Immune complex–mediated antigen presentation induces tumor immunity

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Antigen uptake receptors on dendritic cells (DCs) provide efficient entry for the initiation of antigen-specific adaptive immunity. Here we show that targeting of antigen to Fc receptors on DCs accomplishes combined activation of Th1 CD4 and CD8 effector responses in vivo, namely delayed-type hypersensitivity and tumor immunity. Tumor immunity specific for ovalbumin-expressing tumors was provided by immunization with wild-type but not $Fc\gamma R\gamma^{-/-}$ DCs loaded with ovalbumin-containing immune complexes. Tumor protection was eliminated when immune complex–loaded DCs lacked β_2 microglobulin, TAP, or MHC class II, demonstrating that Fc receptor–targeted antigenic uptake led to both MHC class I– and class II–restricted responses, which together are required for effector tumor immunity. Thus the cross-presentation pathway accessed by antigens acquired endocytically through Fc receptors links humoral and cellular immunity. These data suggest that administration of antitumor antibodies may enhance tumor-specific T cell responses in vivo and provide the rationale for Fc receptor targeting in vaccine development.

Introduction

Adaptive tumor immunity is provided by collaborative MHC class II–restricted CD4 helper and MHC class I–restricted CD8 cytolytic effector responses. Thus, ideally, antigenic processing of tumor antigenic targets by antigen-presenting cells (APCs) should access both the MHC class I and MHC class II pathways. Yet, endocytosis or phagocytosis of extracellular antigens by antigen uptake receptors is processed primarily by APCs via the exogenous endosomal/lysosomal pathway, which ultimately delivers peptide onto surface MHC class II but not MHC class I molecules. However, CD8+ MHC class I responses can be initiated through the "cross-presentation pathway" (1, 2) via dendritic cells' (DCs') uptake of exogenous material, including transplanted (3), tumor (4–8), or virally infected cells (9). Previous efforts to exploit this cross-presentation pathway enabling access of extracellular antigens to the endogenous pathway have focused on DC phagocytosis of dying cells. Recent observations with in vitro systems (10–12) suggest that uptake of antigen through Fc

receptors (FcγRs) may represent an alternative method for cross-presentation. Schuurhuis (13) has shown that this pathway can induce antigen-specific CD8 responses in vivo, but the physiological relevance and potency of this pathway in effector immunity, including tumor immunity, have not yet been demonstrated.

The physiological consequences of cellular bound IgG and immune complexes (ICs) are mediated by FcγRs and include immunoregulatory and inflammatory responses. In particular, FcγRs on effector cells, including monocytes, neutrophils, mast cells, and NK cells, mediate phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), and release of cytokines, chemokines, and other inflammatory mediators central to the protective and injurious properties of antibodies (14). Genetic evidence in humans (15) and mice (16, 17) supports a general requirement for FcγR engagement for the efficacy of antitumor antibodies in vivo, implicating Fc-dependent effector cell ADCC as a common mechanism underlying tumor-specific humoral immunity. However, a requirement for CD8 cellular cytotoxicity for the efficacy of an antitumor mAb (18) suggests that in addition to mediating ADCC, FcγR–mediated enhancement of antigen presentation may also contribute to tumor immunity.

Capture of ICs by APCs likely contributes to the characteristic rapid appearance of high-titered antigen-specific IgGs during secondary exposures to antigen (19–21). This enhancement is due to the trapping, retention, and facilitated uptake of exogenous antigen by cellular complement and FcγRs on APCs and the subsequent intracellular targeting to the endosomal processing pathway for presentation onto membrane-expressed MHC class II molecules (22). Studies with in

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Nonstandard abbreviations used: dendritic cell (DC); antigen-presenting cell (APC); immune complex (IC); antibody-dependent cell-mediated cytotoxicity (ADCC); ovalbumin (OVA); wild-type (WT); horseradish peroxidase (HRP); delayed-type hypersensitivity (DTH); bone marrow–derived dendritic cell (BMDC); sheep red blood cell (SRBC); Fc receptor (FcγR).

vitro systems have shown that FcγR uptake through the endosomal system, in addition to enhancing MHC class II antigenic processing and presentation, provides access, through undetermined mechanisms, to the cytosolic or endogenous pathway (10–12). Furthermore, in some systems, engagement of activating FcγRs induces DC activation and maturation (23). Thus, in vitro observations have suggested that antigenic targeting to FcγRs on DCs accomplishes several important aspects of T cell priming in cancer: facilitated uptake of antigen, class I and II presentation of antigen, and induction of DC activation and maturation. Our studies demonstrate, for the first time to our knowledge, that antigens acquired through endocytosis by DCs, in this case IC uptake, can facilitate tumor immunity in vivo, a situation likely reflective of the combined induction of T cell effector immunity resulting from Th1- and class I–restricted CD8 cell priming.

Methods

Mice. C57BL/6 mice and *β₂M⁻/⁻*, *TAP1⁻/⁻*, and *MHC class II⁻/⁻* C57BL/6 congenic mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). The *FcγRγ⁻/⁻* knockout mice were obtained from Taconic Farms (Germantown, New York, USA). The C57Bl.6 congenic *OT-I* and *OT-II* mice, carrying T cell receptor transgenes recognizing ovalbumin (OVA) peptides restricted to H-2ᵇ MHC class I and class II, respectively, were provided by Janko Nikolic-Zugic (Oregon Health Sciences University, Portland, Oregon, USA) and Alan Frey (New York University School of Medicine, New York, New York, USA). All mice entered the study at 6 to 8 weeks old.

