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CD11c+ Dendritic Cells and B Cells Contribute to the Tumoricidal Activity of Anti-DR5 Antibody Therapy in Established Tumors

Nicole M. Haynes,* Edwin D. Hawkins,* Ming Li,* Nicole M. McLaughlin,* Günter J. Hämerling,† Reto Schwendener,‡ Astar Winoto,§ Allen Wensky,¶ Hideo Yagita,∥ Kazuyoshi Takeda,∗ Michael H. Kershaw,∗‖ Phillip K. Darcy,∗‖ and Mark J. Smyth∗,‖

The selective targeting of the tumor-associated death-inducing receptors DR4 and DR5 with agonistic mAbs has demonstrated preclinical and clinical antitumor activity. However, the cellular and molecular mechanisms contributing to this efficacy remain poorly understood. In this study, using the first described C57BL/6 (B6) TRAIL-sensitive experimental tumor models, we have characterized the innate and adaptive immune components involved in the primary rejection phase of an anti-mouse DR5 (mDR5) mAb, MD5-1 in established MC38 colon adenocarcinomas. FcR-mediated cross-linking of MD5-1 significantly inhibited the growth of MC38 colon adenocarcinomas through the induction of TRAIL-R-dependent tumor cell apoptosis. The loss of host DR5, TRAIL, perforin, FasL, or TNF did not compromise anti-DR5 therapy in vivo. By contrast, anti-DR5 therapy was completely abrogated in mice deficient of B cells or CD11c+ dendritic cells (DCs), providing the first direct evidence that these cells play a critical role. Importantly, the requirement for an intact B cell compartment for optimal anti-DR5 antitumor efficacy was completely abrogated in mice deficient of B cells or CD11c+ dendritic cells (DCs), providing the first direct evidence that these cells play a critical role. Importantly, the requirement for an intact B cell compartment for optimal anti-DR5 antitumor efficacy was also observed in established AT-3 mammary tumors. Interestingly, MD5-1-mediated apoptosis as measured by early TUNEL activity was completely lost in B cell-deficient μMT mice, but intact in mice deficient in CD11c+ DCs. Overall, these data show that Ab-mediated targeting of DR5 triggers tumor cell apoptosis in established tumors in a B cell-dependent manner and that CD11c+ DCs make a critical downstream contribution to anti-DR5 antitumor activity. The Journal of Immunology, 2010, 185: 532–541.

The therapeutic targeting of tumors or components of the immune system with molecule-specific mAbs is now considered a viable treatment option for cancer patients. Trastuzumab (Herceptin), which binds the breast cancer-associated growth factor receptor ErbB2, and rituximab (Rituxan) that targets B cells for elimination by binding the B cell-associated marker CD20, are two such prototypic Abs that are currently in clinical use. Indeed, these Abs have been used successfully in combination with chemotherapy for the treatment of patients with breast cancer and non-Hodgkin’s lymphoma, respectively (1, 2). Tumor-targeting mAbs exert their antitumor effects by modulating basic processes, such as proliferation, differentiation, and/or survival.

Through Fc-mediated Ab-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity, opsonisation, and/or phagocytosis mechanisms, mAbs may also trigger tumor cell death resulting in the liberation of tumor-associated Ags in a manner that can promote tumor immunity (3–5). These cell death-inducing and immune-stimulating properties of mAbs have made them valuable therapeutic tools in cancer treatment and with current advances in the fields of immunology and oncology, novel molecular targets are being elucidated that will significantly broaden the therapeutic application of Ab-based immunotherapies.

Tumor-targeted mAbs that have generated a considerable amount of interest in oncology are those that bind the receptors for a death-inducing ligand, TRAIL (6, 7). Unlike other death-inducing ligands, TRAIL preferentially kills malignant cells via activating the death receptors DR4 and DR5, while sparing most normal cells. However, although many tumors express TRAIL death receptors, they also typically express TRAIL decoy receptors that have the potential to limit the therapeutic efficacy of recombinant TRAIL (8). M Abs that specifically bind the death receptors of TRAIL bypass this problem, and offer the advantage of having a longer half-life in vivo (9), higher affinity for the target receptor, and the potential to promote the recruitment and activation of immune effector cells (10). Furthermore, selective targeting of the TRAIL receptors signals cell death in the absence of p53 function; the loss of which has been shown to disable chemotherapeutic drug responses (11). With encouraging preclinical data and validation that the TRAIL/TRAIL-receptor pathway is important in tumor immunosurveillance (12–14), the therapeutic efficacy and toxicity of agonistic mAbs to human DR4 and DR5 are now being evaluated in early phase clinical trials, alone and in combination with first-line chemotherapy and irradiation (15). Results from these trials have

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Abbreviations used in this paper: ADCC, Ab-dependent cellular cytotoxicity; cIg, control Ig; DC, dendritic cell; DT, diphtheria toxin; MHC I, MHC class I; mTRAIL, mouse TRAIL; pIp, perfom; WT, wild-type.

