Immunotherapeutic Potential of B7-DC (PD-L2) Cross-Linking Antibody In Confining Antitumor Immunity

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ABSTRACT

A naturally occurring human antibody potentiates dendritic cell function on cross-linking B7-DC (PD-L2), supporting robust T-cell responses in vitro. Moreover, treatment of dendritic cells with B7-DC cross-linking antibody resulted in secretion of interleukin-12, suggesting a TH1 polarization of this response. Here we show an in vivo immunotherapeutic effect of this B7-DC cross-linking antibody using a poorly immunogenic B16 melanoma tumor model. Treatment of mice systemically with antibody at the time of tumor cell engraftment prevented tumor growth in a CD4 and CD8 T-cell-dependent manner. The protective effect of B7-DC cross-linking antibody treatment was independent of endogenous antibody responses. Tumor-specific CTL precursors could be isolated from lymph nodes draining the tumor site in animals treated with B7-DC cross-linking antibody, but not from those treated with isotype control antibodies. The elicited antitumor responses in vivo were specific and long-lasting. More strikingly, treatment of mice with B7-DC cross-linking antibody after the tumors were established in the lungs resulted in protection in a CD4- and CD8- deficient fashion. Depletion of natural killer cells did not block the effects of treatment with B7-DC cross-linking antibody. Together, these findings demonstrate that cross-linking B7-DC with the human IgM antibody sHlgH12 can induce a protective immune response against a weakly antigenic experimental tumor and therefore has potential as a novel immunotherapeutic approach for treating cancer.

INTRODUCTION

We recently discovered that the immune-regulatory properties of dendritic cells (DCs) can be potentiated in vitro by treatment with an IgM antibody that cross-links the B7 costimulatory family member B7-DC (PD-L2) on their cell surface. After antibody treatment in vitro, the DCs display increased (a) survival, (b) processing of exogenous protein into antigenic peptides for presentation on the class I pathway, (c) production of cytokines such as interleukin-12, and (d) ability to activate naïve T cells (1, 2). Delivery of small amounts of the IgM antibody i.v. to animals at the time of DC transplantation enhanced the ability of the distantly implanted cells to migrate to the draining lymph nodes and effectively activate naïve T cells in vitro after recovery from the nodes (3). The observation that endogenous DCs, freshly isolated from mice, are bound by the B7-DC cross-linking antibody (2) and the accompanying secretion of IL-12 in vitro (1) raised the possibility that system treatment of animals with antibody might be used to potentiate the immune response to growing tumors.

Developing tumors express sets of antigens that are potential targets for the immune system. When tumors become established, these antigens have either been ignored or ineffective in generating an immune response that can protect the host from tumor development. Tumor resistance mechanisms include down-modulation of MHC class I (3), loss of tumor-associated antigens (4), and expression of immnosuppressive molecules such as transforming growth factor β (5). Tumors also inhibit T-cell response by inducing peripheral tolerance (6) or by inducing T regulatory cells (7). Immunotherapy strategies seek to provide the necessary stimuli to overcome factors limiting the immunogenicity of tumor-associated antigens to promote effective antitumor immunity. Among strategies that have proven effective are protocols designed to raise antigen dose, recruit and mobilize antigen-presenting cells, and promote the activation and survival of effective T cells (8, 9).

Antigen-specific T-cell responses are initiated by DCs. DCs are capable of capturing antigens that are secreted or shed by tumor cells, resulting in presentation of peptides on both class I and class II MHC molecules. Response to maturation signals, DCs up-regulate their expression of costimulatory molecules and cytokines, which efficiently prime CD4 and CD8 T cells (10–12). Techniques have been established for generating DCs ex vivo, resulting in the development of DC-based vaccines for tumors (13, 14). Because costimulatory signals expressed by antigen-presenting cells are critical in determining the outcome of T-cell-dependent immune responses, improved responses have been achieved by introducing costimulatory molecules such as B7-1, B7-2, and B7-DC into tumors (15–17) and then using the engineered cells to induce effective immune responses. B7-DC/PD-L2 belongs to the expanding family of B7 proteins. B7-DC, like other family members, contains immunoglobulin-like extracellular domains, a transmembrane domain, and a short cytoplasmic tail. PD-1 has been identified as a cognate receptor for B7-DC, PD-1 is expressed on activated T cells. Data exist to support both positive and negative modulation of T-cell responses after interaction between B7-DC and its receptors (18, 19). These seemingly contradictory findings might be explained by the possible existence of receptors other than PD-1 that can interact with B7-DC. The ability of B7-DC to bind to PD-1-deficient T cells supports this possibility. Moreover, mutagenesis and modeling studies indicate that the binding site for B7-DC is distinct from that of B7-H1, another ligand for PD-1 (20, 21). Potential differences in the interaction of B7-DC and B7-H1 with different receptors may influence whether positive or negative immune modulation is elicited.

In this report, we document the potentiation of the immune response against B16 melanoma after systemic treatment of mice with small amounts (30 μg) of B7-DC cross-linking antibody in both prophylactic and therapeutic models. A number of different effector mechanisms involving cytotoxic cells and protective lymphokines have been defined in other immunotherapeutic schemes for treatment of B16 melanoma (22–24). Here, we provide evidence that the enhanced immunity induced with B7-DC cross-linking antibody is mediated by T cells and dependent on key components of their cytotoxic granules.