Cell lines. The MO-4 cell lines are stable OVA cDNA transfectants of B16F10 (provided by Alan Houghton, Memorial Sloan-Kettering Cancer Center, New York, New York, USA), and B16 cell lines (a C57BL/6 melanoma cell line) were obtained from the American Type Culture Collection (Manassas, Virginia, USA). Cell culture media consisted of RPMI-1640 (Cellgro, Herndon, Virginia, USA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated fetal calf serum (FCS). Geneticin (1 mg/ml) was added every week for maintenance of selective pressure on stable transformants.

Antigens and other reagents. The H-2Kᵇ–restricted peptide SIINFEKL, corresponding to amino acid residues 257–264, and the H-2Dᵇ–restricted 323–339 peptide (ISQAVHAAHAEINEAGR) of chicken OVA were synthesized by American Peptide Co. (Sunnyvale, California, USA) and by New England Peptide (Fitchburg, Massachusetts, USA) and highly purified (>95%) as assessed by HPLC. Crystallized and lyophilized OVA was obtained from Worthington Biochemical Corp. (Lakewood, New Jersey, USA). The lyophilized rabbit IgG fraction to chicken albumin (anti-OVA) was purchased from Cappel ICN (Aurora, Ohio, USA). Peroxidase/antiperoxidase ICs were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). All reagents were tested for measurable

endotoxin activity with the Limulus Amebocyte Lysate kit (Biowhittaker, Walkersville, Maryland, USA) and were assessed directly for DC maturation activity. No reagent induced any change in the immunophenotype of DCs following 48 hours of incubation.

Generation of bone marrow DCs. The procedure used for the generation of DCs was that described by Lutz et al. (24). After removing all muscle tissues from the femurs and tibiae, the bones were placed in a 100-mm dish with RPMI-1640. Both ends of the bones were cut with scissors in the dish, and then the marrow was flushed out using 2 ml of RPMI-1640 with a syringe and 25-gauge needle. The cell suspension was passed through nylon mesh to remove small pieces of bone and debris, and red cells were lysed with ammonium chloride. After washing, 2×10^5 cells were placed in a non-tissue-culture-coated Petri dish per ml of medium (RPMI, 10% FCS, penicillin, streptomycin, and L-glutamine) supplemented with either 20 ng/ml rGM-CSF (PeproTech Inc., Rocky Hill, New Jersey, USA) or 10% GM-CSF containing supernatant obtained from the GM-CSF transfectant J558L B cell hybridoma cell line (provided by Alan Houghton). At day 3, equal volume of fresh medium and GM-CSF was added to the plate. At day 6, 50% of the medium was aspirated and replaced with equal volume of fresh medium containing GM-CSF. DCs were harvested between days 7 and 10 of culture based on the morphological accumulation of medium-sized 10- to 50-cell DC aggregates, which appear at this time loosely attached to adherent cells. Immaturity of cells was confirmed by flow cytometric determination of low-level expression of both MHC class II and CD86. DCs were collected from plates by gentle aspiration and collection of nonadherent cells, which uniformly bore the DC immunophenotypic signature CD11c⁺CD14⁻.

Endocytosis and phagocytosis assays

Cellular immunofluorescence assessment of OVA uptake. Immature wild-type (WT) or *FcγRγ⁻/⁻* bone marrow–derived dendritic cells (BMDCs) were plated on lysine-coated coverslips and incubated with 10 µg/ml OVA alone, 50 µg/ml anti-OVA, or OVA-ICs (50 µg/ml anti-OVA and 10 µg/ml OVA) for 30 minutes. Cells were fixed in 4% paraformaldehyde and permeabilized with 1% Triton X-100. Cells were blocked in PBS/1% BSA, then stained with rabbit anti-OVA IgG (1:100 in PBS/1% BSA), washed, and visualized with FITC–anti-rabbit IgG (1:100 in PBS/1% BSA) using an E500 fluorescent microscope (Nikon Inc., Melville, New York, USA) and the RT SPOT digital camera/software package (Diagnostic Instruments, Sterling Heights, Michigan, USA).

Phagocytosis assays. 1% sheep red blood cells (SRBCs) were incubated with 1:500 dilutions of an IgG fraction of rabbit anti-SRBC (Cappel ICN) for 30 minutes at room temperature. Washed opsonized SRBCs were added to adherent BMDCs plated on tissue culture–coated glass slides (Becton Dickinson), Franklin Lakes, New Jersey, USA) 30 minutes earlier and incubated at 37°C for another 30 minutes. Unincorporated

SRBCs were removed by rapid hypotonic lysis in water and fixed in 0.025% glutaraldehyde/PBS.

Adoptive transfer with antigen-pulsed DCs and tumor challenge. Donors BMDCs were derived from either syngeneic WT C57BL/6 mice or congenic C57Bl.6 $\beta_2 M^{-/-}$, $TAP1^{-/-}$, $class\ II^{-/-}$, and $Fc\gamma R\gamma^{-/-}$ mice. Immature DCs were cultured with OVA and anti-OVA (10 and 50 µg/ml, respectively) or OVA alone (10 µg/ml). The antigen-pulsed DCs were washed three times in PBS and administered to naive WT mice at a dose of 1×10^6 cells per mouse in 200 µl PBS intravenously through the tail vein. One week later, 2×10^5 tumor cells (B16 or MO-4) were injected subcutaneously. Mice were monitored every 3 days for palpable tumors.