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indicated that these anti-TRAIL receptor mAbs can be administered safely and are capable of promoting disease stabilization and/or partial responses without apparent toxicity (15). Currently, however, there is scant clinical data available on the impact of these therapeutic mAbs on immune parameters.

Because the preclinical development of anti-DR4 and -DR5 agonistic mAbs for cancer therapy has largely involved examining the tumoricidal activity of humanized reagents in immune compromised xenogeneic mouse models of human cancer, the immunological consequences of anti-DR5 therapy on the host and tumor microenvironment have remained poorly defined. Previously we demonstrated an anti-mDR5 mAb (MD5-1) could exhibit potent antitumor effects against TRAIL-sensitive mouse tumors in syngeneic BALB/c mice through FcγR-dependent induction of tumor cell apoptosis (10). When administered at the time of 4T1 mammary tumor cell implantation, anti-DR5 (MD5-1) was found to promote the recruitment of dendritic cells (DCs) and stimulate adaptive tumor immunity that could be raised by secondary rechallenge (10). In this study, we have developed C57BL/6 TRAIL-sensitive tumor models with the aim of being able to use gene-targeted mice to better evaluate the innate and adaptive immune cells contributing to the tumoricidal activity of the MD5-1 mAb in more clinically relevant established tumors. C57BL/6 gene-targeted or immune cell-depleted mice were used to examine the antitumor activity of MD5-1 against the TRAIL-sensitive mouse MC38 colon adenocarcinoma and AT-3 mammary carcinoma. In these tumor models, an intact B cell compartment was critical for the therapeutic activity of MD5-1 against established tumors. B cells were confirmed to trigger tumor cell apoptosis by FcγR-mediated cross-linking of the MD5-1 mAb in vitro and in vivo B cells were critical for directly triggering MD5-1-mediated tumor cell apoptosis. By contrast, although DC subsets could also trigger tumor cell apoptosis in vitro, CD11c+ DCs were not necessary for MD5-1-mediated apoptosis in vivo, but were important downstream for the antitumor activity of MD5-1. These data are intriguing and establish an important platform on which to further explore the mechanism by which small numbers of B cells entering tumors might enable anti-DR5–mediated tumor apoptosis and the direct or indirect role CD11c+ DCs might contribute to the antitumor activity of Ab-based immunotherapeutics. The findings also create new possibilities in the rational design of improved combination cancer therapies comprising the death-inducing anti-DR5 mAb.

Materials and Methods

Mice

C57BL/6 (B6) and BALB/c mice at 8–12 wks of age were from The Walter and Eliza Hall Institute of Medical Research. The B6 RAG-1–deficient (RAG-1−/−), TRAIL−/−, gld−/−, perforin−/− (pfp), TNF−/−, and μMT mice were derived from B6 embryonic stem cells and bred and maintained at the Peter MacCallum Cancer Centre. The B6 and BALB/c DR5-deficient mice (DR5−/−) were generated as described (16). The B6 CD11c.DOG mice (17) were kindly provided by Dr. Christian Engwerda (Queensland Institute of Medical Research, Queensland, Australia). All mice were maintained under specific pathogen-free conditions and used in accordance with the institutional guidelines of the 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.

Cell lines

The B6-derived TRAIL-sensitive MC38 colon adenocarcinoma and AT-3 mammary carcinoma tumor lines, 2PK-3 mock and mouse TRAIL-(mTRAIL)-transfected B cell lymphoma lines and the P815 mastocytoma line have been described (10, 18, 19). These lines were cultured in DMEM supplemented with 10% heat-inactivated FCS (Moregate Biotech, Bulimba, Queensland, Australia), 100 μg/ml penicillin/streptomycin (Sigma-Aldrich, Castle Hill, New South Wales, Australia), and 2 mM l-glutamine (JRH Biosciences, Brooklyn, Victoria, Australia). The BALB/c-derived 4T1 mammary carcinoma has been previously described (18) and was cultured in RPMI 1640 media containing the aforementioned supplements. The MC38-c-FLIP line was generated using retroviral gene-transfer technology as previously described (18). Briefly, a standard calcium phosphate transfection method was used to introduce the murine stem cell virus-IRE5-GFP containing the FLIPlong encoding sequence (kindly provided by Dr. Thomas Sayers, National Cancer Institute, Frederick, MD) and the amphipathic helper plasmid into the 293T packaging cell line. Viral supernatant collected from these 293T cell cultures, was supplemented with 4 μg/ml polybrene (Millipore, Billerica, MA) and used to transduce the MC38 tumor cell line. Seventy-two hours after transduction, GFP-positive tumor cells were selected for by flow cytometry-mediated cell sorting (Vantage-DIVA; BD Biosciences, San Jose, CA).