MATERIALS AND METHODS

Mice. Wild-type C57BL/6J, B6.129S2-Cd4tm1Mak/J (CD4−/−), B6.129S2-Cd8tm1Mak/J (CD8−/−), B6.129S7-Rag1tm1Mom/J (Rag1−/−), B6.129S2-GzmBtm1Ley/J (granzyyme B−/−) strains of mice, all on the C57BL genetic
background, were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129P2-B2m<sup>tm1Unc/J</sup> (β2-microglobulin<sup>−/−</sup>) mice were bred in the inmu-
nonegenic mouse colony (C. David, Mayo Clinic). B-cell-deficient μMT mice (25) were obtained from M. Cascallo (Mayo Clinic), C57BL/6-
Prf1<sup>tm1Sider</sup>/J (perforin<sup>−/−</sup>) mice were originally obtained from The Jackson Laboratory and subsequently bred in the mouse colony of M. Rodriguez (Mayo Clinic).

Reagents. The B7-DC cross-linking antibody sHIgM12 was purified from serum of a patient with Waldenström’s macroglobulinemia as described previously (2). A preparation of polyclonal human IgM antibody (2) and an IgM antibody (sHIgM39) purified from the serum of a patient with chronic lymphoproliferative disorder were used as isotype control antibodies. Bouin’s fixative was obtained from Sigma-Aldrich (St. Louis, MO). B16, B16-F10, and EL-4 lines of tumors were maintained in RPMI 1640 (Cambrex Bioscience, Walkersville, MD) containing 10% calf serum (GIBCO Invitrogen, Grand Island, NY). Phycocerythrin-coupled antibody to pan-natural killer (NK) cell marker DX-5 was obtained from Pharmingen (San Diego, CA). NK1.1 (PK136) was obtained from L. Chen (Mayo Clinic). Lipopolysaccharide and polynonsic polyclidicylic acid (poly I:C) were purchased from Calbiochem. Cpg (TCCATGACGTTCCTGACGTT) was synthesized in the Mayo Clinic molecular biology core facility.

Prophylactic Regimen. In this protocol, mice received injection with 2 × 10⁶ B16 cells on the right flank in a 100-μl volume. Animals received injection with 10 μg of sHIgM12 or control antibody in 100 μl of PBS the day before, the day of, and the day after tumor challenge. Mice were monitored for the development of tumor and euthanized when tumor size reached 225 mm². The size of the tumors was determined in two dimensions using calipers (Dyer, Lancaster, PA). The mice that failed to develop tumors for 30 days were rechallenged with 2 × 10⁶ B16 cells on the left flank. Rechallenged mice were euthanized for another 30 days. Separate sets of mice that resisted B16 melanoma grafts were challenged with 2 × 10⁶ EL-4 cells on the opposite flank and monitored for tumor growth. Naïve mice injected with 2 × 10⁶ EL-4 cells served as controls for this test of specificity of tumor resistance.

Therapeutic Regimen. In this protocol, mice received injection with 5 × 10³ B16-F10 cells i.v. Mice were treated with 10 μg of sHIgM12 as a control antibody on days 3, 4, and 5. Sentinel mice received injection with the B16 tumor cells and received no further treatments. Tumor load in sentinel animals was monitored by examining the lungs of individual animals on a weekly basis to ascertain when a significant tumor load was present in the tumor-bearing groups. When a sentinel was detected that had developed 50 or more tumor nodules in its lungs, the animals in the experimental treatment groups were euthanized, and tumor nodules in their lungs were counted. For depletion experiments, 200 μg of NK1.1 monoclonal antibody was injected i.v. 24 h before tumor injection on day 0 and 48 h before tumor injection on day 3. Tumor engraftment was monitored in 3–4 days thereafter for the duration of the experiment. Depletion of the appropriate cell subset was confirmed by flow cytometry using anti-NK1.1 and anti-DX-5 antibody. In our hands, the sentinel mice developed nodules in the lungs by days 17–21. Mice from all of the experimental groups were then euthanized, and the lungs were fixed with Bouin’s fixative. The number of nodules was determined using a dissection microscope.

Flow Cytometry. Sera were collected from mice that had been engrafted with B16 melanoma on the flank and treated using the prophylactic regimen with either control polyclonal IgM antibody or sHIgM12 antibody. Dilutions of the sera were assayed for the presence of anti-tumor antibodies using standard flow cytometry (1) to assess binding to B16 melanoma cells. DCs derived from bone marrow precursors in vitro were isolated directly from the spleens of animals as described previously (2) were analyzed by multicolor flow cytometry to assess binding to B16 melanoma cells. DCs derived from the host were stained with anti-CD11c-APC.

Cytotoxicity Assay. Briefly, 5 × 10³ B16 melanoma cells were injected in the right flank of C57BL/6 mice. Mice received i.v. injection with 10 μg of either control antibody or B7-DC cross-linking antibody on the day before, the day of, and the day after tumor injection. Seven days later, cells from the draining lymph nodes from 5 mice/group were harvested, pooled, and further stimulated by mitomycin C (Calbiochem)-treated B16 melanoma cells (2 × 10⁶ cells/ml) for an additional 4 days. The effector cells were harvested and titrated in triplicate against ⁵¹Cr (Amersham)-labeled B16 or EL-4 cells in a standard 4-h cytotoxicity assay.

