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Bone Marrow-Derived IFN-Producing Killer Dendritic Cells Account for the Tumoricidal Activity of Unpulsed Dendritic Cells

Nourredine Himoudi,* Stephen Nabarro,* Jo Buddle,† Ayad Eddaoudi,† Adrian J. Thrasher,† and John Anderson2*†

The CD11c\textsuperscript{int}B220\textsuperscript{+}NK1.1\textsuperscript{+}CD49\textsuperscript{+} subset of cells has recently been described as IFN-producing killer dendritic cells (IKDC), which share phenotypic and functional properties of dendritic cells and NK cells. Herein we show that bone marrow-derived murine dendritic cell preparations contain abundant CD11c\textsuperscript{int}B220\textsuperscript{+}NK1.1\textsuperscript{+}CD49\textsuperscript{+} cells, the removal of which results in loss of tumoricidal activity of unpulsed dendritic cells in vivo. Moreover, following s.c. injection, as few as $5 \times 10^3$ highly pure bone marrow-derived IKDC cells are capable of shrinking small contralateral syngeneic tumors in C57BL/6 mice, but not in immunodeficient mice, implying the obligate involvement of host effector cells in tumor rejection. Our data suggest that bone marrow-derived IKDC represent a population that has powerful tumoricidal activity in vivo. The Journal of Immunology, 2008, 181: 6654–6663.

Dendritic cells (DC) are pivotal to the induction of innate and adaptive immune responses. In vivo DC are attracted to sites of infection or inflammation where they can take up Ag. Subsequent maturation of DC results in the upregulation of costimulatory molecules and MHC class II and their migration to sentinel lymph nodes where they present Ags to T cells and can stimulate innate responses through secretion of inflammatory cytokines. For example, NK cells and DC can exchange bidirectional activating signals in a positive feedback, referred to as cross-talk (1, 2). Both myeloid DC and plasmacytoid DC promote cytotoxicity and cytokine production by NK cells, while activated NK cells can reciprocate by providing immunoregulatory helper function to DC, stimulating them to produce proinflammatory cytokines that stimulate cytotoxic lymphocyte (CTL) and Th1 responses.

It has long been recognized that adoptive transfer of DC has the potential to inhibit cancer growth through stimulation of both innate and adaptive immune responses (3–6). Innate responses induced by DC can result, for example, from the stimulation of tumor-reactive NK cells. Adaptive anticancer immune responses result from the presentation of tumor Ags by DC. In adoptive transfer studies stimulation of adaptive immunity has generally resulted from the priming of DC ex vivo with tumor Ags (7, 8), although unprimed DC have shown effective tumoricidal activity in animal models following direct intratumoral injection (9–11). In tumor models involving injection of DC at distant sites, unpulsed DC usually show low or no antitumor activity compared with Ag-pulsed DC (12–16). However, some studies have shown tumoricidal activity of unpulsed DC injected at sites distant to a tumor, attributed, at least in part, to stimulation of innate NK responses (4, 17–19).

Heightened interest of the role of DC in cross-talk of innate and adaptive responses follows the recent description of IFN-γ-producing killer dendritic cells (IKDC), which were reported to show both NK and Ag presenting cell surface phenotype and function (20–22). IKDC were originally described as having intermediate expression of CD11c coupled with high expression of B220, NK1.1, and CD49b, and absence of Gr-1, to distinguish them from conventional NK cells (NK1.1\textsuperscript{+}CD49b\textsuperscript{−}CD11c\textsuperscript{−}B220\textsuperscript{−}) and plasmacytoid DC (pDC; NK1.1\textsuperscript{+}CD49b\textsuperscript{−}Gr-1\textsuperscript{−}B220\textsuperscript{+}) (20, 21). However, the distinction between IKDC, DC, and NK cells has been challenged in recent reports that have described the presence of CD11c and B220 on conventional NK cells, and failed to demonstrate presentation of Ag by IKDC, leading to the suggestion that IKDC represent an activated form of NK cells (23–25).

Herein we provide further evidence for the antitumor properties of cells with the IKDC phenotype. We show that the IKDC can be readily isolated in large numbers from conventional bone marrow DC preparations, but that remarkably low numbers of bone marrow-derived IKDC administered by s.c. injection are required for effective adoptive transfer immunotherapy of distant tumors. Both innate NK response and adaptive T cell responses are observed in mice undergoing tumor rejection following administration of unpulsed DC.