DR5 and MHC class I expression

Cell surface DR5 and MHC class I (MHC I) levels were assessed by flow cytometry. Staining of cells involved treating 106 cells with biotinylated anti-mouse DR5 (MD5-1, eBioscience, San Diego, CA), anti-mouse H-2Kb (AF6-88.5; BD Pharmingen, San Jose, CA) or anti-mouse I-Aα (KH74; BD Pharmingen) in the presence of normal mouse serum (Jackson Immunoresearch Laboratory, West Grove, PA). Cells were subsequently treated with allopurinol-conjugated streptavidin (eBioscience). During this staining procedure, tumor cells were incubated on ice and washed and resuspended in FACS buffer (2% FCS in PBS with 0.1% sodium azide and 1 mM EDTA) prior to being examined on a LSRII analyzer (BD Biosciences). Cell viability was assessed through the addition of 100 ng/ml DAPI (4′,6-diamidino-2-phenylindole; Invitrogen, Eugene, OR).

Abs and reagents

Agnostic mAbs to mouse DR5 (MD5-1), neutralizing/depleting Abs to asialoGM1 (NK cell depletion; Wako Pure Chemicals, Osaka, Japan), mouse CD11b (5C6), mouse CD8α (53.6.7, and control IgG1 [Hamster] (UC8-I89) or rat (MAC4) Abs were prepared as described (10). For tumor therapy, 100 μg MD5-1 or UC8-I89 clg were administered into the peritoneal cavity (i.p.) of mice once every 4 d for a total of three treatments. In experiments using μMT and RAG-1−/− mice, 50 μg MD5-1 or UC8-I89 clg was used to limit the onset of toxic side effects in the immune compromised mice. Neutralizing/depleting Abs to CD11b (300 μg), NK cells (asialoGM1; 50 μg), CD8+ cells (200 μg), CD4+ cells (200 μg), or MAC4 clg were administered i.p. day −2, −1, 3, 7, and 11 relative to anti-DR5 treatment. In experiments examining the immune cell subsets involved in the natural suppression of MC38 tumor growth, mice were treated with the aforementioned neutralizing/depleting Abs 1 d prior to treatment and subsequent injections of these mAbs were administered every 4 d throughout the course of the experiment. Clodronate-containing liposomes for the depletion of phagocytic cells and empty-control liposome preparations was used as described [clodrolip; sodium clodronate tetrahydrate, Farchemis SRL, Treviglio, Italy (20)]. Clodrolip and control liposomes were diluted in PBS and injected i.p. at 2 mg per 20 g body weight, 2 d prior to anti-DR5 therapy. Subsequent doses of depleting and control liposome preparations (1 mg/20 g body weight) were administered every 2 d. CD11c.DOG mice were treated with 8 ng/g body weight of diptheria toxin (DT) i.p. on day −2, 0, 4, 8, 12, and 16 relative to anti-DR5 treatment.

Cytotoxicity assay

The cytotoxic activity of the MD5-1 mAb was tested in a 20 h [51Cr] release assay as previously described (21). Tumor cell susceptibility to TRAIL-mediated cytotoxicity was assessed using the murine TRAIL-transfected 2PK-3 or mock-transfected 2PK-3 lines as effector cells at the indicated effector:tumor target cell ratios. In the case of MD5-1–mediated cytotoxicity, tumor cells were 1) cultured for 20 h on Protein A plates (Pierce, Rockford, IL) coated with 1–10 μg/ml of MD5-1 or UC8-I89 clg or 2) cultured in the presence of FcγR+ P815 cells, enriched naive splenic B cells, bone marrow-derived DCs or enriched splenic CD8+ or CD4+ DCs at the indicated effector/target ratios. B cell enrichment from wild-type (WT) B6 mouse spleens was performed as previously described (22). Briefly, collagenase-treated single-cell splenic preparations were fractionated on a cold Percoll gradient of 50, 65, and 80%. Naïve B cell enrichment (90–95%) was subsequently achieved using a B cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Naïve B cell purity was verified by flow cytometric analysis using Ab mixtures containing anti-mouse CD19-FITC (ID3; BD Pharmingen), together with B220-PE (RA3-
Figure 1. Characterization of the DR5 expression levels and TRAIL sensitivity of the B6-derived colon adenocarcinoma line MC38 in vitro. A. Surface expression of DR5 and MHC I on the MC38 and 4T1.2 tumor lines was assessed by flow cytometry. The open/bold histograms represent DR5 and MHC I on their surface (Fig. 1A). The MC38 tumor cells were sensitive to the cytotoxic activity of recombinant mTRAIL, presented on the surface of the B cell lymphoma line 2PK-3 (Fig. 1B) and MD5-1 when cross-linked by FcR on P815 cells in vitro (Fig. 1C). The B6-derived, DR5⁺ melanoma line, B16-F10 proved resistant to the cytotoxic activity of both mTRAIL and MD5-1 (Fig. 1B, 1C). Importantly, we were able to demonstrate the sensitivity of the MC38 tumor line to the tumoricidal actions of MD5-1 in vivo. Against established MC38 tumors, MD5-1 mAb treatment induced significant tumor regression, which in some instances resulted in complete tumor regression (Fig. 2A). Strikingly, the tumor suppressive effects of MD5-1 were observed as early as 24 h after Ab administration. The cytotoxic effects of MD5-1 in these established MC38 tumors was primarily mediated through DR5 receptor signaling of apoptosis, as the antitumor activity of MD5-1 was completely abrogated in mice bearing established MC38 tumors overexpressing cFLIP (Fig. 2A); a protein that can inhibit caspase-8-mediated death receptor signaling. Thus, the contribution of ADCC during the primary rejection phase of anti-DR5 therapy in established MC38 tumors was minimal. Consistent with this, we did observe that the antitumor activity of MD5-1 was not diminished in mice deficient of pfp: a key component of NK cell-mediated ADCC (Fig. 2B); however, we cannot formally exclude the possible involvement of other modes of ADCC.