Statistical Analysis. Statistical analysis was performed on normally distributed data using ANOVA for multiple comparisons or Student’s test for comparisons of two groups. For data that were not distributed normally, the Whitney rank-sum test was used. For analysis of two treatment groups and a rank ANOVA was used for comparison of more than two treatment groups.

RESULTS

Systemic Treatment with sHIgM12 B7-DC Cross-Linking Antibody Induces Resistance to B16 Melanoma Transplanted s.c. in the Flank of B6 Mice. B16 melanoma is an aggressive tumor derived from C57BL mice that kills immunocompetent animals receiving a s.c. inoculum of as few as 2 × 10⁵ cells. In this model, palpable tumors develop between 10 and 12 days after tumor transplantation. Typically, tumors progress to surface areas in excess of 225 mm² by day 17, at which time animals are euthanized according to Institutional Animal Care and Use Committee protocol. Mice received treatment with an isotype control antibody, PBS, or sHIgM12 B7-DC cross-linking antibody injected i.v. at a distant site on days −1, 0, and +1 relative to tumor transplant. Whereas only 1 of 13 mice (7%) treated with an isotype control polyclonal IgM antibody and 0 of 13 treated animals were tumor free at day 17, 11 of 16 (69%) mice receiving sHIgM12 antibody remained tumor free (P < 0.001, Table 1). For mice that did develop palpable tumors, sHIgM12 antibody treatment significantly inhibited the growth of the tumor as compared with tumors growing in mice treated with PBS or polyclonal human IgM antibody (P < 0.001; Table 1). The delay in growth was transient because tumors that did develop in sHIgM12-treated mice eventually progressed to 225 cm² in size. In a separate experiment, mice treated with sHIgM12 antibody on days 9, 8, and 7 before challenge with B16 melanoma displayed no treatment effects on tumor growth; 100% of the animals treated with B7-DC cross-linking antibody (n = 8) or isotype control antibody (n = 8) developed tumors with the same kinetics (data not shown). This finding indicates that the timing of treatment relative to tumor engraftment is an important factor in determining treatment outcome.

Induced Resistance to B16 Melanoma by Systemic sHIgM12 Antibody Treatment Is Immune Mediated. We first considered the possibility that the sHIgM12 antibody acts directly on the B16 tumor cells. Some murine tumor cell lines, particularly those of hematopoietic origin, have been reported to express B7-DC mRNA message (26). Using flow cytometry, we evaluated whether the antibody sHIgM12 binds directly to tumor cells and found no binding, suggesting that the antibody may be acting indirectly on the tumor cells by binding to cells derived from the host.

Our earlier studies demonstrated that sHIgM12 antibody binds to

<table>
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<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Tumor-free (day 17)</th>
<th>Statistic</th>
<th>Average tumor size (mm²)</th>
<th>SE (mm²)</th>
<th>Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6δ</td>
<td>pHIgM</td>
<td>1/13</td>
<td>Reference</td>
<td>167.0 ± 21.2</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>C57BL/6δ</td>
<td>PBS</td>
<td>0/13</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>182.1 ± 21.3</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>C57BL/6δ</td>
<td>sHIgM12</td>
<td>11/16</td>
<td>P &lt; 0.001</td>
<td>13.2 ± 7.5</td>
<td>P &lt; 0.001</td>
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</table>

<sup>a</sup> NS, no statistical difference.
DCs derived from bone marrow precursors in vitro. This binding activity suggests that the induced resistance to the lethal tumor challenge may be mediated by antibody interactions with endogenous DCs. Modulation of DC function could promote changes in the immune response and be the underlying mechanism determining antibody-induced tumor resistance. We explored this possibility using two approaches. First, we evaluated whether sHIgM12 antibody administered systemically to mice modulated the phenotype of endogenous DCs. Second, we evaluated whether in vivo administration of the antibody induced tumor resistance by potentiating an antitumor response.

Using DCs generated in vitro from bone marrow precursors, we had previously shown that treatment with sHIgM12 B7-DC cross-linking antibody potentiates the ability of these cells to activate naïve antigen-specific T cells but does not induce traditional maturation markers. As DCs mature, they lose the ability to acquire antigen from their surroundings and increase their expression of the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) on their cell surfaces. We demonstrated previously that B7.1 and B7.2 expression levels do not substantially increase after treatment of bone marrow-derived myeloid DCs with sHIgM12 antibody in vitro (2). To evaluate functional changes associated with activation after B7-DC antibody treatment, we assessed changes in pinocytic activity by monitoring the ability of DCs to take up FITC-tagged bovine serum albumin (BSA) in vitro. As shown in Table 2, DCs treated with the toll-like receptor 4 (TLR-4) agonist lipopolysaccharide accumulated significantly lower amounts of FITC-BSA compared with BSA acquired by DCs treated with control polyclonal IgM antibody. Because engagement of TLR-4 induces the maturation of DCs, this is an expected result. In contrast, DCs treated with the B7-DC cross-linking antibody sHIgM12 accumulated significantly more FITC-BSA than did DCs treated with isotype control antibody. This finding provides additional evidence that activation of DCs with B7-DC cross-linking antibody does not induce a traditionally defined maturation response but rather induces a distinct activation phenotype.