Cell proliferation assay. For restimulation of lymphocytes obtained after in vivo immunization, BMDC standard protocols were performed with a minor modification to reduce xenoprotein immunoreactivity upon restimulation with FCS in vitro. On day 6 of culture, bone marrow–derived DCs were removed from serum-containing media and cultured with fresh AIM V medium (GIBCO BRL; Life Technologies Inc., Grand Island, New York, USA) supplemented with 20 ng/ml GM-CSF for their final 6 days in culture prior to footpad immunization of recipients. Twelve days later, draining lymph node cells (2×10^6 per well) were incubated in RPMI-1640 with 1% FCS in 96-well flat-bottom microculture plates (Fisher Scientific Co., Pittsburgh, Pennsylvania, USA) in the presence of 10 µM OVA–peptide I (amino acid residues 257–264) or 10 µM OVA–peptide II (amino acid residues 323–339). After 2 days, cells were pulsed with 1 µCi ^{3}H-thymidine. Sixteen hours later, cells were harvested and thymidine incorporation was counted in a liquid scintillation counter. Proliferation of lymph node cells is expressed as the mean cpm of triplicate wells.

For in vitro stimulation of naive transgenic T cells, CD4 and CD8 T cells were positively selected with anti-CD4 and anti-CD8 beads (Miltenyi Biotec Inc., Auburn, California, USA) CD4 and CD8 T cell splenocytes were cultured at a cell density of 2×10^6/ml with 1×10^5/ml pulsed DCs in 96-well flat-bottom microculture plates. At day 3, cells were pulsed with 1 µCi ^{3}H-thymidine. Sixteen hours later, cells were harvested and thymidine incorporation was measured in a liquid scintillation counter. Proliferative responses are expressed as the mean cpm of triplicate wells.

For cytokine production, cells were plated at a cell density of 2×10^6/ml with 1×10^5/ml pulsed DCs in 24-well plates for 4 days prior to harvest of supernatants. For polyclonal activation, control T cells were plated on anti-CD3–coated plates (Pharmingen, San Diego, California, USA) in the presence of 10 µg/ml anti-CD28 (Pharmingen).

Anti-OVA Ig ELISA. Blood was collected 15 days after primary immunization of mice with pulsed DCs. Sera were tested by ELISA for total IgG, IgG1, or IgG2a. Maxisorp plates (Fisher Scientific Co.) were coated overnight at 4°C with 10 µg/ml OVA in coating buffer.

Fifty microliters of dilutions of both experimental samples and standard sera (obtained from mice immunized with OVA in CFA or Alum) were added to the plates and incubated overnight at 4°C. Plates were then incubated for 2 hours at room temperature with 50 µl of biotinylated anti-mouse IgG, IgG1, or IgG2a (diluted at 1:1,000) (Pharmingen), followed by incubation for 1 hour with 50 µl avidin–horseradish peroxidase (avidin-HRP) (diluted at 1:10,000). Finally, 50 µl 3,3′,5,5′-tetramethylbenzidine dihydrochloride (Sigma-Aldrich) was added, and OD at 450 nm was measured 10–20 minutes later. ELISAs were standardized using sera obtained from mice immunized with OVA in either CFA or Alum. OVA-specific IgE was not detectable in any BMDC-immunized mice.

Cytokine concentrations in culture supernatants were determined with a sandwich ELISA technique, using Cytoscreen IL-4 and IFN-γ ELISA kits to measure different epitopes of each cytokine (BioSource International Inc., Camarillo, California, USA).

Delayed-type hypersensitivity responses. Seven days after primary immunization, the mice were challenged with 30 µg OVA in 30 µl PBS injected into the right footpad. The left footpad was injected with 30 µl PBS alone. The thickness of the foot was measured with a Pocket Thickness Gauge (Mitumoyo Corp., Aurora, Illinois, USA) 24 hours after the challenge. Foot swelling as a result of OVA-specific delayed-type hypersensitivity (DTH) is expressed as the mean difference in swelling (mm) between the OVA- and PBS-challenged feet.

Results

Immunization with IC-loaded DCs provides tumor immunity. To demonstrate the physiological significance of IC-augmented T cell priming to the initiation of a relevant immune response in vivo, the ability of IC-loaded DCs to induce tumor immunity was tested in prevention, treatment, and recall responses (Figure 1). C57BL/6 mice were injected intravenously with BMDCs alone, OVA-pulsed BMDCs, or OVA/IC–pulsed BMDCs. In tumor prevention studies, 7 days after DC immunization, MO-4 cells (a syngeneic melanoma cell line transfected with the cDNA encoding OVA [ref. 25]) were injected subcutaneously into these mice (Figure 1a and Table 1) and mice were monitored for the development of palpable tumors. All mice developed large tumors when they were immunized with either BMDCs alone (9 of 10 tumor takes) or OVA-pulsed BMDCs (24 of 25 tumor takes). In contrast, only one tumor was palpable at 4 weeks in 25 mice immunized with OVA/IC–pulsed BMDCs. The OVA specificity of the antitumor immune response was confirmed since OVA/IC–pulsed BMDCs failed to offer any protection in mice injected with B16 melanoma cells (10 of 10 tumor takes) that did not express OVA. In the treatment studies, DC immunization was delayed until 7 days following injection with MO-4 cells. All mice receiving either BMDCs alone or BMDCs loaded with OVA developed tumors (10 of 10
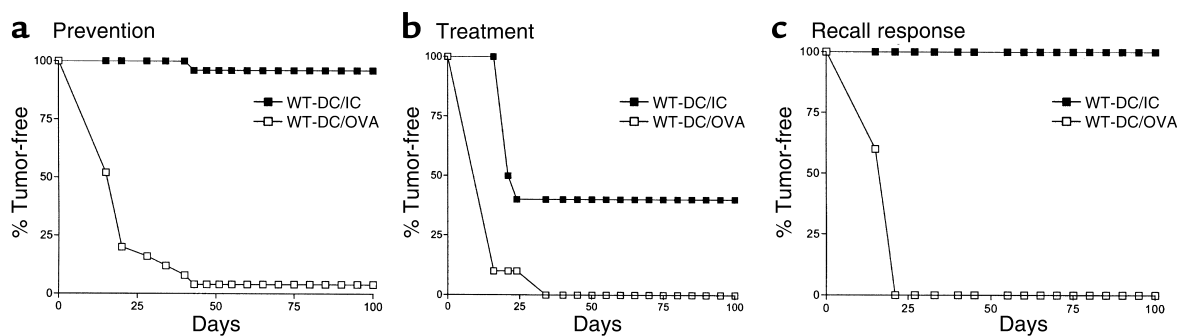
Figure 1
Immunization with BMDCs loaded with tumor antigen containing–ICs provides long-lasting and potent tumor immunity. (**a**) Prevention. Antigen-pulsed DCs (1×10^6 cells per mouse) were administered intravenously to naive WT mice ($n = 25$ per group). One week later, 2×10^5 MO-4 cells were injected subcutaneously and mice were monitored for the development of palpable tumor. No protection was provided by immunization with OVA-loaded BMDCs; 24 of 25 mice developed tumors. Near-complete protection was seen after immunization with OVA/IC–loaded BMDCs (only 1 of 25 mice developed tumors). (**b**) Treatment. 2×10^5 MO-4 cells were injected subcutaneously; 1 week later, antigen-pulsed DCs (1×10^6 cells per mouse) were administered intravenously to naive WT mice ($n = 10$ per group). (**c**) Recall response. Six months after intravenous immunization with antigen-pulsed BMDCs, WT mice ($n = 5$) were challenged subcutaneously with 2×10^5 MO-4 cells. No protection was provided by immunization with OVA-loaded BMDCs, but mice were fully protected 6 months after immunization with OVA/IC–loaded BMDCs. No significant differences in tumor protection were found between mice immunized with BMDCs alone and with BMDCs loaded with soluble OVA; for simplicity, the BMDC-alone group is not presented.