Results

Sensitivity of the mouse adenocarcinoma line, MC38 to anti-DR5-mediated apoptosis

Previously, we demonstrated that FcR-expressing effector cells were necessary for the anti-DR5 mAb, MD5-1, to induce TRAIL-receptor oligomerization and tumor cell apoptosis in vitro and in vivo (10). To perform a comprehensive analysis of the specific innate and/or adaptive FcR⁺ immune cells that may facilitate the tumoricidal actions of anti-DR5 mAb in vivo, we searched for a TRAIL-sensitive tumor line derived from the C57BL/6 (B6) strain, so as to take advantage of a broad range of gene-targeted mice available on this background. Of the B6-derived tumor lines that we screened for TRAIL sensitivity, the colon adenocarcinoma tumor line MC38 proved highly sensitive to this death-inducing signaling pathway. Similar to the BALB/c-derived 4T1.2 tumor line, which was used for the initial characterization of the antitumor activity of the MD5-1 mAb (10), the MC38 tumor line expressed equivalent levels of the TRAIL death receptor DR5 and MHC I on its surface (Fig. 1A). The MC38 tumor cells were sensitive to the cytotoxic activity of recombinant mTRAIL, presented on the surface of the B cell lymphoma line 2PK-3 (Fig. 1B) and MD5-1 when cross-linked by FcR on P815 cells in vitro (Fig. 1C). The B6-derived, DR5⁺ melanoma line, B16-F10 proved resistant to the cytotoxic activity of both mTRAIL and MD5-1 (Fig. 1B, 1C). Importantly, we were able to demonstrate the sensitivity of the MC38 tumor line to the tumoricidal actions of MD5-1 in vivo. Against established MC38 tumors, MD5-1 mAb treatment induced significant tumor regression, which in some instances resulted in complete tumor regression (Fig. 2A). Strikingly, the tumor suppressive effects of MD5-1 were observed as early as 24 h after Ab administration. The cytotoxic effects of MD5-1 in these established MC38 tumors was primarily mediated through DR5 receptor signaling of apoptosis, as the antitumor activity of MD5-1 was completely abrogated in mice bearing established MC38 tumors overexpressing cFLIP (Fig. 2A); a protein that can inhibit caspase-8-mediated death receptor signaling. Thus, the contribution of ADCC during the primary rejection phase of anti-DR5 therapy in established MC38 tumors was minimal. Consistent with this, we did observe that the antitumor activity of MD5-1 was not diminished in mice deficient of pfp: a key component of NK cell-mediated ADCC (Fig. 2B); however, we cannot formally exclude the possible involvement of other modes of ADCC. A...
role for the death-inducing ligands, TRAIL, FasL, and TNF were also discounted, as mice deficient of these ligands still demonstrated MD5-1–mediated suppression of MC38 tumor growth (Fig. 2C–E).

Having established tumor cell sensitivity to anti-DR5 therapy in vivo, we next examined whether host endogenous expression of DR5 might impact the therapeutic efficacy of MD5-1 treatment. In DR5-deficient mice, we found that the antitumor activity of MD5-1 was similar to that observed in WT mice, discounting any contribution of host DR5 to the antitumor effects of MD5-1 in vivo (Fig. 2F). A similar comparative study in MD5-1–treated BALB/c WT and DR5−/− mice bearing established s.c. 4T1.2 lesions confirmed these findings (data not shown). It should be noted that no signs of mAb-related hepatotoxicity were observed in these experiments, in which a total of 300 μg MD5-1 was administered over three separate treatments.

Contribution of NK cells, CD11b+ cells and macrophages to the tumoricidal effects of MD5-1 in established s.c. tumors

In an earlier study, we proposed that CD11b+ cells, such as macrophages, were the primary mediators of MD5-1 activity against freshly implanted 4T1.2 tumors cells (10). In this study, we examined whether such cells might facilitate MD5-1–mediated primary rejection of established MC38 tumors. Like 4T1.2 tumors (Fig. 3B), the tumoricidal activity of MD5-1 in established MC38 lesions was retained following anti-asialoGM1 Ab-mediated depletion of NK cells (Fig. 3A). In contrast, anti-CD11b mAb treatment, which completely abrogated the tumoricidal effects of MD5-1 treatment in developing 4T1.2 tumors (Fig. 3D), had no effect on the efficacy of MD5-1 treatment in the MC38 tumors (Fig. 3C).

