoculated with R331-mock cells. The mice were then treated with MD5-1 (solid symbols) or control Ig (open symbols). (F) Wild-type (squares), FcRγ$^{-/-}$ (circles), or FcγRII$^{-/-}$ (triangles) BALB/c mice were s.c. inoculated with 4T1 cells. The mice were then treated with MD5-1 (solid symbols) or control Ig (open symbols). Some groups of wild-type mice were treated with both anti-ASGM1 Ab and anti-CD11b mAb (upside down triangles). All data are represented as the mean ± SE of 5–10 mice. Similar results were obtained in two or three independent experiments.

441   Takeda et al.
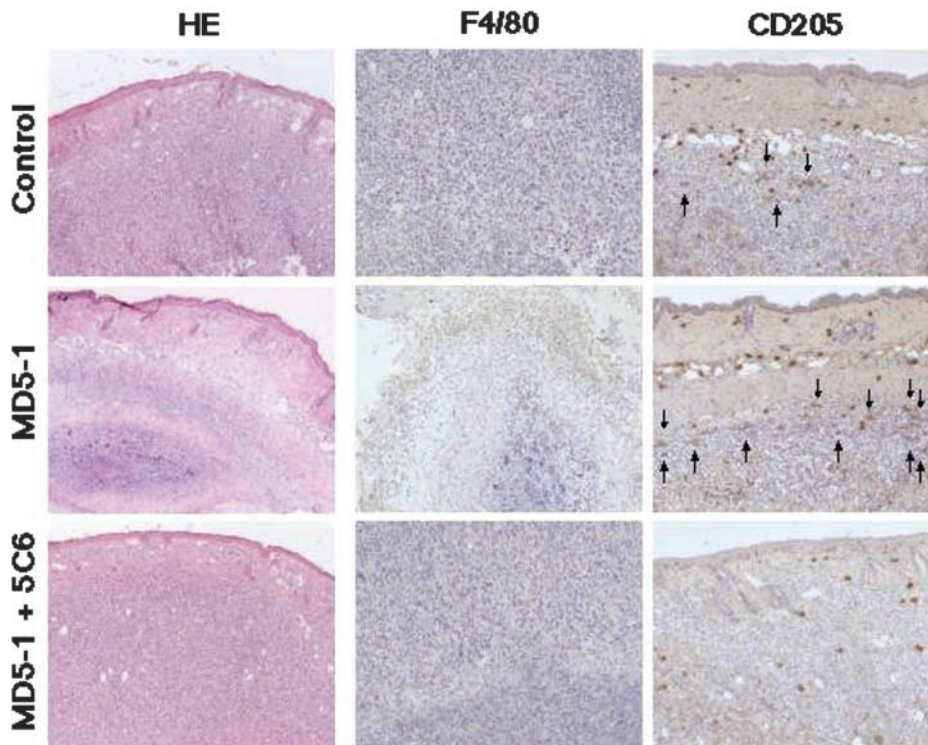
Figure 5. MD5-1 induces infiltration of macrophages and DCs in tumor site. Tumor was removed 8 d after tumor inoculation from control Ig–, MD5-1–, or MD5-1– and anti-CD11b mAb (5C6)–treated mice, and then stained with H/E (×5), anti-F4/80 mAb (×10), or anti-CD205 mAb (×10). The marginal region of s.c. tumor is shown. A massive infiltration of F4/80^{+} macrophages (stained brown) was observed in the marginal region of tumor from MD5-1–treated mice. The arrows are indicated to distinguish CD205^{+} DCs from nonspecific staining of mast cells. Representative of five tumors in each group.

pletion did not affect the natural protection, but significantly impaired the tumoricidal effect of MD5-1 (Fig. 4 C). Again, the anti-CD11b treatment mostly abrogated the effect of MD5-1. We also examined the effect of MD5-1 against established tumors. The MD5-1 treatment commencing on day 15 (tumor size was ~10 mm²) or 25 (tumor size ~35 mm²) still significantly inhibited the growth of 4T1, although the MD5-1 treatment starting on day 0 was most effective (Fig. 4 D).