tumor takes in each group; Figure 1b and data not shown). In contrast, treatment with BMDCs loaded with ICs cured 40% of mice bearing early-established tumors. Protection mediated by BMDC/IC immunization was long-standing and induced memory responses, as MO-4 tumor takes were prevented in all mice challenged with tumor 6 months after initial immunization with IC-loaded BMDCs (Figure 1c).

IC-mediated tumor immunity results from FcR uptake of ICs and MHC class I and class II antigen presentation. To determine the mechanism of protection mediated by IC-loaded DCs, syngeneic DCs obtained from *FcγRγ*$^{-/-}$, *MHC class II*$^{-/-}$, *TAP1*$^{-/-}$, and *β₂M*$^{-/-}$ mice were used for immunization (Table 1). Uptake through activation FcγRs was required for induction of tumor immunity, as use of *FcγRγ*$^{-/-}$ BMDCs loaded with ICs failed to induce tumor immunity. Thus, uptake of OVA-containing ICs through the remaining FcγRs, FcγRII, does not result in T cell responses capable of rejecting tumors. We evaluated the contribution of both CD4 and CD8 responses to antitumor immunity by assessing recipients of IC-loaded *class II*$^{-/-}$, *β₂M*$^{-/-}$, and *TAP1*$^{-/-}$ DCs for tumor protection. Tumor immunity induced by IC/BMDC vaccination required that DCs contain both an intact MHC class I and MHC class II antigen presentation pathway, demonstrating that full protection requires combined CD4 and CD8 cellular responses. The impaired ability of *TAP1*$^{-/-}$ or *β₂M*$^{-/-}$ BMDCs to induce tumor immunity eliminates the possibility that recipient APCs were responsible for cross-presentation of transferred cells or material in vivo and instead confirms that transferred BMDCs were responsible for cross-priming MHC class I–restricted recipient T cells. Anti-OVA humoral responses were induced in recipients of IC-loaded *TAP1*$^{-/-}$ and *β₂M*$^{-/-}$ BMDCs (data not shown), indicating that anti-OVA IgG alone

was insufficient for protection. The impaired ability of *MHC class II*$^{-/-}$ BMDCs to provide tumor immunity implies an additional requirement for CD4 responses, although it does not distinguish between CD4-mediated contributions to either priming of OVA-specific CD8 cells (sensitization phase) or enhancement of the CD8 cytotoxic effector function (effector phase). Thus tumor immunity was significantly impaired in recipients of IC-loaded BMDCs harboring disrupted *MHC class II*, *β₂M*, or *TAP* genes, consistent with a requirement for FcγR–mediated access to both MHC class I and class II antigenic presentation pathways and for combined CD4 and CD8 T cell responses for antitumor effector immunity.

IC-loaded BMDCs induce antigen-specific humoral and CD4 and CD8 cellular responses in vivo. To investigate the mechanism of protection further, T cell responses and antibody responses were directly assessed in recipient mice of BMDCs alone, OVA-pulsed BMDCs, or OVA/IC–pulsed BMDCs (Figure 2). Total IgG was not affected by any immunization (data not shown), but specific anti-OVA IgG responses were detectable in BMDCs loaded with OVA, and these IgG responses were significantly enhanced after immunization with OVA/IC–loaded BMDCs (Figure 2b). Strong Th2 responses were not apparent with BMDC immunization, as IgE specific for OVA was not detectable in any treatment group. To directly assess T cell priming in vivo, C57BL/6 mice were injected intravenously with DCs loaded with OVA or OVA/ICs. Two weeks later, lymph node cells were obtained from these animals and restimulated in vitro with OVA MHC class I– or class II–restricted immunodominant peptides for measurement of OVA-specific CD8 and CD4 responses. In this assay, T cells from naive mice are not activated and proliferative responses upon in vitro restimulation are

indicative of in vivo priming. Mice that received DCs loaded with OVA or anti-OVA alone did not generate OVA-specific T cell responses. In contrast, T cells from mice that had received IC-loaded DCs had strong CD4 and CD8 OVA-specific proliferative responses upon restimulation with class II– and class I–restricted OVA peptides, respectively (Figure 2a). Thus, antigens acquired through FcγR–mediated endocytosis by DCs can prime both CD4 and CD8 responses in vivo.