Because flow cytometric analysis of immune cell subsets within established MC38 tumors revealed the presence of F4/80+ macrophages (19 ± 5% of live tumor cells; n = 6), we next...
assessed to what extent these cells might contribute to the antitumor effects of MD5-1 in established MC38 tumors. For these experiments, mice bearing established tumors were treated with clodrolip, a reagent that has proven effective in the selective depletion of phagocytic cells, such as macrophages, including CD11b+ tumor-associated macrophages (24). As a control, some groups of mice were treated with an empty liposome preparation. Treatment of tumor-bearing mice with i.p. injections of clodrolip was found to be sufficient to promote the natural suppression of established MC38 lesions, because these tumors grew out at a slower rate than those in mice treated with a hamster cIg alone (Fig. 3E). These data suggested that macrophages present within the established MC38 tumors were supporting tumor progression. Thus, depletion of these cells from the tumor microenvironment might account for the enhanced therapeutic efficacy of MD5-1 when delivered postclodrolip treatment. Indeed, a tumor rejection rate of 50% was recorded in the MD5-1/clodrolip treated group; whereas, no tumor free mice were reported for any of the other treatments. It should be noted that the empty liposome preparation had no effect on the antitumor activity of MD5-1. The maintained efficacy of anti-DR5 therapy in clodrolip-treated mice (Fig. 3E) suggested that tumor-associated macrophages were not the primary mediators of the cytotoxic effects of MD5-1 in established MC38 tumors. Clodrolip was also administered to mice bearing established 4T1.2 tumors (Fig. 3F). In this tumor setting, macrophages were found to contribute minimally to the control of 4T1.2 tumor growth. However, clodrolip treatment did partially inhibit the antitumor activity of MD5-1 verifying that macrophages can play a role in facilitating the tumoricidal effects of this death inducing mAb in some tumor microenvironments.

**MD5.1 is ineffective in B cell-deficient mice and CD11c+ DC-deficient mice**

In the past, the lack of appropriate mAbs or BALB/c gene-targeted mice has restricted our ability to examine the contribution of FcR+ leukocytes, such as B cells and CD11c+ DCs, to MD5-1–mediated tumor suppression. Using gene-targeted mice on a B6-background we looked to identify whether such adaptive or innate immune cells could help in facilitating the tumoricidal activity of MD5-1 in established MC38 tumors. Initially, we chose to explore whether MD5-1–mediated primary rejection was dependent on T and B cells. Having identified the immunogenic nature of the MC38 tumors and that CD8+ T cells, but not CD4+ T or NK cells, have a significant role to play in the natural suppression of these tumors (Fig. 4A), we first examined whether depletion of CD8+ cells would limit the tumoricidal activity of MD5-1 in established s.c. tumors. Although depletion of CD8+ T cells significantly enhanced MC38 tumor growth as compared with cIg-treated mice (Fig. 4B), MD5-1 treatment still inhibited tumor growth in a similar manner to that observed in WT mice with an intact T cell compartment (Fig. 4B). These data suggested that CD8+ T cells...
were not critical for the tumor growth inhibitory effects of MD5-1. Surprisingly, however, in RAG-1−/− mice, the tumoricidal effects of MD5-1 were completely abrogated, reaffirming the likely contribution of adaptive immune components to MC38 growth suppression post–anti-DR5 therapy (Fig. 4C). Again, this finding was in contrast to what we observed in the 4T1.2 tumors where MD5-1 treatment was equally effective in WT and SCID mice (data not shown).

Given that B cells have been reported to express the inhibitory low-affinity FcR for IgG, FcγRIIB, which regulates activating signals transduced by the B cell receptor (25), we next examined the tumoricidal effects of MD5-1 in B cell-deficient, μMT mice. Strikingly in these mice, MD5-1 treatment had no therapeutic benefit and the MC38 tumors grew out in a similar manner to that observed for the cIg-treated tumors (Fig. 4C). It is unlikely that the observed loss of MD5-1 antitumor activity was due to altered pharmacokinetics of the MD5-1 mAb in μMT mice (lacking Ig), because similar doses of an anti-mouse CD137 (4-1BB) mAb was reported to be comparatively effective in both WT and μMT mice (26). However, these findings were intriguing and somewhat counterintuitive to the low frequency of B cells in established MC38 tumors (0.05 ± 0.02% total live CD19+ B220− cells; n = 6).

A possible role for DCs in MD5-1 antitumor activity in vivo was speculated based on their infiltration into 4T1.2 tumors post Ab-treatment (10); however, confirmation of their involvement has not been possible in the BALB/c strain. Certainly, DCs do express both activating and inhibitory FcγRs and thus have the potential to facilitate MD5-1–mediated cytotoxicity (27). For this we employed the use of CD11c.DOG transgenic mice, which can largely be depleted (>90%) of CD11c+ MHC class II+ conventional DCs and 50–60% of plasmacytoid DCs by continuous injections of DT (17) and communication with Prof. G. Hämmerling. In these experiments, CD11c.DOG mice bearing established MC38 tumors were treated with DT 2 d prior to MD5-1 or hamster cIg treatment. Strikingly in the DT-treated CD11c.DOG mice, which possess an intact B cell compartment, the tumoricidal activity of MD5-1 was almost completely abrogated, with the tumors growing out in all mice in a similar manner to that seen in cIg-treated groups (Fig. 4D). These data identified an important role for CD11c+ DCs in MD5-1–mediated antitumor activity.