To further explore the effector mechanisms, we examined the antitumor effect of MD5-1 in perforin$^{-/-}$, TRAIL$^{-/-}$, IFN-$\gamma^{-/-}$, FcR$\gamma^{-/-}$, and FcγRIIB$^{-/-}$ mice. In the perforin$^{-/-}$, TRAIL$^{-/-}$, or IFN-$\gamma^{-/-}$ mice, the antitumor effect of MD5-1 was not impaired (Fig. 4 E). Similarly, in the FcγRIIB$^{-/-}$ mice, MD5-1 exerted an antitumor effect equivalent to that in wild-type mice. By contrast, the antitumor effect of MD5-1 was abolished in the FcR$\gamma^{-/-}$ mice (mice that lack FcγR1 and FcγRIII), just as in the anti-ASGM1 Ab and anti-CD11b mAb–treated wild-type mice (Fig. 4 F).

To confirm the recruitment of macrophages in tumor rejection, we examined the tumor sections by immunohistochemistry. Significantly larger numbers of infiltrating CD11b$^+$ mononuclear cells (unpublished data), including F4/80$^+$ macrophages and CD205$^+$ DCs, were observed at the tumor edge in the MD5-1–treated mice as compared with the control Ig–treated mice (Fig. 5). The MD5-1–induced macrophage and DC infiltration into tumor was almost completely inhibited by the anti-CD11b mAb (5C6) treatment (Fig. 5). These results suggested that the antitumor effect of MD5-1 was mainly mediated by the FcR on

macrophages, but did not depend on endogenous perforin, TRAIL, or IFN-γ.

Induction of Tumor-specific T Cell Immunity by MD5-1–mediated Tumor Rejection. Given the accumulation of APCs such as macrophages and DCs in the tumor, we next examined whether eradication of primary tumors by the MD5-1 treatment could induce tumor-specific T cell immunity. MD5-1 administration afforded complete rejection of low doses of 4T1 cells in wild-type BALB/c mice (Fig. 6 A, left). When rechallenged with 4T1 or R331-mock cells on the opposite flank, these mice rejected 4T1 cells (Fig. 6 A, middle), but not R331-mock cells (Fig. 6 A, right). Depletion of either CD8$^+$ T cells or CD4$^+$ T cells abrogated the secondary rejection, although some suppression of tumor growth was still observed in the CD4$^+$ T cell–depleted mice (Fig. 6 A, middle). In contrast, BALB/c mice preimmunized with the same number of irradiated 4T1 cells did not reject the secondary challenge with live 4T1 cells (unpublished data).