Materials and Methods

Mice and cell lines

Studies were conducted on 8-wk-old C57BL/6 or immunodeficient RAG–/–/common γ-chain–/–/complement C5–/– mice. Tumor cells suspended in 150 μl PBS were injected s.c. into the flanks of groups of at least six mice, and DC subtypes were injected s.c. at the contralateral flank. Tumor size
was monitored every 2 days using calipers, and mice were killed when tumors reached a maximum diameter of 1.2 cm. Tumors were mechanically disaggregated before staining for flow cytometric analysis, or fixed in 10% formalin for frozen sections. 76-9 and 76-9-P3F (76-9:C23) have been previously described (26). B16F10 melanoma and LL/2 lung cancer cell lines, which are syngeneic to C57BL/6, and NK-sensitive YAC-1 cells, were from the American Type Culture Collection. Mouse experiments were performed in accordance with a U.K. Home Office-approved project license held by one of the authors (J.A.).

Abs and flow cytometry analyses

All the Abs used in this study are listed in Table I. Stained cells were analyzed using CyAn ADP flow cytometer (Dako) using Summit 4.3 software.

Preparation of bone marrow-derived DC subsets and NK cells

Recombinant murine GM-CSF (800 IU/ml) was added to freshly isolated bone marrow cells of C57BL/6 mice, and the cells were cultured for 7 days at 37°C in 5% CO₂. On day 3, nonadherent cells were discarded and fresh RPMI 1640 containing 10% FCS, 800 IU/ml recombinant murine (rm)GM-CSF and 1000 IU/ml rmIL-4 (PeproTech) was added. On day 6, the non-adherent cells were replated. On day 7, nonadherent immature DC were harvested, resuspended in fresh media, and matured with 10 μg/ml keyhole limpet hemocyanin (Calbiochem) and prostaglandin E2 (1 μg/ml). In some experiments DC were matured in the presence of 500 ng LPS, with 800 IU/ml rmGM-CSF and 1000 IU/ml rmIL-4 (Promega). Tetrazolium inner salt) assay according to the manufacturer’s instructions (Promega).

Statistical analyses

We compared groups using ANOVA followed by multiple comparisons of means. Nonparametric statistical methods were used when the variables studied were not normally distributed. Wilcoxon two-sample rank-sum test was used to compare the values of continuous variables between two groups. Paired comparisons were made using a Wilcoxon paired test. p-values were determined and considered significant when p < 0.05.

Results

Unpulsed DC are capable of shrinking small established tumors

In initial experiments designed to assess tumor lysate-pulsed DC vaccination in alveolar rhabdomyosarcoma, we made use of murine 76-9 methylcolanthrene-induced rhabdomyosarcoma cells stably transfected with the PAX3-FKHR oncogene, referred to as 76-9:C23 cells (26). To our surprise, subcutaneously administered unpulsed bone marrow-derived DC were as effective as tumor-lysate-pulsed DC in inhibiting outgrowth of subsequently administered tumor (Fig. 1A). Furthermore, unpulsed DC had a similar effect on the growth of syngeneic B16F10 melanoma and LL/2 lung cancer cells (Fig. 1B). Unpulsed DC could also inhibit growth of small established tumors (Fig. 1C), and splenocytes from DC-vaccinated tumor-bearing mice secreted large amounts of IFN-γ following addition of tumor cells in vitro (Table II). Furthermore, these splenocytes demonstrated a high degree of cytotoxicity against 76-9:C23 cells, and the cytotoxicity was abrogated by antagonistic