Importantly, we were able to further validate the importance of an intact B cell compartment to the antitumor activity of MD5-1 using the B6-derived mammary carcinoma line AT-3, which like the MC38 line, expresses both DR5 and MHC I (Fig. 5A) and is sensitive to anti-DR5–mediated cytotoxicity in vitro (Fig. 5B). In both μMT mice (Fig. 5A) and is sensitive to anti-DR5–mediated cytotoxicity in vitro (Fig. 5B).

**FIGURE 4.** The tumoricidal activity of MD5-1 therapy against immunogenic MC38 tumors is retained in mice depleted of CD8+ T cells but lost in B cell-deficient and CD11c+ DC-deficient mice. A, B6 WT mice were injected i.p. with mAbs to CD4, CD8, or asialoGM-1. Control groups of mice were treated with PBS or MAC4 cIg. One-day post-Ab treatment 10^6 MC38 tumor cells were injected s.c. in the hind flank. Results are presented as mean tumor growth ± SEM. Statistical differences in tumor sizes between MAC4 cIg and anti-CD8 treated mice were determined by the Mann-Whitney U test (p < 0.0008). B, Mice were injected s.c. with 10^6 MC38 tumor cells and treated with an anti-CD8α mAb or MAC4 cIg on day 6 posttumor inoculation. Mice were subsequently treated with MD5-1 or UC8-1B9 cIg at the indicated time points (designated by arrows). Results are presented, as mean tumor growth ± SEM. Statistical differences in mean tumor sizes between the UC8-1B9 cIg and MD5-1–treated WT mice were detected (p = 0.02). C, WT, RAG-1−/−, and μMT mice were injected s.c. with 10^6 MC38 parental tumor cells. On day 6 after tumor inoculation mice were injected with MD5-1 or UC8-1B9 cIg at the designated time points (indicated by arrows). Results are presented as mean tumor growth ± SEM. Statistical differences in mean tumor size between the UC8-1B9 cIg and MD5-1–treated WT (p = 0.02) and CD8-depleted (p = 0.02) mice were detected. D, WT, RAG-1−/−, and μMT mice were injected s.c. with 10^6 MC38 parental tumor cells. On day 6 after tumor inoculation mice were injected with MD5-1 or UC8-1B9 cIg at the designated time points (designated with arrows). Results are presented as mean tumor growth ± SEM. All in vivo results are representative of two experiments.

**FIGURE 5.** The tumoricidal activity of MD5-1 against AT-3 mammary tumor cells is dependent on adaptive immune components. A, Surface expression of DR5 and MHC I on the AT-3 tumor line was assessed by flow cytometric analysis. The open/bold histograms represent DR5 and MHC I staining and the solid histograms represent the unstained tumor controls. B, The comparative sensitivity of the MC38 and AT-3 tumor cells to MD5-1–mediated apoptosis was assessed in a 20 h [51Cr] release assay, in which the tumor cells were cocultured with FcR+ P815 cells and MD5-1 or UC8-1B9 cIg. Each group was performed in triplicate. Results are representative of three experiments. C and D, WT, μMT, or RAG-1−/− mice were injected s.c. with 10^6 AT-3 tumor cells. On day 8 after tumor inoculation mice were injected with MD5-1 or UC8-1B9 cIg at the indicated time points (designated with arrows). Results are presented as mean tumor growth ± SEM. Statistical differences in tumor sizes between MD5-1– and cIg-treated mice were determined by the Mann-Whitney U test (p < 0.008). No statistical differences between the cIg- and MD5-1–treated μMT and Rag1−/− mice were detected.
CD8+, CD4+, and pDC subsets was assessed by flow cytometry. Fc on CD8+ DCs, the primary mediators of Ag cross-presentation, inhibitory FcR
Interestingly, of these DC subsets, expression of the activating and cytotoxic activity in vitro.

Based on these in vivo observations, we were interested in determining whether B cells and CD11c+ DC could directly trigger MD5–1–mediated apoptosis in vitro. Indeed, we detected FcR on ex vivo splenic CD8+, CD4+, and plasmacytoid DCs (Fig. 6A). Interestingly, of these DC subsets, expression of the activating and inhibitory FcγRII/FcγRIII receptors (CD16/32) was only detected on CD8+ DCs, the primary mediators of Ag cross-presentation (Fig. 6A) (28). Given the differential expression of FcRs on CD8+ and CD4+ DCs we chose to compare the capacity of these two DC subsets to facilitate FcR-dependent MD5–1–mediated cytotoxicity against the MC38 tumor line in vitro (Fig. 6B). As expected, tumor cell death was dictated by the level of FcR expression on the DC subsets. Consistent with the need to cross-link the MD5–1 mAb to signal apoptosis, Ab-mediated blockade of FcRs on both DC subsets effectively inhibited the cytotoxic activity of this death-inducing mAb. BMDCs were also observed to be capable of mediating MD5–1 cytotoxicity against the MC38 tumor line (data not shown). Although in vivo, it is unlikely that CD8+ DCs contribute to the antitumor effects of MD5–1, because Ab-mediated depletion of CD8+ cells did not impair the ability of MD5–1 to suppress tumor growth (Fig. 4B), it remained possible that other CD11c+ DC subsets were important (Fig. 6A). We were also able to demonstrate that enriched splenic B cells could facilitate MD5–1–mediated apoptosis of the MC38 tumor cells and this was completely abrogated by Ab-mediated blockade of FcR on the B cells (Fig. 6C). Overexpression of cFLIP in the MC38 tumor line was also shown to block the cytotoxic effects of MD5–1 in this in vitro system (Fig. 4C). These results confirmed that B cell-mediated cross-linking of the MD5–1 mAb was sufficient to induce DR5- and caspase-mediated cell death.