The ability of endogenous DCs to pinocytose was evaluated to determine whether systemic treatment with B7-DC cross-linking antibody affects DCs in vivo, as well. C57BL/6 mice were treated i.v. on two successive days with either isotype control IgM antibody s39, B7-DC cross-linking antibody sHIgM12, or poly I:C and CpG-ODN relative to the time of i.p. administration of 100 µg of FITC-OVA. Splenic DCs were harvested after tissue digestion with collagenase and DNase and identified by cell surface expression of CD11c. CD11c-positive DCs were evaluated for incorporation of FITC-OVA and cell surface expression of the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) by flow cytometry. Data are represented as mean fluorescent intensity in relative units.

<table>
<thead>
<tr>
<th>Source of DCs</th>
<th>Treatment</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro⁵</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow GM-CSF/L-4</td>
<td>Polyclonal human IgM; 10 µg/ml FITC-BSA</td>
<td>141.4 ± 0.5 (P &lt; 0.001) ND ND</td>
</tr>
<tr>
<td>Bone marrow GM-CSF/L-4</td>
<td>sHIgM12; 10 µg/ml FITC-BSA</td>
<td>583.5 ± 6.5 (P &lt; 0.001) ND ND</td>
</tr>
<tr>
<td>Bone marrow GM-CSF/L-4</td>
<td>LPS; 10 µg/ml FITC-BSA</td>
<td>10.0 ± 1.0 (P &lt; 0.001) ND ND</td>
</tr>
<tr>
<td>In vivo⁶</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>sHIgM39; isotype control; 100 µg of FITC-OVA</td>
<td>56.0 ± 10.7 (P &lt; 0.001) 52.01 ± 3.2 (NS) 194.7 ± 2.4 (NS)</td>
</tr>
<tr>
<td>Endogenous DCs</td>
<td>sHIgM12; 100 µg of FITC-OVA</td>
<td>116.0 ± 7.0 (P = 0.001) 53.0 ± 2.4 (NS) 200.0 ± 8.5 (NS)</td>
</tr>
</tbody>
</table>

⁵ DC, dendritic cell; MFI, mean fluorescent intensity; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; ND, not done; LPS, lipopolysaccharide; FITC-OVA, FITC-conjugated chicken ovalbumin; poly I:C, polyinosinic-polycytidylic acid; ODN, oligodeoxynucleotides.

⁶ Bone marrow-derived DCs were treated with 10 µg/ml control polyclonal human IgM, B7-DC cross-linking antibody sHIgM12, or LPS for 24 h before the addition of 10 µg/ml FITC-BSA to the cultures. After overnight incubation (16 h), the cells were harvested, washed, and analyzed for incorporation of the FITC label as an assessment of pinocytic activity.

Mice were treated i.v. on days −1 and 0 with 10 µg of control human IgM antibody s39, B7-DC cross-linking antibody sHIgM12, or poly I:C and CpG-ODN relative to the time of i.p. administration of 100 µg of FITC-OVA. Splenic DCs were harvested after tissue digestion with collagenase and DNase and identified by cell surface expression of CD11c. CD11c-positive DCs were evaluated for incorporation of FITC-OVA and cell surface expression of the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) by flow cytometry. Data are represented as mean fluorescent intensity in relative units.

Table 3: B7-DC cross-linking antibody is not protective in immunocompromised host

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Tumor free (day 11)</th>
<th>Statistic</th>
<th>Average tumor size (mm²)</th>
<th>SE (mm²)</th>
<th>Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6-JAG−/−</td>
<td>phIGM</td>
<td>0/5</td>
<td>Reference</td>
<td>150.0</td>
<td>22.2</td>
<td>Reference</td>
</tr>
<tr>
<td>C57BL/6-Jβ1−/−</td>
<td>sHIgM12</td>
<td>0/5</td>
<td>NS⁷</td>
<td>192.6</td>
<td>15.8</td>
<td>NS</td>
</tr>
<tr>
<td>C57BL/6-CDC4−/−</td>
<td>sHIgM12</td>
<td>0/6</td>
<td>Reference</td>
<td>136.5</td>
<td>29.3</td>
<td>Reference</td>
</tr>
<tr>
<td>C57BL/6-JAG−/−</td>
<td>phIGM</td>
<td>0/9</td>
<td>Reference</td>
<td>162.3</td>
<td>13.0</td>
<td>Reference</td>
</tr>
</tbody>
</table>

⁷ NS, no statistical difference.
were measured on day 17. The product of width /H11003 /H9262 melanoma engraftment, mice received either 10 mm2 in size. Animals treated with B7-DC cross-linking antibodies isotype control antibodies had developed tumors approaching 225 Serum was collected from mice on day 17, when animals treated with palpable tumors akin to mice receiving control treatment (Table 3).