ICs induce DC maturation and activation of naive CD8 cells and CD4 cells in vitro. Without exogenous inflammatory cytokines, bone marrow–derived DCs cultured for 10 days with GM-CSF are uniformly phenotypically immature and are highly endocytic and phagocytic, including through FcγRs. Amigorena and colleagues (10) reported that addition of ICs to these immature BMDC cultures resulted in cellular maturation and activation as seen by upregulation of costimulatory molecules (CD86, CD80, and CD40) and MHC class II. We similarly observed that OVA-containing ICs induced immuophenotypic maturation of WT but not activating FcγR-deficient cells (data not shown). Specifically, flow cytometric analysis revealed that 24 hours of IC stimulation of BMDCs led to increased surface expression of CD40, CD54, CD86, CD80, ICAM-1, and MHC class II. Neither OVA nor anti-OVA IgG alone enhanced expression of these immunophenotypic markers of maturation (data not shown) by either WT or $FcγRγ^{-/-}$ BMDCs. Interestingly, the upregulation of the adhesion molecule ICAM-1 (CD54) has been noted to be a Th1-driving influence (26, 27).

As seen in Figure 3, WT BMDCs exhibited efficient phagocytosis of antibody-opsonized SRBCs that was abrogated in $FcγRγ^{-/-}$ BMDCs, demonstrating a requirement for γ chain signaling for IgG-mediated phagocytosis, as previously reported for macrophages (28). In contrast, endocytosis of OVA/ICs was comparable by both WT and $FcγRγ^{-/-}$ BMDCs. Thus, although endocytosis of ICs through FcγRII is efficient, it is not accompanied by the induction of maturation and does not lead to effector tumor immunity.

To evaluate the contribution of DC maturation to the enhanced antigen presentation mediated by ICs, OVA-specific T cell receptor transgenic OT-I and OT-II naive OVA-specific CD8+ or CD4+ T cells, respectively,

were cultured in vitro with BMDCs alone or loaded with OVA or OVA-containing ICs. BMDCs loaded with OVA-containing ICs stimulated potent CD4 and CD8 proliferative responses, demonstrating the ability of IC-loaded antigen to prime both class I– and class II–restricted naive T cells (Figure 4a). Interestingly, this enhancement of antigen presentation appears specific for the antigen contained within the IC. Thus, addition of "irrelevant" HRP/rabbit anti-HRP (PAP) containing ICs to soluble OVA succeeded in inducing DC maturation (data not shown) but failed to enhance "bystander" OVA-specific class I– or II–restricted antigen presentation (Figure 4a). Thus, IC-mediated maturation is not likely to prime T cell responses to other self and non-self antigens to which the DC is exposed at the time of IC stimulation. Further, these data imply that DC maturation induced by ICs is not sufficient to fully explain their enhancement of T cell priming capacities. Other IC-mediated pathways must also contribute, including their facilitated entry into the antigen processing/presentation MHC class I and II pathways.
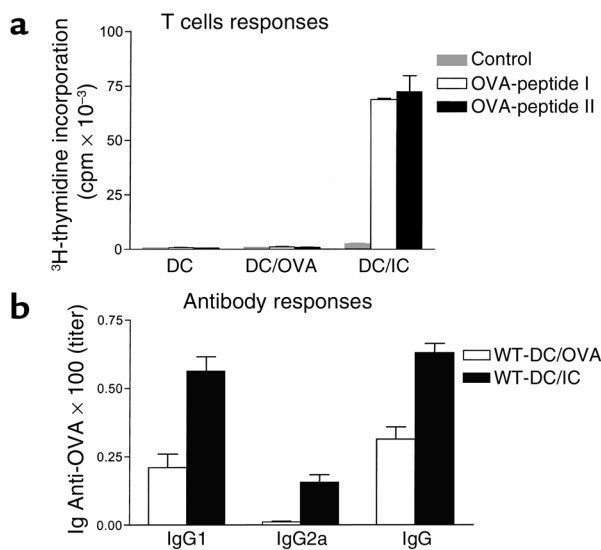
IC-loaded BMDCs induce Th1 responses in vitro and sensitize for DTH response in vivo. DCs critically regulate the initial steps in the immune response by providing a number of signals including chemokines and cytokines that polarize the developing T cell response into either a Th1 or a Th2 pathway (29). As the development of a cytolytic CD8 response would be enhanced by a simultaneous polarized Th1 CD4 helper response (30, 31), we sought to determine whether IC-loaded DCs were functionally polarized to drive Th1, rather than Th2, CD4 responses by analysis of the T cell production of Th1 and Th2 cytokines (Figure 4b). Under nonpolarizing conditions, OT-II transgenic CD4+ T cells produced considerable amounts of both IL-4 and IFN-γ when stimulated by αCD3/αCD28, consistent with activation of a heterogenous population of both Th1- and Th2-polarized T cells. Immature BMDCs loaded with 10 μM class II–restricted OVA peptide 323–339 preferentially induced the production of Th2 cytokines by stimulated OT-II cells. In contrast, stimulation of naive CD4+ OT-II T cells by IC-loaded BMDCs induced T cell activation and production of three times more IFN-γ than IL-4, consistent with a Th1 polarized response. IFN-γ was also elaborated by OT-I CD8+ T cells upon