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**Table I. Abs used in this study**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Conjugated</th>
<th>Isotype</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11c</td>
<td>N418</td>
<td>PE, FITC, allophycocyanin, PE-Cy5</td>
<td>Armenian hamster IgG</td>
<td>FC</td>
</tr>
<tr>
<td>CD11b</td>
<td>NM1/70</td>
<td>PE, FITC</td>
<td>Rat IgG2b</td>
<td>FC</td>
</tr>
<tr>
<td>B220</td>
<td>RA3-6B2</td>
<td>PE-Cy7, PE-Cy5, allophycocyanin</td>
<td>Rat IgG2α</td>
<td>FC</td>
</tr>
<tr>
<td>CD86</td>
<td>GL1</td>
<td>PE-Cy5, PE-Cy7</td>
<td>Rat IgG2α</td>
<td>FC</td>
</tr>
<tr>
<td>CCR7</td>
<td>4B12</td>
<td>Allophycocyanin, PE-Cy5, PE-Cy7</td>
<td>Rat IgG2α</td>
<td>FC</td>
</tr>
<tr>
<td>MHC class II (I-A/E-E)</td>
<td>M5/114.15.2</td>
<td>Allophycocyanin, PE-Cy5, PE-Cy7</td>
<td>Rat IgG2α</td>
<td>FC</td>
</tr>
<tr>
<td>MHC class II (I-A/E-E)</td>
<td>M5/114.15.2</td>
<td>No, functional grade</td>
<td>Rat IgG2b</td>
<td>Block</td>
</tr>
<tr>
<td>Ly-6G (Gr-1)</td>
<td>RB6-8C5</td>
<td>PE, FITC</td>
<td>Rat IgG2b</td>
<td>FC</td>
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<td>F4/80</td>
<td>BM8</td>
<td>FITC</td>
<td>FC</td>
<td></td>
</tr>
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<td>Armenian hamster IgG</td>
<td>FC</td>
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<td>145-2C11</td>
<td>No</td>
<td>Armenian hamster IgG</td>
<td>Block</td>
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<tr>
<td>CD4</td>
<td>GK1.5</td>
<td>PE, FITC, allophycocyanin, PE-Cy5</td>
<td>Rat IgG2b</td>
<td>FC</td>
</tr>
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<td>CD8</td>
<td>YTS169.4</td>
<td>No, functional grade</td>
<td>Rat IgG2α</td>
<td>Block</td>
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<tr>
<td>NK1.1</td>
<td>PK136</td>
<td>PE, FITC, allophycyanin, biotin</td>
<td>Mouse IgG2α</td>
<td>FC</td>
</tr>
<tr>
<td>NKG22/CD314</td>
<td>C7</td>
<td>Affinity purified</td>
<td>Armenian hamster IgG</td>
<td>Block splenic NK cells</td>
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<td>NKG22/CD314</td>
<td>C7</td>
<td>No, functional grade</td>
<td>Rat IgG1α</td>
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<td>CD49b/CD314</td>
<td>DX5</td>
<td>PE, FITC, allophycocyanin, biotin</td>
<td>Rat IgMx</td>
<td>FC</td>
</tr>
<tr>
<td>TRAIL</td>
<td>N2B2</td>
<td>No, functional grade</td>
<td>Rat IgG1, κ</td>
<td>FC</td>
</tr>
</tbody>
</table>

a All mAbs were purchased from eBioscience. FC indicates flow cytometry.
anti-CD3, anti-CD8, or anti-NKG2D Abs, suggesting that unpulsed DC induced both CTL- and NK-mediated cytotoxicity (Fig. 1D). This was confirmed by analysis of tumor-infiltrating lymphocytes, which showed a large increase in NK cells and CTL (but not NKT cells) as well as other inflammatory cells in unpulsed DC-treated mice (Table III). In *beige* mice lacking...
functional NK cells, 76-9:C23 tumor growth was more rapid, but unpulsed DC prepared from wild-type mice still inhibited tumor growth (Fig. 1E), indicating that 76-9:C23 are NK targets but that host NK cells are not essential for tumor inhibition mediated by unpulsed DC. Furthermore, the role of T cells in beige mice was suggested by the high number of activated tumor-infiltrating CD4 and CD8 T cells compared with untreated mice (Fig. 1F).

Unpulsed DC cause an adaptive T cell immune response, which is dependent on the presence of tumor at a distant site in immunized mice

To confirm that unpulsed DC had induced an adaptive T cell-mediated immune response, we investigated whether the activated T cells were specific for the tumor implanted in the immunized mice. Purified FACS-sorted T cells and NK cells, but not NKT cells, from unpulsed DC-treated 76-9:C23 tumor-bearing mice showed markedly enhanced in vitro cytotoxicity against 76-9:C23 targets (Fig. 2A), but the T cells did not show significant killing of B16 or LL2 targets (Fig. 2B). Moreover, in an MTS assay these purified T cells showed enhanced proliferation following addition of tumor lysate from 76-9:C23 but not from LL2 or B16 tumors (Fig. 2C). This proliferation correlated with increased IFN-γ secretion following addition of 76-9:C23 tumor lysate to splenocytes from tumor-bearing unpulsed DC-treated mice (Fig. 3A). T cells and NK cells were also purified from spleens of non-tumor-bearing DC-treated mice, and here the T cells showed no killing of 76-9:C23 cells, although NK cells had been similarly activated (Fig. 3B). Therefore, a mixed NK and adaptive T cell-mediated immune response occurs following unpulsed DC treatment, and the adaptive T cell component is dependent on the presence of tumor at a distant s.c. site.