Although these knockout mice have an intact CD4 T-cell response to the tumors was established using /H11002 /H9273 fluorescein (E) i.v. Serum was collected on day 17 from both groups. Antibodies in the sera were evaluated by serial dilution and flow cytometry for their ability to bind to B16 melanoma cells. Data are represented as mean fluorescent intensity (n = 5/group).

To determine whether treatment of mice with shHgM12 B7-DC cross-linking antibody induces tumor resistance by potentiating an immune response, we evaluated the ability of antibody to induce tumor resistance in immunodeficient B6-RAG1−/− mice lacking B and T cells. Treatment of these animals with shHgM12 antibody using the prophylactic treatment protocol had no effect on B16 melanoma appearance or growth, demonstrating that an intact immune system is essential for the induction of tumor resistance (Table 3). The failure of shHgM12 antibody treatment to protect Rag-deficient mice also provides additional evidence that the antibody does not act directly on the tumor. The importance of the CD8 T cells in the host immune response to the tumors was established using β2-microglobulin−/− mice. Although these knockout mice have an intact CD4 T-cell repertoire, the deficiency in CD8 T cells ablated the protective effect of shHgM12 antibody treatment (Table 3). Likewise, the absence of a helper response in CD4 knockout mice ablated the protective effect of shHgM12 because all of the mice developed palpable tumors akin to mice receiving control treatment (Table 3).

The B-cell immune response, as measured by tumor levels of antitumor antibodies, was indistinguishable between C57BL/6 mice receiving shHgM12 and those receiving polyclonal IgM treatment. Serum was collected from mice on day 17, when animals treated with isotype control antibodies had developed tumors approaching 225 mm2 in size. Animals treated with B7-DC cross-linking antibodies were tumor free. Serial dilutions of the sera were assessed for tumor-binding antibodies by flow cytometry. As shown in Fig. 1, levels of tumor-reactive antibodies were indistinguishable in animals receiving tumor protective treatment with B7-DC cross-linking antibody or nonprotective treatment with polyclonal human IgM antibody. To further evaluate whether B-cell responses are important contributors to the antitumor response induced by B7-DC cross-linking, B-cell-deficient μMT animals were treated with B7-DC cross-linking antibodies and challenged with B16 melanoma using the prophylactic treatment model. Wild-type C57BL/6 mice treated with isotype control human IgM antibody developed rapidly growing tumors that reached 225 mm2 by day 17 after tumor engraftment (n = 4). In contrast, C57BL/6 (n = 5) and μMT (n = 5) mice treated with shHgM12 B7-DC cross-linking antibody were strongly protected; one of five of the C57BL/6 mice eventually developed a tumor, whereas no tumors developed in the B-cell-deficient μMT animals. B16 melanoma grew rapidly in the μMT mouse line after treatment with isotype control antibody, demonstrating histocompatibility of the tumor with the μMT subline. These results demonstrate that an antitumor antibody response is not a critical factor distinguishing susceptible from resistant mice to this tumor model. Furthermore, the finding that B-cell-deficient mice are protected from B16 melanoma after treatment with the human antibody shHgM12 excludes the possibility that an antihuman IgM antibody response by antibody-treated animals is an integral component of the treatment effect elicited with B7-DC cross-linking antibody.

The hallmark of an effective adaptive immune response is a vigorous memory response on secondary challenge. To study whether a memory response against B16 tumor antigens was established after treatment with shHgM12 antibody, we rechallenged the surviving mice with a lethal dose of B16 melanoma cells in the opposite flank. As shown in Table 4, mice that had survived for at least 30 days after initial tumor challenge displayed significant resistance to a secondary challenge with tumor cells (P < 0.001). Because none of the surviving mice received additional treatments with shHgM12, the resistance to secondary challenge indicates that an effective antitumor immune response was established in mice treated with shHgM12 after the initial challenge. In contrast, animals that resisted B16 melanoma after shHgM12 antibody treatment showed no increased resistance to the unrelated tumor, EL-4.

To determine whether treatment with B7-DC cross-linking antibody potentiates tumor-specific CTLs, the draining lymph nodes of mice bearing day 7 tumors were assayed for tumor-specific CTL precursors. The animals were treated with shHgM12 antibody or control antibody as described before (one day before, the same day, and 1 day after tumor challenge). Harvested lymph node cells were cultured in the presence of mitomycin-treated B16 melanoma cells for an additional 4 days and then assessed for tumor-specific cytotoxic activity in a standard 51Cr release assay. Cytotoxic activity against B16 target cells was only observed in cultures of cells derived from animals treated with B7-DC cross-linking antibody (Fig. 2). Killing was specific for the B16 melanoma targets because EL-4 tumor cells were not killed. Lymph node cells from B16-challenged mice treated

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Tumor free (day 17)</th>
<th>Statistic</th>
<th>Average tumor size (mm2)</th>
<th>SE (mm2)</th>
<th>Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>Naive, pHgM</td>
<td>0/5</td>
<td>Reference</td>
<td>206.0</td>
<td>25.2</td>
<td>Reference</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>Naive, shHgM12</td>
<td>4/5</td>
<td>P = 0.048</td>
<td>13.6</td>
<td>13.6</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>Tumor survivors (shHgM12)</td>
<td>6/9</td>
<td>P = 0.031</td>
<td>35.6</td>
<td>20.2</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>Naive, EL-4 challenge</td>
<td>0/4a</td>
<td>Reference</td>
<td>171.5</td>
<td>17.7</td>
<td>Reference</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>B16 survivor, EL-4 challenge</td>
<td>0/4a</td>
<td>NSb</td>
<td>158.5</td>
<td>20.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

a Animals were evaluated for growth of EL-4 tumors on their flanks on day 13.
b NS, no statistical difference.
with control antibody displayed no activity, a finding consistent with the known weak innate antigenicity of the B16 melanoma tumor line.