MD5-1 administration afforded complete rejection of low doses of 4T1 cells in SCID mice as well as in wild-type BALB/c mice (Fig. 6 B, left), confirming the innate immune cell-mediated primary rejection. However, these SCID mice were no more capable of resisting the secondary 4T1 tumor challenge than naive SCID mice (Fig. 6 B, middle). Adoptive transfer of splenic T cells isolated from wild-type BALB/c mice that had rejected cells after MD5-1 treatment, but not those isolated from naive BALB/c mice, protected SCID mice from a lethal dose of 4T1 tumor challenge (Fig. 6 B, right). These data indicated that memory T cells reactive with 4T1 had developed during the MD5-1–mediated primary tumor rejection.
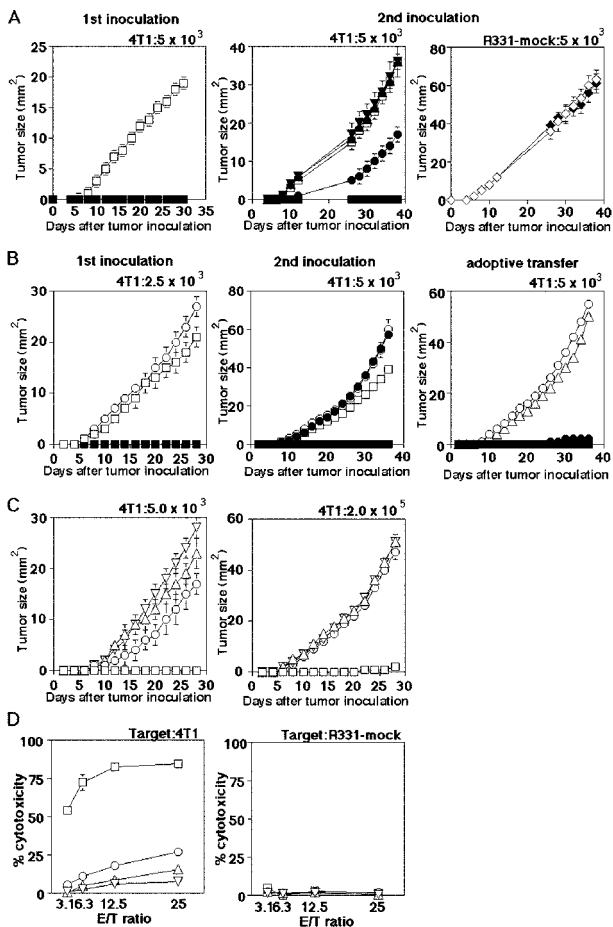
Figure 6. Induction of tumor-specific T cell memory by MD5-1–mediated tumor rejection. (A) BALB/c mice were inoculated with 4T1 cells and then treated with MD5-1 mAb (■; $n = 25$) or control Ig (□; $n = 5$) (left). Simultaneously to secondary challenge with 4T1 cells, some mice were treated with anti-CD4 mAb (●), anti-CD8 mAb (▲), anti-CD4 and anti-CD8 mAbs (▼), or control Ig (■) (middle). Naive mice were inoculated with 4T1 cells as the control (□). Irrelevant R331-mock cells were inoculated into 4T1-rejecting mice (◆) or naive mice (◇) (right). (B) BALB/c (squares) and SCID (circles) mice were inoculated with 4T1 cells and then treated with MD5-1 mAb (solid symbols; $n = 25$) or control Ig (open symbols; $n = 5$) (left). 5 wk after the first inoculation, the MD5-1–treated BALB/c (■) and SCID (●) mice that had rejected 4T1 were secondarily inoculated with 4T1 cells (middle). Naive BALB/c (□) and SCID (○) mice were inoculated with 4T1 cells as the control. 2 d after inoculation of 4T1 cells, some SCID mice were i.v. transferred with splenic T cells from the MD5-1–treated BALB/c mice that had rejected 4T1 (●) or naive BALB/c mice (△) (right). Naive SCID mice (○) and MD5-1–treated wild-type BALB/c mice that had rejected 4T1 (■) were inoculated with 4T1 cells as the control. (C) Naive BALB/c mice (▽), MD5-1–treated wild-type BALB/c mice that had rejected live 4T1 (□), BALB/c mice immunized by irradiated 4T1 cells precoated with control Ig (△), or BALB/c mice immunized by irradiated 4T1 cells precoated with MD5-1 (○) were inoculated with the indicated number of live 4T1 tumor cells. (D) Cytotoxic activity of splenic T cells against 4T1 (left) or R331-mock (right) target cells. Effector splenic T cells were prepared from BALB/c mice that had rejected live 4T1 cells by MD5-1 treatment (□), BALB/c mice that had been immunized by irradiated 4T1 cells precoated with MD5-1 (○) or control Ig (△), or naive BALB/c mice (▽). All data are represented as the mean ± SE of 5–10 mice. Similar results were obtained in three to five independent experiments.

Wild-type BALB/c mice that had been immunized with 100-fold greater number of irradiated 4T1 cells precoated with MD5-1 developed a very poor secondary response compared with that developed in the mice that had rejected live 4T1 tumor cells after MD5-1 treatment (Fig. 6 C). Moreover, 4T1 tumor cell–specific CTLs were substantially induced in the mice that had rejected live 4T1 tumor cells after MD5-1 treatment, but not in the mice that had been immunized with irradiated 4T1 cells precoated with either MD5-1 or control Ig (Fig. 6 D). These results suggested a critical contribution of MD5-1–mediated apoptosis of the primary tumor to the development of tumor-specific CTLs.