Table 1
Tumor takes in recipients of OVA/IC–loaded BMDCs: tumor immunity requires MHC class I and II antigen presentation and activating FcγRs

| Loaded antigen | WT DCs/MO-4 tumors | DC Genotype | | | | WT DCs/B16 tumors |
		$FcγRγ^{-/-}$	MHC class II$^{-/-}$	TAP1$^{-/-}$	$β_2M^{-/-}$	
-	9/10	5/5	5/5	5/5	5/5	10/10
OVA	24/25	5/5	5/5	5/5	5/5	ND
OVA/ICs	1/25	4/5	4/5	4/5	4/5	10/10

Mice were intravenously immunized with either 10^6 BMDCS alone or 10^6 BMDCs loaded with either OVA or OVA/ICs, and then subcutaneously challenged 7 days later with 2×10^5 MO-4 B16 OVA transfectant cells. Recipients of WT DCs were challenged with either MO-4 cells (far left) or nontransfected B16 parental cells (far right). Data are expressed as the number of mice containing palpable tumors after 6 weeks over the total number of mice injected with tumor cells. IC-loaded WT BMDCs offered significant protection only for MO-4 tumors, not for B16 parental tumors. IC-loaded BMDCs lacking either activating Fcγ receptors or MHC class II, TAP, or $β_2M$ genes provided no significant protection compared with WT DCs (*P* = 0.0008, Fisher's exact test). ND, not determined.

Figure 2

IC-loaded BMDCs induce OVA-specific cellular and humoral immunity in vivo. (**a**) OVA–peptide MHC class I– and II–restricted cellular immune responses result from OVA/IC–loaded BMDC immunization. Proliferative responses occur after addition of MHC class I– or class II–restricted peptides to lymph node cell populations from mice immunized with BMDCs loaded with OVA/ICs but not OVA alone. (**b**) Antibody responses are enhanced after immunization with OVA/IC–loaded BMDCs. OVA-specific ELISA reveals higher OVA-specific total IgG, IgG1, and IgG2a titers in diluted sera from mice immunized 15 days earlier with IC-pulsed rather than OVA-pulsed BMDCs (ten mice per group).

activation during exposure to IC-loaded BMDCs, demonstrating that CD8 expansion and effector cell differentiation could be induced by IC-loaded BMDCs. In each case, intracellular staining confirmed that the cellular origin of IFN-γ production were CD4⁺ or CD8⁺ cells rather than cocultured CD11c⁺ DCs (data not shown). Notably, although minor proliferative responses were observed upon exposure of naive transgenic T cells to BMDCs loaded with OVA alone, no detectable IFN-γ production could be found in these cultures.

The effector phase of the DTH responses is the result of a Th1-driven inflammatory response (32) initiated upon a recall response to antigen. To address the relevance of the production of Th1 cytokines by OT-II cells in vitro to the situation in vivo, DTH responses were evaluated in normal mice sensitized with either BMDCs alone or BMDCs loaded with OVA or OVA-containing ICs (Figure 5). Seven days after sensitization, recall responses were elicited by subcutaneous footpad challenge with soluble OVA. Inflammatory recall responses were not induced in recipients of BMDCs loaded with OVA alone. In contrast, all mice immunized with IC-loaded BMDCs developed footpad swelling, consistent with the induction of a Th1-polarized CD4 effector response in vivo. Thus, immunization with IC-loaded BMDCs induces both Th1 and CD8 effector immunity. Through engagement of FcγRs on DCs, antibody can induce CD4 and CD8 cellular

effector immunity, and this suggests new strategies for the induction of tumor immunity.

Discussion

Passive immunization with antitumor antibodies represents a new class of therapeutics in the treatment of human malignancy. Mechanistically, efficacy of antitumor antibodies has been attributed to direct growth-inhibitory effects of tumor cells, complement-mediated cytotoxicity, or ADCC resulting from FcγR activation on myeloid and NK effector cells (33). Genetic evidence in humans (15) and mice (16, 17) supports a general requirement for FcγRs engagement for the efficacy of antitumor antibodies in vivo, implicating Fc-dependent effector ADCC as a common mechanism underlying tumor-specific humoral immunity. However, a requirement for CD8 cellular cytotoxicity for the efficacy of an antitumor mAb (18, 34) suggests that, in addition to mediation of ADCC, FcγR–mediated enhancement of antigen presentation is another mechanism contributing to tumor immunity. The data presented here directly demonstrate that FcγR targeting of DCs results in tumor immunity through the activation of antigen-specific naive CD4 and CD8 T cells. Thus, an additional component of antitumor antibody efficacy, not previously appreciated, may be the induction of tumor antigen–specific T cell responses.

The priming of naive T cells in vivo by extracellular antigen is orchestrated by DCs, which have multiple pathways for antigen uptake, including macropinocytosis, endocytosis, and phagocytosis (29). Processing of extracellular antigen via the exogenous pathway cul-
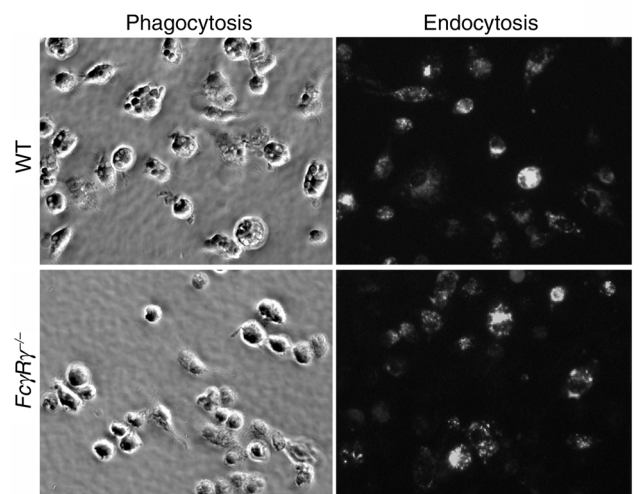
Figure 3