We hypothesized that the recently described IKDC (20, 21) might account for the apparent ability of unpulsed DC to trigger regression of tumor implanted at a distant site. We first demonstrated the presence of cells with the IKDC phenotype in the bulk bone marrow DC preparation that had been used in the DC vaccinations, and refer to these hereafter as bone marrow-derived IKDC (BM-IKDC: CD11c<sup>+</sup>B220<sup>−</sup>NK1.1<sup>+<sup>). These cells represented between 2 and 10% of the viable cells in DC preparations depending on the method of enrichment (Table IV). As previously described (20, 21), FACS-sorted BM-IKDC stained brightly for the NK marker CD49b but low for MHC class II, and did not express Gr-1 (Fig. 4B).

The removal of BM-IKDC from the DC bulk culture caused loss of most of the tumoricidal effect. However, add-back of the FACS-sorted BM-IKDC to the sorted negative fraction of DC (CD11c<sup>+</sup>B220<sup>−</sup>NK1.1<sup>−<sup> and CD11c<sup>+</sup>B220<sup>−</sup>NK1.1<sup>−<sup>) caused a dose-dependent increase of tumoricidal activity that exceeded that observed with unsorted DC (Fig. 4C, left panel). Furthermore, in a separate in vivo experiment, as few as 5 × 10<sup>5</sup> purified CD11c<sup>+</sup>B220<sup>−</sup>NK1.1<sup>+</sup>CD49<sup>+</sup> cells significantly slowed down the growth of established tumors (Fig. 4C, right panel). Therefore, BM-IKDC are necessary and sufficient for a strong tumoricidal effect in vivo, and are the principal cells involved in tumor rejection when unpulsed DC are administered at a distant site.

### Table II. Unpulsed DC vaccine induces IFN-γ-secreting cells in tumor- or non-tumor-bearing mice<sup>a</sup>

<table>
<thead>
<tr>
<th>Cells From</th>
<th>Target Cells</th>
<th>E:T Ratio of 30:1 (Median and Range)</th>
<th>E:T Ratio of 10:1 (Median and Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol 2</td>
<td>76-9</td>
<td>168 ± 37 48 ± 12</td>
<td>10 ± 22 55 ± 13</td>
</tr>
<tr>
<td>Protocol 2</td>
<td>76-9:C23</td>
<td>165 ± 32 55 ± 13</td>
<td>10 ± 22 55 ± 13</td>
</tr>
<tr>
<td>Protocol 3</td>
<td>76-9</td>
<td>180 ± 44 52 ± 7</td>
<td>10 ± 22 55 ± 13</td>
</tr>
<tr>
<td>Protocol 3</td>
<td>76-9:C23</td>
<td>175 ± 39 49 ± 9</td>
<td>10 ± 22 55 ± 13</td>
</tr>
<tr>
<td>Protocol 4</td>
<td>76-9</td>
<td>217 ± 34 75 ± 21</td>
<td>10 ± 22 55 ± 13</td>
</tr>
<tr>
<td>Protocol 4</td>
<td>76-9:C23</td>
<td>198 ± 37 83 ± 23</td>
<td>10 ± 22 55 ± 13</td>
</tr>
<tr>
<td>Protocol 1 (tumor only)</td>
<td>76-9</td>
<td>38 ± 15 25 ± 16</td>
<td>10 ± 22 55 ± 13</td>
</tr>
<tr>
<td>Protocol 1 (tumor only)</td>
<td>76-9:C23</td>
<td>41 ± 17 27 ± 15</td>
<td>10 ± 22 55 ± 13</td>
</tr>
<tr>
<td>Naive</td>
<td>76-9</td>
<td>39 ± 15 25 ± 9</td>
<td>10 ± 22 55 ± 13</td>
</tr>
<tr>
<td>Naive</td>
<td>76-9:C23</td>
<td>42 ± 17 27 ± 13</td>
<td>10 ± 22 55 ± 13</td>
</tr>
</tbody>
</table>

<sup>a</sup> Splenic cells from C57BL/6 naive or mice in protocol 1, 2, 3, or 4 were mixed with 76-9 or 76-9:C23 target cells and incubated in the presence of 10 IU/ml of murine IL-2 for 72 h in an IFN-γ ELISPOT-release assay.