Eradication of TRAIL-resistant Variants after CTL Induction. Next, we examined whether eradication of primary R331 tumors by MD5-1 treatment enabled the induction of R331-specific T cell immunity. Administration of MD5-1 resulted in complete rejection of 10^4 R331-mock cells, but not R331-FLIP cells, in BALB/c mice (Fig. 7 A, left). Notably, when rechallenged with R331-mock or R331-FLIP, these mice rejected not only R331-mock, but also R331-FLIP cells (Fig. 7 A, middle). A rechallenge with irrelevant 4T1 tumor cells was not rejected in these mice (unpublished data). In contrast, BALB/c mice preimmunized with 50-fold greater number of irradiated R331-mock cells did not reject the secondary challenge with live R331-mock cells. Depletion of CD8+ T cells abrogated the secondary rejection of both R331-mock (Fig. 7 A, right) and R331-FLIP (unpublished data). These results indicated that the MD5-1–mediated primary rejection of R331-mock cells induced CD8+ T cell–mediated immunity that could also eradicate MD5-1–resistant R331-FLIP cells.

Next, we examined the kinetics of the development of T cell immunity after MD5-1 treatment. All mice receiving 10^4 R331-mock cells rejected their primary tumor after MD5-1 treatment. These mice were additionally inoculated with R331-FLIP cells in the opposite flank to evaluate the T cell–mediated rejection (Fig. 7 B). The growth of R331-FLIP tumor was somewhat reduced in the mice rejecting R331-mock cells in the opposite flank compared with the mice receiving MD5-1 and the same dose of R331-FLIP cells alone (Fig. 7 B, 0 day). A more significant inhibition of R331-FLIP tumor growth was observed when R331-FLIP was injected 1 wk after the inoculation of R331-mock cells and commencement of MD5-1 treatment (Fig. 7 B, 1 week). Complete rejection was observed when R331-FLIP was inoculated at least 2 wk later (Fig. 7 B, 2 weeks or 3 weeks). These results suggested that MD5-1–mediated rejection of R331-mock tumors induced R331-specific T cell immunity within 1 to 2 wk.

Next, we examined the strength of the T cell–mediated secondary rejection in comparison with the MD5-1–mediated primary rejection. The T cell–mediated secondary rejection was more powerful than the MD5-1–mediated primary rejection in eliminating greater numbers of R331-mock cells (Fig. 7 C). Interestingly, R331-FLIP cells were somewhat more resistant to the secondary rejection, possi-
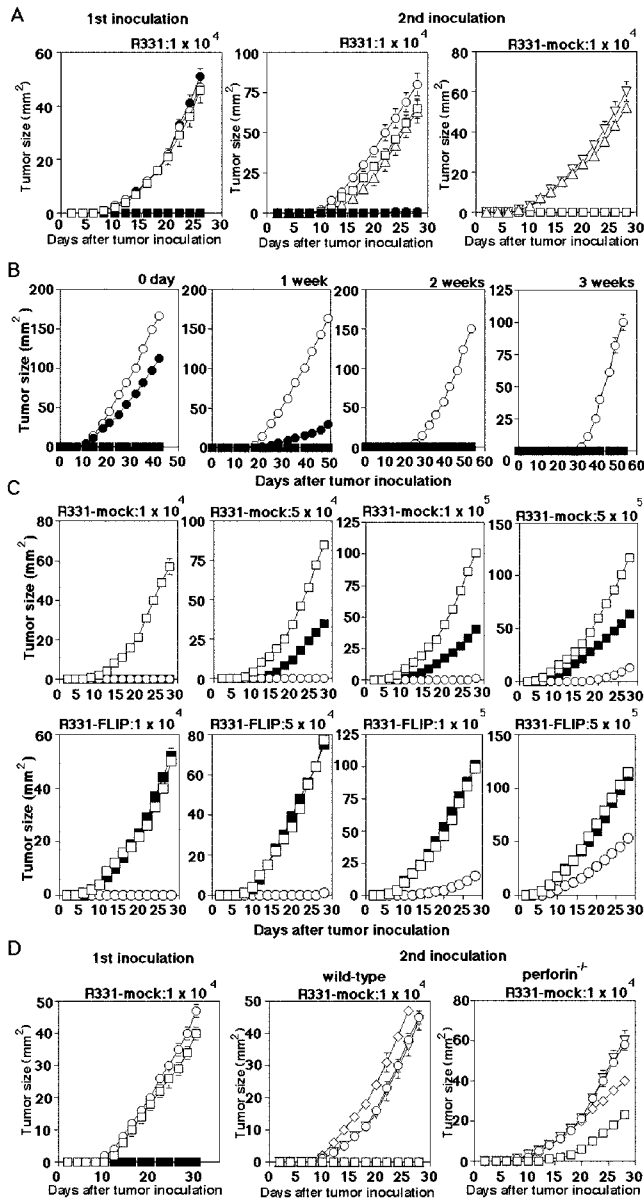
Figure 7. Induction of perforin- and FasL-mediated T cell cytotoxicity that can also eliminate TRAIL-resistant variants. (A) BALB/c mice were inoculated with R331-mock (squares) or R331-FLIP (circles) cells, and then treated with MD5-1 mAb (solid symbols; $n = 20$) or control Ig (open symbols; $n = 5$) (left). The cured mice were secondarily challenged with R331-mock (■) or R331-FLIP (●) cells (middle). Naive mice were inoculated with R331-mock (□) or R331-FLIP (○) cells, and the mice preimmunized by irradiated R331-mock cells were inoculated with R331-mock cells (△) as the controls (middle). Simultaneously to the secondary challenge with R331-mock cells, some mice were treated with anti-CD8 mAb (△), anti-CD4 and anti-CD8 mAbs (▽), or control Ig (□) (right). (B) R331-FLIP cells were inoculated into the left flank (●) at the indicated time point after R331-mock cell inoculation into the right flank (■) and commencement of MD5-1 treatment on day 0. As a control, R331-FLIP cells were inoculated into naive mice (○) at the same time as the R331-FLIP inoculation into the treated mice. (C) BALB/c mice that had rejected R331-mock by MD5-1 treatment were secondarily challenged with the indicated number of R331-mock or R331-FLIP cells (○). Naive mice were inoculated with the same numbers of R331-mock or R331-FLIP cells and treated with MD5-1 (■) or control Ig (□) for comparison. (D) Wild-type (squares) or perforin$^{-/-}$ (circles) BALB/c