Together, these findings demonstrate that systemic treatment with the B7-DC cross-linking antibody shHgM12 potentiates a cellular immune response against B16 melanoma causing acute tumor rejection and long-term immunity.

Systemic Treatment of Mice with shHgM12 B7-DC Cross-Linking Antibody Protects Mice in a B16 Lung Metastasis Model. The B16-F10 subline of B16 melanoma has been selected for its efficient ability to metastasize to the lungs. This particular tumor line is highly virulent and weakly antigenic; moreover, it is characterized by depressed MHC class I gene expression. A useful modification of the metastasis model has been to introduce the B16-F10 melanoma i.v. as a cell suspension. This results in 50–200 tumor nodules in the lungs of all challenged animals within 3–4 weeks to evaluate the effectiveness of shHgM12 antibody treatment on the induction of tumor resistance in this model, animals were seeded with lung metastases 3 days before antibody treatment. Animals received 10 μg of shHgM12 B7-DC cross-linking antibody i.v. on days 3, 4, and 5 after tumor challenge. Untreated sentinel mice that received identical tumor challenges were monitored for tumor burden. When tumor burdens exceeding 50 nodules were detected in the sentinel mice, the experiments were terminated, and the lungs of animals from all treatment groups were analyzed for the presence of tumor.

As shown in Fig. 3A, the lungs of animals receiving shHgM12 antibody treatment contained significantly fewer tumor nodules than mice receiving control polyclonal IgM antibody. In the illustrated experiment, all eight animals treated with shHgM12 antibody developed fewer tumor nodules than the least number observed in the eight animals treated with control isotype-matched antibody (Fig. 3B; P < 0.001). In the experiment shown, three of the eight protected animals developed resistance. Our overall experience is that approximately half the animals (4 of 29) treated with B7-DC cross-linking antibodies remained free of tumor. These findings demonstrate that systemic administration of B7-DC cross-linking antibody confers resistance to a highly lethal, weakly immunogenic tumor, even after the tumor is allowed to establish itself for 3 days before initiation of treatment.

Induced Resistance to B16-F10 Melanoma Is Mediated by CD8+ T Cells and Is Perforin and Granzyme B Dependent. Because the B16-F10 tumor expresses reduced amounts of MHC class I molecules on its cell surface, we considered the possibility that induced resistance by treatment with B7-DC cross-linking antibody might be mediated by NK cells. To evaluate this possibility, animals were treated with anti-NK1.1 antibody before tumor challenge. The efficiency of the NK cell depletion protocol was monitored by flow cytometry using NK1.1- and DX-5-specific antibodies to visualize NK cells. Spleenic NK cells were reduced by >90% in animals treated with NK1.1-specific antiserum. As shown in Fig. 4, direct comparison of shHgM12-treated, NK cell-depleted animals with shHgM12-treated mice suggests a minor but statistically significant (P < 0.001) contribution of NK cells to the antitumor response induced by systemic shHgM12 treatment. However, NK cell-depleted mice were still responsive to the immunotherapeutic effects of B7-DC cross-linking antibody administered 3 days after i.v. challenge with B16-F10 melanoma (P = 0.002). In contrast, CD8 knockout mice were not responsive to treatment with B7-DC cross-linking antibody, indicating that CD8+ T cells are critical mediators of antitumor immunity in this model. NK-deficient or CD8-deficient mice that did not receive B7-DC cross-linking antibodies were no more susceptible or resistant to development of lung tumors than were untreated animals, findings...
CD8 T cells with B7-DC cross-linking antibody potentiates cytolytic responses. The most consistent with the view that treatment of tumor-bearing mice with B7-DC cross-linking antibody potentiates cytolytic responses are most consistent with the view that treatment of tumor-bearing mice with B7-DC cross-linking antibody potentiates cytolytic responses. These findings are consistent with the view that treatment of tumor-bearing mice with B7-DC cross-linking antibody potentiates cytolytic responses. Because we have shown that NK cells are not the primary effectors of cytotoxicity in the sHIgM12 B7-DC cross-linking antibody treatment model, we performed an independent experiment to determine whether the protective effect induced by systemic antibody treatment was perforin dependent. As shown in Fig. 5, sHIgM12 treatment was ineffective in perforin-deficient animals but highly effective in wild-type B6 mice. In an independent experiment, B7-DC cross-linking with sHIgM12 antibody also was not protective in granzyme B-deficient mice. Using the therapeutic treatment model, an average of 98.2 ± 4.9 (n = 5) tumor nodules were found in the lungs of granzyme B-deficient mice treated with control polyclonal human IgM antibody, as compared with 99.2 ± 15.0 (n = 5) tumor nodules in the lungs of granzyme B-deficient mice treated with sHIgM12 B7-DC cross-linking antibody (data not shown). Because we have previously demonstrated that NK cells are not the primary mediators of the antitumor resistance induced by treatment with B7-DC cross-linking antibody, it is not surprising that resistance is associated with an increase in the number of tumor nodules. As shown in Fig. 5, sHIgM12 antibody on days 3, 4, and 5 after i.v. tumor challenge, significantly increased on cross-linking B7-DC (1). It is possible that antibody treatment enhances tumor rejection by augmenting antigen presentation, resulting in better priming and effector responses. Our findings that treatment with B7-DC cross-linking antibody induces protective antitumor treatment effects in comparison with animals receiving isotype-matched control human IgM antibody. Approximately 70% of the animals treated with B7-DC cross-linking antibody and challenged with B16 melanoma remained tumor free; the remaining 30% grew tumor more slowly. Animals that resisted the growth of the initial B16 melanoma challenge faced a secondary challenge with B16 melanoma, indicating a classical memory response. Resistance was antigen specific because an unrelated tumor, EL-4, grew unabatedly in mice that had previously resisted B16 melanoma as a result of sHIgM12 treatment. We interpret these findings as evidence that systemic treatment of animals with B7-DC cross-linking antibody potentiates a long-lasting, protective, adaptive, cellular immune response against weak tumor antigens expressed by B16 melanoma.