Phagocytic but not endocytic uptake of antibody-complexed antigen requires activating FcγRs on DCs. Immature BMDCs were incubated for 30 minutes with either OVA-containing ICs for endocytosis or antibody-opsonized SRBCs for phagocytosis. Uptake of soluble OVA or anti-OVA alone was negligible (data not shown), while uptake of OVA/ICs was dramatically enhanced for both WT and *FcγRγ⁻/⁻* BMDCs, indicating that inhibitory FcRs are capable of efficiently endocytosing OVA/ICs. In contrast, phagocytic uptake of antibody-opsonized SRBCs was seen in WT but not in *FcγRγ⁻/⁻* BMDCs.

minates in the presentation of antigenic peptides on MHC class II for the activation of CD4 helper T cell responses and humoral responses in vivo (35–37). Indeed, our data are consistent with other studies (19, 38–40) in demonstrating that DC uptake of immune-complexed antigen through activating FcγR–mediated endocytosis results in augmented CD4 priming in vitro and in vivo. Furthermore, IC-loaded BMDCs preferentially polarized Th1 effector cell differentiation of naive OT-II transgenic CD4 cells. The quality of the CD4 effector Th1-polarized response in vivo is demonstrated by the DTH responses seen in OVA-challenged BMDC OVA/IC–sensitized mice. The DTH response, dependent on CD4 cell elaboration of Th1 cytokines, was abrogated in *FcγRγ*$^{–/–}$ mice (38), but this previous study could not distinguish between requirements for FcγR on subtypes of FcγR-bearing cells, including APCs and cellular effectors during sensitization and/or challenge phases. Our data suggest that APC expression of activating FcγRs contributes to the induction of antibody-enhanced Th1 responses.

In addition to enhancing CD4 responses, FcγR targeting accomplishes a goal of effective tumor immunization, namely the induction of potent CD8 effector immunity specific for tumor-specific antigens. Recent in vitro studies have shown that DC FcγR targeting of soluble ICs (10) and opsonized human tumor cells (11) can facilitate class I–restricted antigen presentation to antigen-experienced CD8 T cells. In our studies we show that DC uptake of ICs can directly induce the proliferation and activation of effector cell differentiation of MHC class I–restricted naive transgenic OT-I CD8 cells. Tumor protection studies showed that protective immunity required DCs to harbor intact MHC class I and II antigen presentation pathways. This eliminates the possibility that transferred DCs were cross-presented by host DCs and demonstrates that the FcγR-mediated cross-presentation pathway is required for CD8 responses in vivo. Further, a requirement for DC MHC class II expression suggests that both CD4 and CD8 responses collaborate in vivo for full tumor protection. Our data cannot distinguish whether IC-induced Th1 CD4 responses collaborate with CD8 cellular cytotoxicity by providing IFN-γ to augment CD8 responses during sensitization (41) and/or at the effector level (30, 31). Thus, FcγR targeting has the potential to accomplish several important features for vaccine design; efficient antigen uptake, and processing of antigen for both MHC class I and class II antigenic presentation for priming of both CD4 and CD8 cells.

IC-loaded BMDCs induced potent and long-lasting effector tumor immunity in vivo, curing 40% of early-established MO-4 tumors and providing effector memory responses for at least 6 months. Tumor immunity was antigen-specific, as no protection was seen when using the parental cell line B16F10, which lacks OVA expression. This dismisses the possibility that presentation of FCS-derived xenogenic antigens by cultured DCs is solely responsible for tumor immunity; however, xenogenic responses may still contribute to rejection of OVA-expressing tumors (42).