mice were inoculated with R331-mock cells, and then treated with MD5-1 mAb (solid symbols; $n = 20$) or control Ig (open symbols; $n = 5$) (left). The cured mice were secondarily challenged with R331-mock cells. Simultaneously to the secondary challenge, some wild-type (middle) and perforin$^{-/-}$ (right) mice were treated with anti-FasL neutralizing mAb (○), anti-TRAIL neutralizing mAb (△), anti-FasL and anti-TRAIL mAbs (▽), or control Ig (□). Naive wild-type or perforin$^{-/-}$ mice were inoculated with the same number of R331-mock cells as a control (◇). All data are represented as the mean ± SE of 5–10 mice. Similar results were obtained in two independent experiments.

bly due to their resistance to FasL-mediated cytotoxicity (see below).

We finally examined the effector mechanisms responsible for T cell–mediated secondary rejection (Fig. 7 D). Although MD5-1 treatment eradicated primary R331-mock tumors in perforin$^{-/-}$ mice just as in wild-type mice, the secondary rejection of R331-mock cells was abrogated in perforin$^{-/-}$ mice (Fig. 7 D, right). Administration of a neutralizing anti-FasL mAb also abrogated the secondary rejection of R331-mock cells both in wild-type and perforin$^{-/-}$ mice. In contrast, administration of a neutralizing anti-TRAIL mAb did not effect the secondary rejection of R331-mock cells in either group of mice (Fig. 7 D, middle and right). These results indicated that the T cell–mediated secondary rejection was mediated by perforin- and FasL-mediated cytotoxicity.

Discussion

In this study, we have characterized the antitumor effects of an agonistic anti–mouse DR5 mAb, MD5-1, in vivo. The anti–mouse DR5 mAb not only inhibited s.c. growing metastases, but also lung and liver metastases of TRAIL-sensitive mouse tumors, without apparent systemic toxicity. Moreover, for the first time, we have revealed that anti-DR5 mAb-mediated primary rejection of TRAIL-sensitive tumors efficiently induced tumor-specific T cell immunity in immunocompetent hosts, which could also eradicate TRAIL-resistant tumor variants. These results substantiated the ability of apoptosis-inducing mAb to stimulate adaptive antitumor immunity and the potential utility of anti-DR5 mAb as a cancer therapeutic.