**DISCUSSION**

We have devised a novel immunotherapeutic strategy for the treatment of cancer. Systemic administration of a human B7-DC cross-linking antibody induced protective cellular immune responses against the weakly antigenic B16 and B16-F10 melanoma lines using both prophylactic and therapeutic treatment schemes. Animals received i.v. treatments with the human B7-DC cross-linking antibody sHIgM12, without further manipulation of tumor or administration of antigen. This antibody binds and cross-links B7-DC expressed on mouse DCs and induces prolonged DC survival, up-regulation of cytokines, enhanced antigen processing, and potentiation of the ability of bone marrow-derived DCs to activate naive T cells in vitro (1, 2). Here we show that systemic administration of the B7-DC cross-linking antibody enhances acquisition of the experimental antigen FITC-OVA by endogenous DCs and does not induce increased expression of the maturation markers B7.1 and B7.2. Thus, the activation phenotype induced by the sHIgM12 B7-DC cross-linking antibody is distinct from that of the defined maturation responses induced by TLR and tumor necrosis factor receptor agonists. Mature DCs characteristically have down-regulated their ability to acquire soluble antigen and up-regulated their cell surface expression of B7.1 and B7.2.

All of the wild-type mice in our study treated with B7-DC cross-linking antibody showed protective antitumor treatment effects in comparison with animals receiving isotype-matched control human IgM antibody. Approximately 70% of the animals treated with B7-DC cross-linking antibody and challenged with B16 melanoma remained tumor free; the remaining 30% grew tumor more slowly. Animals that resisted the growth of the initial B16 melanoma challenge faced a secondary challenge with B16 melanoma, indicating a classical memory response. Resistance was antigen specific because an unrelated tumor, EL-4, grew unabatedly in mice that had previously resisted B16 melanoma as a result of sHIgM12 treatment. We interpret these findings as evidence that systemic treatment of animals with B7-DC cross-linking antibody potentiates a long-lasting, protective, adaptive, cellular immune response against weak tumor antigens expressed by B16 melanoma.

**Fig. 4.** B7-DC cross-linking antibody-mediated protection is CD8 T-cell dependent. Wild-type or CD8-deficient mice received injection with B16-F10 cells followed by antibody treatment with either isotype control antibody (polyclonal human IgM antibody, pHIgM) or B7-DC cross-linking antibody (sHIgM12). In parallel, two separate groups of mice were treated with NK cell-depleting antibody before injection of B16-F10 cells, followed by B7-DC cross-linking antibody treatment or control antibody treatment. Sentinel animals were used as described in the Fig. 1 legend. All mice were sacrificed at day 17. The lungs were harvested, and the number of nodules was counted. Data represent the number of nodules from five to seven mice in each group. Data were analyzed using rank-sum methods. Pairwise comparisons are shown. Comparison of animals receiving sHIgM12 antibody with those receiving sHIgM12 antibody and NK1.1 NK-depleting antibody revealed a significant difference (P < 0.001). A comparison of the three treatment groups receiving control antibodies (with or without other treatments) was also performed. No significant difference was evident (P = 0.116).