### Table III. Tumor-infiltrating cells<sup>a</sup>

<table>
<thead>
<tr>
<th>Tumor-Infiltrating Cells</th>
<th>Tumor Only</th>
<th>Tumor/DC/DC (Protocol 3, Fig. 1)</th>
<th>DC/DC/Tumor (Protocol 2, Fig. 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>4–7</td>
<td>9.5–13</td>
<td>16–31</td>
</tr>
<tr>
<td>TCRαβ&lt;sup&gt;+&lt;/sup&gt;NK1.1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>3–5</td>
<td>9–12</td>
<td>18–20</td>
</tr>
<tr>
<td>CD3, CD8</td>
<td>3–6</td>
<td>9–11</td>
<td>9–14</td>
</tr>
<tr>
<td>CD3, CD4</td>
<td>2–4</td>
<td>7–9</td>
<td>8–13</td>
</tr>
<tr>
<td>NK1.1&lt;sup&gt;−&lt;/sup&gt;TCRαβ&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>0.4–0.6</td>
<td>1.3–1.6</td>
<td>1.2–1.4</td>
</tr>
<tr>
<td>NKT (TCRαβ&lt;sup&gt;−/+&lt;/sup&gt;NK1.1&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>1.4–1.5</td>
<td>1.3–1.5</td>
<td>1.6–1.8</td>
</tr>
<tr>
<td>CD69</td>
<td>3–7</td>
<td>ND</td>
<td>23–29</td>
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<td>Proinflammatory cells</td>
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</tr>
<tr>
<td>F4/80</td>
<td>6–8</td>
<td>11–13</td>
<td>20–25</td>
</tr>
<tr>
<td>CD11b</td>
<td>8–11</td>
<td>15–18</td>
<td>18–24</td>
</tr>
<tr>
<td>Ly6G</td>
<td>4–5</td>
<td>7–9</td>
<td>11–13</td>
</tr>
<tr>
<td>CD11c&lt;sup&gt;+&lt;/sup&gt; cells</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CD40</td>
<td>0.8–1.2</td>
<td>2.5–4</td>
<td>3–6</td>
</tr>
<tr>
<td>CD80</td>
<td>0.5–1</td>
<td>5–7.5</td>
<td>13–25</td>
</tr>
<tr>
<td>CD86</td>
<td>0.5–0.7</td>
<td>0.5–1</td>
<td>1.7–2.4</td>
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<td>MHC class II</td>
<td>0.3–0.5</td>
<td>1–1.4</td>
<td>4.5–5.2</td>
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</tbody>
</table>

<sup>a</sup> Results represent mice treated with tumor only (protocol 1, Fig. 1), in tumor-bearing mice and treated with two DC vaccinations (protocol 3), or in mice challenged with tumor after two DC vaccinations (protocol 2).
We next investigated whether the BM-IKDC in the bulk bone marrow DC culture shared the same in vivo killing activity as the IKDC described previously (20, 21). We hypothesized that the activating receptor NKG2D might be essential for killing because it was consistently present in the previously published IKDC, and it was present in the CD11c<sup>int</sup>B220<sup>-</sup>NK1.1<sup>-</sup> cells that we purified from bone marrow DC (Fig. 5A). NKG2D ligands RAE1 and H60 were present on 76-9:C23 target cells. Unlike plasmacytoid and conventional DC (CD11c<sup>+</sup>B220<sup>-</sup>NK1.1<sup>-</sup> and CD11c<sup>-</sup>B220<sup>-</sup>NK1.1<sup>-</sup> negative

In vitro killing capacity of BM-IKDC

We next investigated whether the BM-IKDC in the bulk bone marrow DC culture shared the same in vivo killing activity as the IKDC described previously (20, 21). We hypothesized that the activating receptor NKG2D might be essential for killing because it was consistently present in the previously published IKDC, and it was present in the CD11c<sup>int</sup>B220<sup>-</sup>NK1.1<sup>-</sup> cells that we purified from bone marrow DC (Fig. 5A). NKG2D ligands RAE1 and H60 were present on 76-9:C23 and LL2 targets but not on B16. Moreover, IFN-γ release by BM-IKDC following addition of these targets correlated with the presence of the cognate ligands and was blocked by anti-NKG2D blocking Abs (Fig. 5B). Unlike plasmacytoid and conventional DC (CD11c<sup>+</sup>B220<sup>-</sup>NK1.1<sup>-</sup> and CD11c<sup>-</sup>B220<sup>-</sup>NK1.1<sup>-</sup> negative