A number of previous studies have evaluated the mechanism of action of antitumor mAbs, but in general the in vivo mechanism of action remains poorly understood. Very comprehensive in vivo studies have been performed with mAbs reactive with erbB2 (Herceptin), CD20 (rituxan), and melanoma antigens (17, 35, 36). These have demonstrated the importance of FcR, ADCC, and macrophages in primary tumor rejection, with more conjecture concerning the possible role of complement (37). By contrast, evidence for direct cytotoxic effects of these and other mAbs in vivo is scarce. Our present approach was to target a tumor antigen (DR5) that was capable of triggering tumor cell death directly via caspase activation. Regardless of whether we used noncytotoxic or cytotoxic effector cells

or effector cells that only expressed the nonactivatory Fcγ-RII, cross-linking of DR5 by MD5-1 was sufficient to cause caspase-dependent tumor cell death in vitro. As demonstrated in vivo, the cytotoxic activity of MD5-1 against TRAIL-sensitive tumor cells required cross-linking by FcR-expressing innate immune cells, such as NK cells and macrophages. Although NK cells are well-known effectors of ADCC (31), MD5-1 did not trigger perforin-dependent ADCC in vitro and the antimetastatic effect of MD5-1 was not impaired in perforin$^{-/-}$ mice. Moreover, the depletion of NK cells only weakly impaired the tumoricidal effect of MD5-1. These results suggested a minor contribution of NK cells and NK cell–mediated ADCC to the antitumor effects of MD5-1. In contrast, anti-CD11b mAb treatment suggested a major contribution of CD11b$^+$ macrophages. The CD11b$^+$ effector cells might also include myeloid DCs, which also express FcR (38). Consistent with this notion, augmented infiltration of F4/80$^+$ macrophages and CD205$^+$ DCs was observed in the MD5-1–treated tumors. The anti-CD11b treatment does not deplete macrophages but rather inhibits Mac-1 function (34, 39), and from other studies it is clear that Mac-1 is required for efficient ADCC (40). We cannot formally exclude a role for ADCC in vivo because the presence of activating FcR (FcγRI or FcγRIII containing the FcRγ), but not FcγRII, was required for primary tumor rejection by MD5-1 in vivo. Nevertheless, macrophages generally mediate ADCC via their production of nitric oxides and superoxides (32). Thus, the protection of R331-FLIP tumors from MD5-1 in vivo strongly suggested that the antitumor effects of MD5-1 were primarily mediated by the induction of caspase-dependent apoptosis via DR5 as demonstrated in vitro, rather than the triggering of ADCC by macrophages. Given the inhibitory effects of FLIP expression in vitro and in vivo and the ability of MD5-1 to engage FcγRII to trigger tumor cell death in vitro, we suggest the possibility that activation via the FcRγ chain (and in conjunction with Mac-1) in vivo may enhance the function and further recruitment of FcR-expressing innate immune cells, which then contribute to the development of adaptive immunity.

More importantly, no prior studies have assessed and characterized the development of adaptive immunity subsequent to primary antibody-mediated tumor rejection. One previous study demonstrated that simply coating tumor cells with antibodies to surface molecules (like syndecan-1) promoted cross-presentation of cellular antigens by DCs to T cells, but these studies were restricted to in vitro analysis and the mechanism here was most likely opsonized tumor cell phagocytosis (41, 42). It is also noteworthy that primary induction of apoptosis in tumor cells and secondary induction of tumor-specific immunity has been conceptually implicated, but not proven, in the therapeutic effects of anti-Her2 mAb and anti-CD20 mAb in clinical settings (43, 44). We have now provided new and rigorous evidence that antibody-based strategies targeting a death receptor lead to the induction of tumor-specific T cell immunity. Importantly, we demonstrated that death recep-