**Only B cells are critical for MD5–1–mediated tumor cell apoptosis in vivo**

To determine whether B cells and CD11c+ DCs were directly involved in MD5–1–mediated apoptosis in vivo, we used a TUNEL staining assay to quantitatively compare the levels of MD5–1–mediated tumor cell apoptosis in solid MC38 tumors isolated from WT, μMT, and DT-treated CD11c.DOG mice, 8 and 24 h post-Ab treatment (Fig. 7; data not shown). Any observed loss in TUNEL staining within tumor sections from B cell

![Figure 6. B cells and CD11c+ DC subsets are capable of MD5–1–mediated cytotoxicity in vitro.](image)

![Figure 7. Detection of MD5–1–mediated apoptosis in established MC38 tumors.](image)
and/or CD11c+ cell-deficient mice, compared with that observed in tumors isolated from MD5-1-treated WT mice, would identify whether one or both of these immune cell subsets was key in MD5-1-mediated apoptosis in vivo. Interestingly, quantitative analysis of TUNEL positive staining in random whole tumor sections from MD5-1- and clg-treated tumors, 8 h posttreatment, revealed a significant loss of MD5-1-induced TUNEL activity when B cells, but not CD11c+ DCs, were absent (Fig. 7). Similar results were also evident at 24 h post-Ab treatment; however, consistent with a rapid apoptotic effect, the level of detectable apoptosis was considerably reduced at that later time point (data not shown). Ultimately, these data identify B cells as being directly involved in, or upstream of, the first primary event in tumor suppression—MD5-1-mediated apoptosis in vivo. DT-treated CD11c.DOG mice that possess a fully competent B cell compartment, displayed the same quantity of TUNEL positive MC38 tumor cells as WT mice, post-MD5-1 treatment. Importantly, these data rule out a direct role for CD11c+ DCs in triggering MD5-1-mediated apoptosis in vivo, however, suggest that the profound loss of MD5-1 activity in these mice is a result of a downstream role for DCs in promoting and/or sustaining the antitumor effects of MD5-1 therapy in vivo.

Discussion
Based on the cell death and immune stimulating properties of mAbs, these targeted therapeutics have the capacity to significantly alter the immunogenicity of the tumor microenvironment and in turn increase tumor cell sensitivity to immune attack. Certainly, mAbs that kill tumor cells, like anti-DR5, have proven to be an effective means of priming tumor-specific cell-mediated immunity (10). Yet little is known about how this immunity is primed or even the innate and adaptive cellular components responsible for initiating the tumoricidal actions of anti-DR5 mAb therapy. Such information would ensure that the full therapeutic power of death inducing mAbs is best capitalized on in future combination strategies. Herein we have identified FeR2+ immune cell subsets that are responsible for MD5-1-mediated tumor cell apoptosis and MD5-1 antitumor activity in vivo. We report for the first time that B cells and CD11c+ DCs can play a critical role in MD5-1-mediated primary suppression of established MC38 tumors; whereas, CD11b+ and NK cells were not critical. Importantly, the contribution of B cells to the antitumor efficacy of MD5-1 was also validated in the B6-derived mammary carcinoma model, AT-3. Both B cells and DCs were identified as being capable of binding MD5-1 via their FeRs and permitting DR5-mediated apoptosis of tumor cells in vitro. These findings contrasted somewhat with our previous and present analysis of 4T1.2 tumors in which CD11b+ cells and, to a lesser extent, NK cells were important in the antitumor activity of MD5-1 (10). Interestingly, we identified that host DR5 expression, which is tightly regulated on immature DCs (29) and naive and activated B cells (30), did not influence the antitumor activity of MD5-1 in vivo. We have further shown that B cells, but not CD11c+ DCs, were critical for MD5-1-mediated tumor cell apoptosis in vivo, thus suggesting that CD11c+ DCs play an important role in the antitumor activity of MD5-1 downstream of primary tumor cell apoptosis.