**Fig. 5.** The protective effect of B7-DC cross-linking antibody is perforin dependent. Wild-type or perforin-deficient mice received injection with B16-F10 cells followed by antibody treatment. Mice were sacrificed, and lungs were harvested on day 17, when sentinel animals contained ≥50 tumor nodules. Data represent the mean number of nodules in the lungs of four mice in the wild-type (WT) or perforin-deficient (PPF-/-) control antibody-treated (polyclonal human IgM antibody, pHIgM) or B7-DC cross-linking antibody-treated (sHIgM12) groups. n = 4 in all groups except for the wild-type, sHIgM12-treated group, where n = 6. Statistical evaluations were performed using rank-sum comparisons.
antibody enhances pinocytic activity by DCs in vitro and in vivo is consistent with this view. A second, mutually nonexclusive mechanism by which the tumors might be rejected could involve IL-12 and IFN-γ secretion leading to a pro-inflammatory tumor microenvironment. The enhanced production of IL-12 by DCs treated with B7-DC cross-linking antibody is consistent with this view (1). Indeed, previous studies have shown that CD4 T cells present in the tumor microenvironment are mobilized by release of IL-12 to kill tumors, and this activity correlated with the serum levels of human IFN-γ (32). We find that CD4+ cells are required for the induction of antitumor resistance with shHgM12 antibody. Because CD4+ cells were not protective in the absence of CD8+ cells, our findings are most consistent with a helper function of CD4+ T cells in support of a CD8+ CTL response. However, whether CD4+ T cells function as effector cells in this tumor model after treatment with shHgM12 antibody is not known.

NK cells are known to play an important role in the rejection of tumors under some circumstances, and IL-12, perforin, and IFN-γ contribute to the NK-mediated antitumor response (33, 34). Furthermore, NK cell-mediated rejection of tumor can lead to subsequent priming and onset of acquired immunity (34). A protective role for NK cells in resistance to the development of B16-F10 tumor nodules would be consistent with the reduced levels of class I expression by these tumor cells (35, 36). In our study, depletion of NK cells with NK-reactive antibody had little effect on the ability of B7-DC cross-linking antibody to induce resistance to tumor. Expression of both perforin and granzyme B was shown to be critical for the induction of antitumor resistance with shHgM12 B7-DC cross-linking antibody. Our interpretation is that despite the low levels of class I antigen-presenting molecules expressed by B16-F10 cells, an antibody-induced cytolytic T-cell response is protecting the animals.

Our current view, based on these findings, is that B7-DC cross-linking antibody binds to resident DCs, potentiating their ability to acquire tumor antigens in situ and process the antigens for presentation to T cells and enhancing their ability to activate naïve T cells, promoting a protective cellular cytolytic response. We have previously documented the ability of systemically administered antibody to mobilize and potentiate DCs introduced in a distal site to augment their ability to reach draining lymph nodes. Here we have extended these results, demonstrating that systemic antibody treatment increases antigen acquisition by endogenous DCs of antigen administered i.p. These findings suggest the possibility that i.e., the site of tumor growth might also be mobilized by this treatment strategy.

B7-DC is known to interact with the T-cell ligand PD-1, inducing, under some circumstances, negative signals that dampen T-cell activation (19). The extent to which treatment with small amounts of shHgM12 antibody interferes with the regulatory interaction is not known, but any interference could lead to further increases in immune potentiation.

The shHgM12 antibody may have important therapeutic potential for the treatment of human disease. The nature of the epitopes recognized by the IgM antibody and the function in vivo is not known. However, in several instances seems to be conserved across species. In macaques we described that bind to rat oligodendrocytes and spinal cord to human oligodendrocytes and mouse myelin tracts (37). The ability of antibody shHgM12 to bind to both mouse and human DCs (2) generalizes this concept. Because much is known about the biology of DCs, it will be possible to compare how cross-linking B7-DC expressed by human and mouse DCs will influence immune functions. These studies are ongoing, but it is already evident that the shHgM12 antibody induces changes in human myeloid DCs that mirror some of the important changes we have described in mice (1, 2). It will be of great interest to determine whether the human antibody shHgM12 can be used to manipulate human DCs in vivo and to modify the immune response in beneficial ways, just as we have done in the mouse. The induction of a protective immune response against an aggressive, weakly antigenic tumor, as described in this report, is one example of how this antibody might be used.

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19. Latchman Y, Wood CR, Chernova T, et al. CD86 (B7-2) is known to interact with the T-cell ligand PD-1, inducing, under some circumstances, negative signals that dampen T-cell activation (19). The extent to which treatment with small amounts of shHgM12 antibody interferes with the regulatory interaction is not known, but any interference could lead to further increases in immune potentiation.

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The authors wish to retract the article titled "Immunotherapeutic Potential of B7-DC (PD-L2) Cross-Linking Antibody In Conferring Antitumor Immunity," which was published in the July 15, 2004 issue of Cancer Research (1).

In the course of investigating suspicious patterns of experimental results in the laboratory, a systematic and in-depth study of key findings in this article was carried out using blind protocols. In these repeat studies, no evidence was found to support our original conclusions that B7-DCXAb modulates dendritic cell functions. We do not believe our failure to reproduce our earlier findings is the result of a technical problem. A member of the B7-DCXAb investigative team, Dr. Suresh Radhakrishnan, who was involved in or had access to all work on this subject, was found in a formal institutional investigation to have engaged in scientific misconduct in unpublished experiments by manipulating another investigator’s experiment involving the B7-DCXAb reagent. This finding of misconduct together with our inability to reproduce key findings using blinded protocols has undermined our confidence in our published reports. We seek therefore to retract this body of work.

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Immunotherapeutic Potential of B7-DC (PD-L2) Cross-Linking Antibody In Conferring Antitumor Immunity

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