tor–mediated apoptosis efficiently evoked tumor-specific T cell immunity against variants that were resistant to primary rejection. Emergence of TRAIL-resistant variants is a critical problem for TRAIL-based therapy (45). Our results have indicated that anti-DR5 mAb therapy may resolve this problem by inducing tumor-specific CTLs that can eliminate the variants by perforin- and FasL-mediated cytotoxicity. Notably, by comparison, immunization with irradiated tumor cells precoated with the MD5-1 mAb did not significantly immunize mice against the tumor. Although this limitation precluded us from easily further investigating the mechanisms for the induction of adaptive immunity, these data suggested that the active induction of DR5-mediated tumor cell apoptosis in concert with the recruitment of FcR-expressing innate immune cells was required for optimal induction of tumor-specific CTLs. It seems likely that anti-DR5 mAb not only induces apoptosis in tumor cells by recruiting FcR-expressing macrophages and DCs, but also targets the apoptotic tumor cells to these APCs via FcR. Then, the APCs cross-present tumor antigens and induce tumor-specific CTLs (42, 46–48). Consistent with this notion, it has been reported that the FcR-mediated uptake of apoptotic tumor cells by DCs could efficiently induce tumor-specific CTLs in vivo (49, 50). Therefore, the antibody-based therapy targeting DR5 might be an efficient strategy not only to eliminate TRAIL-sensitive tumor cells temporarily, but also to potentiate tumor-specific T cell immunity that affords a long-term protection from tumor recurrence. Such a benefit is yet to be established for therapeutic approaches that use the recombinant TRAIL ligand itself.

A possible concern over the use of anti-DR5 mAb in cancer therapy is its potential toxicity against normal tissue–expressing DR5. Although a previous study has reported tumoricidal activity of an agonistic anti–human DR5 mAb against TRAIL-sensitive human tumor xenografts in SCID mice with no hepatocellular toxicity in vitro (16), the potential toxicity in vivo was not addressed because that mAb did not cross-react with mouse DR5. In addition, it has been reported that some preparations of recombinant TRAIL were cytotoxic against cultured human hepatocytes (33), keratinocytes (51), and thymocytes (52) in vitro. However, other preparations of recombinant TRAIL were not cytotoxic against these cells in vitro (51, 53), and no apparent systemic toxicity was observed in mice and non-human primates when administered in vivo (10–12). In this study, we have been able to evaluate the potential toxicity of targeting DR5 in vivo. We have observed no detectable systemic or organ-specific toxicity of MD5-1 as estimated by the behavior, body weight, gross appearance, blood transaminase levels, and histological examination. In more recent experiments, some mice have been treated with 300 μg MD5-1 weekly for >150 d with no signs of toxicity (unpublished data). Consistently, DR5 expression was not detectable on the surface of freshly isolated murine hepatocytes or thymocytes as estimated by flow cytometry (unpublished data). In concert with our data, it has been re-

ported that normal human hepatocytes did not express DR5 and were totally resistant to anti–human DR5 mAb in vitro (16). Collectively, these observations suggest that anti-DR5 mAb might be safe as a therapeutic agent.

Our results suggested some other possible advantages of anti-DR5 mAb treatment over the antitumor activities of endogenous or recombinant TRAIL. First, although endogenous TRAIL naturally inhibits liver metastasis, but not lung metastasis, due to the lack of TRAIL on lung NK cells (4, 7), anti-DR5 mAb effectively inhibited both liver and lung metastases. Second, although endogenous IFN-γ/TRAIL-mediated tumor surveillance might be disturbed by immunosuppressive cytokines such as IL-10 and TGF-β (54, 55), anti-DR5 mAb can exert antitumor effects in TRAIL- or IFN-γ–deficient conditions. Third, anti-DR5 mAb might be more effective than recombinant TRAIL because it can induce tumor cell apoptosis independently of the decoy receptors that may limit the efficacy of recombinant TRAIL. Moreover, mAbs are quite amenable to engineering to improve their half-life (by humanization; 56–58), their agonistic activity by modification of the antigen-binding portion or the Fc portion (56, 58), and their ADCC by defucosylation (59). Finally and most importantly, the recruitment of FcR–expressing innate immune cells by anti-DR5 mAb can evoke tumor-specific adaptive T cell immunity that also eradicates TRAIL-resistant variants. A direct comparison between recombinant TRAIL and anti-DR5 mAb approaches should be undertaken in mice or humans when both reagents are freely available or approved for clinical trial.

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