Despite the distinct tumor microenvironments of TRAIL-sensitive MC38 and 4T1.2 tumors, we found that anti-DR5 therapy was still capable of inducing significant growth inhibition in both types of established tumors. Previously, we concluded that the antitumor effects of MD5-1 against implanted 4T1.2 tumor cells was primarily mediated by FeR on CD11b+ macrophages (10). This was also observed in established 4T1.2 tumors; however, we could not discount a possible role for FeR on neutrophils, which are present in large numbers within the 4T1.2 tumors (15 ± 8% of live tumor cells, n = 3). Certainly, neutrophils do posses potent cytotoxic activity that can be enhanced in the presence of antitumor mAbs (31). Interestingly, in the MC38 tumor model we identified B cells as critical for MD5-1-mediated apoptosis and antitumor activity. These data were surprising given the low frequency of B cells detectable within established MC38 tumors. However, the ex vivo analysis of a tumor specimen only provides a momentary snapshot of what is occurring within that tumor, and thus we cannot discount that the circulation of B cells into and out of the tumor microenvironment may be sufficient to enable MD5-1-bound B cells to trigger DR5-signaling of tumor cell apoptosis. What factors may promote this preferential interaction between B cells and MD5-1 within the tumor microenvironment and/or post-mAb administration is currently under investigation.

Characterization of DC involvement in MD5-1-mediated antitumor activity was performed using the CD11c.DOG transgenic mouse strain, that when treated with DT can be depleted of >90% of CD11chigh MHC class II+ DCs (17). In these mice, which possess an intact B cell compartment, MD5-1-mediated tumor cell apoptosis was still detectable; however, surprisingly this alone did not result in the therapeutic control of tumor growth. Ultimately, this result both eliminates a direct role for CD11c+ DCs in facilitating MD5-1-mediated apoptosis and implicates an important role of DCs downstream of tumor cell apoptosis mediated by MD5-1. Although DT treatment of these mice has also been reported to deplete a small proportion of F4/80+, MOMA-1+, and ERTR-9+ subpopulations of macrophages in the spleen (17), this is unlikely to have accounted for the loss of MD5-1 efficacy, because clodrolip, which can also target these macrophage populations (24), had no impact on the capacity of MD5-1 to inhibit the growth of established MC38 tumors. The mechanism by which DCs contribute to the antitumor activity of MD5-1 in vivo now remains to be resolved.

In the absence of equivalent strains on the BALB/c background, it remains to be tested whether DCs and B cells can also contribute to MD5-1 suppression of 4T1.2 tumors. However, it should be noted that in FcγRIIB-deficient BALB/c mice, MD5-1 exerted an antitumor effect equivalent to WT mice, possibly discounting a direct role for B cells in this tumor model (10). Certainly, this previously unreported contribution of B cells and DCs to the antitumor effects of MD5-1 is important and may have far-reaching clinical implications, particularly if this type of death induction can also promote DC uptake of tumor Ags and boost tumor immunity. This capacity of death-inducing mAbs like MD5-1 to potentially bridge dying tumor cells with immune cells that can promote tumor-specific immunity may have a number of important immunological consequences. Indeed, although Fc-mediated cross-linking of the MD5-1 mAb is critical to stimulate tumor cell apoptosis, this interaction may in turn help target tumor Ags to APCs and subsequently allow the development of tumor-specific immunity. If this is occurring, and CD8+ CD11c+ DCs are important, it still remains unclear what final effector mechanism causes tumor suppression. Ultimately the, optimization of the targeting of some antitumor mAbs to selective FcγRs on DCs and possibly even B cells will in some cases be beneficial to induce an efficient and durable effect on the tumor.

It is now becoming increasingly evident that many distinct types of apoptosis-inducing agents have the capacity to render tumor cell death immunogenic and thus prime cellular immune responses that can be mobilized to eliminate cancer (32, 33). Currently, little is known about the immunogenicity of TRAIL-mediated killing; however, one study has presented evidence to suggest that high-mobility group box 1 protein release from tumor cells, which through binding of TLR4 on DCs can stimulate the processing
and presentation of Ags to cytotoxic T lymphocytes (34), can be promoted by soluble TRAIL and possibly even by activated NK cells that express TRAIL (35). Given that we did not identify a role for CD8⁺ T cells in the antitumor responses mediated by MDS5-1, it could be argued that Ab-mediated DR5 signaling of tumor cell apoptosis is not a strong inducer of immunogenicity. Certainly, it will be important to consider whether targeting the TRAIL death receptors, DR5 and DR4 alone or in combination will have any bearing on the immunogenic outcomes of signaling TRAIL-mediated apoptosis. However, although killing tumor cells in a manner that generates danger signals and tumor Ags in the right context can promote engagement of innate and adaptive immunity, this response will likely prove insufficient in controlling established cancer because of the immunosuppressive nature of the tumor microenvironment. Indeed, we have evidence demonstrating that MC38 tumors are sensitive to T regulatory cell control and express high levels of the immune inhibitory program of the Fab' tumor cell apoptosis; a phenomenon likely dictated by the affinity development or in clinical trials need to be cross-linked to trigger studies have not been pursued. It is noteworthy though that not all xenografts was found to be markedly influenced by their isotype, and depending on the individual strength of these opposing complexes can trigger activating and inhibitory signaling pathways and present Ags to cytotoxic T cells in the clearance of lymphocytic choriomeningitis virus infection. Exp. J. Immunol. 27: 2626–2633.