Figure 4

OVA/IC–mediated antigen presentation in vitro induces Th1 CD4 and CD8 cellular proliferation and effector differentiation of naive transgenic T cells. (**a**) Anti-OVA T cell responses. IC-mediated antigen presentation efficiently drives OT-I and OT-II proliferative responses in vitro. 2×10^5 CD4 OT-II and 4×10^5 CD8 OT-I cells were cultured with 10^4 or 2×10^4 antigen-pulsed BMDCs in 96-well culture plates. ^{3}H-thymidine incorporation was measured between 36 and 48 hours later and expressed in cpm. OVA (10 μg/ml), OVA-containing ICs (10 μg/ml OVA and 50 μg/ml anti-OVA), or OVA (10 μg/ml) plus "irrelevant" peroxidase/antiperoxidase (PAP) complexes (50 μg/ml) were added to DCs for 48 hours prior to incubation with T cells. The results are representative of five separate experiments. (**b**) Cytokine production. IC-loaded BMDCs induce IFN-γ production by naive OT-I and OT-II transgenic cells. CD4 OT-II or CD8 OT-I transgenic T cells were cultured at 10^6/ml with 5×10^4/ml pulsed DCs in 24-well plates. IL-4 and IFN-γ production was determined by ELISA after 3–5 days of culture. The results are from two independent experiments. The sensitivity of the ELISAs were 1 pg/ml. Culture of OT-I or OT-II transgenic T cells with DCs alone (i.e., without OVA peptides or soluble OVA) did not elaborate detectable IFN-γ or IL-4.
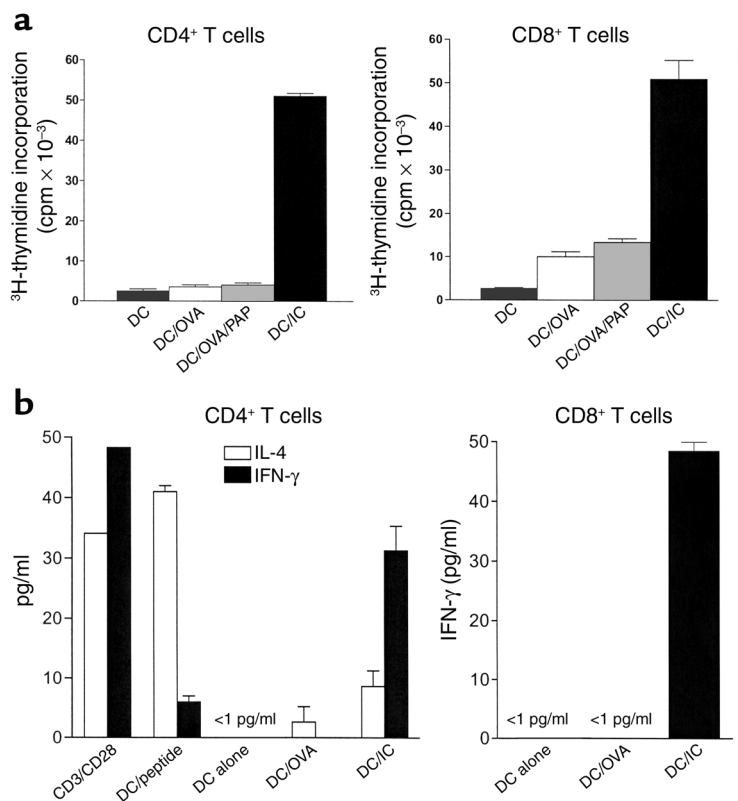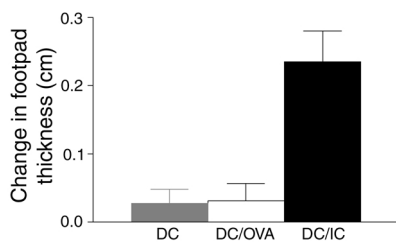
Figure 5
Sensitization with IC-loaded BMDCs induces DTH effector responses. Groups of eight to ten mice were immunized with 1×10^6 cells per mouse of OVA-pulsed DCs or IC-pulsed DCs in the front footpad. One week after primary immunization (sensitization), the mice were challenged with PBS in one footpad and OVA in the other footpad. Change in footpad thickness (OVA-injected footpad vs. PBS-injected control footpad) was measured 9 days after initial immunization. Error bars are the SEM of each group.

Interestingly, this enhancement of antigen presentation appears specific for the antigen contained within the IC. The addition of "irrelevant" HRP/anti-HRP containing ICs induced DC maturation but did not augment the presentation of soluble "bystander" OVA or immunodominant OVA-derived peptides (Figure 4 and data not shown). Thus, DC maturation induced by ICs is not sufficient to fully explain their enhancement of T cell priming capacities. Other IC-mediated pathways must also contribute, including their facilitated entry into the activating FcγR antigen presentation pathways for MHC class I and class II. Others have noted that DC maturation results in an initial burst of antigen processing followed by a rapid shut-down of further processing. This results in a "snapshot" display of antigenic peptides synchronous with the initiation of the maturation stimulus (25). However, this display, even if temporally constrained, would likely result in the display and potential T cell priming of many stimulus-unrelated peptides, including those with self-specificities, if the processing were not further restricted. In the case of ICs it appears that the enhancement of antigen processing is coupled to the antigen within the IC, thus limiting extraneous and perhaps injurious priming of other irrelevant self and non-self antigens. Thus, IC-loaded DCs induce both CD4 and CD8 priming in vitro, in a manner specific for the antigen contained within the IC.

While activating FcγRs are required for tumor immunity, the data presented here do not distinguish between potential roles for the two activating FcγRs, types I and III. Previous studies have demonstrated that targeting FcγRI can result in enhanced humoral responses (40, 43, 44), but class I–restricted responses were not evaluated. Recent in vitro studies with $FcγRI^{-/-}$ and $FcγRIII^{-/-}$-deficient BMDCs have suggested a role for both of these receptors in IC-mediated cross-presentation (45). Furthermore, while uptake by FcγRII alone was insufficient to induce tumor responses, a potential role for this inhibitory receptor (46) in modulating DC function remains to be determined. FcγRII is highly expressed on immature BMDCs and efficiently endocytoses ICs (Figure 3). Entry of ICs through FcγRII may lead to an endosomal processing pathway distinct from that accessed by the γ-associated FcγRI and FcγRIII receptors (47). Preliminary work in our laboratory suggests that IC targeting to lysosomes occurs rapidly after entry by FcγRI and III but not after entry by FcγRII. Indeed, the comparable level of intracellular ICs seen in WT BMDCs (which express all three FcγRs) and $FcγRγ^{-/-}$ BMDCs (which express only FcγRII) is consistent with a delay in degradation and resultant accumulation of ICs in FcγRII-bearing $FcγR^{-/-}$ BMDCs.

Thus, FcγR targeting of antigen provides BMDCs the ability to prime both CD4 and CD8 responses in vivo. The ability of antigen-specific antibody to complex antigen and facilitate its uptake and retention by APCs is well described for the enhancement of MHC class II–restricted responses during secondary responses. The data presented here are consistent with the notion that antigen-containing ICs may also facilitate the activation of MHC class I–restricted responses by CD8 cells. Thus, antibody formed during a primary immune response would augment the potential for CD8-mediated cellular immunity upon secondary exposure to antigen, thus linking humoral and cellular immunity to tumor, auto-, allo-, or microbial antigens. Regarding tumor immunity, our data suggest that antitumor antibody–mediated efficacy may result from antibody-mediated enhancement of tumor antigen presentation to T cells, suggesting alternative approaches to improving efficacy. Finally, activating FcγR targeting may represent a novel approach for vaccine design for active immunization.

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