FIGURE 2. Unpulsed DC cause a specific T cell immune response dependent on the presence of tumor at a distant site. Splenocytes were prepared from unpulsed DC-treated mice 2 wk after 76-9:C23 tumor challenge. CD3 T cells (CD3<sup>+</sup>NK1.1<sup>-</sup>), NK cells (CD3<sup>-</sup>NK1.1<sup>-</sup>), and NKT cells (CD3<sup>+</sup>NK1.1<sup>-</sup>) were FACS sorted. In these experiments, DC were matured with prostaglandin E<sub>2</sub> and keyhole limpet hemocyanin. A. Sorted cells were stimulated for 5 days with bone marrow-derived DC pulsed with 76-9:C23 lysate in the presence of 10 U/ml of murine IL-2, and then incubated with <sup>51</sup>Cr-labeled 76-9:C23 target cells. B. CD3 T cells were stimulated as described in A and incubated with <sup>51</sup>Cr-labeled LL/2 or B16 or 76-9:C23 tumor cell lines in a 4-h <sup>51</sup>Cr-release assay at E:T ratio of 50:1. C. Sorted CD3 T cells were stimulated with DC pulsed with tumor lysate prepared from LL/2 or B16 or 76-9 cell lines for 5 days in the presence of 10 U/ml of murine IL-2, and the proliferation of cells was analyzed by MTS assay. Each assay point was done in triplicate, and the result is representative of mean data ± SEM obtained with six mice per group.

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fraction cells, respectively), purified BM-IKDC were cytotoxic for 76-9:C23, LL2, B16, and YAC1 targets, and cytotoxicity was partially blocked by NKG2D Abs only in the cell lines expressing the cognate ligands. In agreement with previous findings, cytotoxicity against all the targets was inhibited by TRAIL-blocking Ab (Fig. 5C). Therefore, BM-IKDC present in bulk bone marrow DC cultures share cytotoxic properties with the previously described IKDC. To determine whether the inherent cytotoxic function of the BM-IKDC could account for tumor regression, we repeated the tumor growth assays using immunodeficient tumor-bearing host mice lacking functional NK and T cells. Here the BM-IKDC had no measurable effect on tumor growth, although parallel tumor growth assays in C57BL/6 mice showed the same growth inhibition as previously observed (Fig. 5D). Therefore, BM-IKDC are dependent on the presence of host effector cells for tumor regression following injection at a distant site.

Discussion

Previous workers have shown unpulsed immature DC to be capable of tumoricidal activity. Often these studies have involved intratumor injection, although distantly injected unpulsed DC cause some tumoricidal activity in some models (17–19) and no activity in other studies (12–16), presumably reflecting differences in DC subtype and/or inherent antigenicity of the model. In our C57BL/6 model, bone marrow-derived unpulsed DC were generated by short-term culture from plastic-adherent monocytes, following addition of GM-CSF and IL-4 and activation with LPS (Fig. 1) or
FIGURE 4. CD11cintB220^+NK1.1^+ cells are necessary and sufficient for the tumoricidal effect of unpulsed DC vaccination in tumor-bearing mice. A, Bone marrow-derived DC were separated by flow cytometry after staining with FITC-conjugated B220-specific, PE-conjugated CD11c-specific, and allophycocyanin-conjugated-specific NK1.1 Abs. We gated on CD11cint cells and FACS sorted B220^+^ and NK1.1^+^ double-positive cells. B, Sorted cells were further analyzed for their purity and for the expression of CD49b, NK1.1, MHC class II (I-A/I-E) and Gr-1 using PE-Cy7-conjugated-specific Abs. Isotype controls are open histograms. C, Left panel, 76-9-C23 tumor-bearing C56BL/6 mice were left untreated or treated, as specified in protocol 3, with 1 × 10^6^ sorted negative fraction (shown in A) or unsorted total DC, or negative fraction supplemented with 5% or 10% of CD11cintB220^+NK1.1^+CD49b^+. Animals were culled when tumor size reached 1.2-cm diameter. Right panel, Tumor-bearing mice were left untreated or treated as per protocol 3 with 10^5^, 10^4^, or 5 × 10^3^ of FACS-sorted CD11cintB220^+NK1.1^+CD49b^+ cells. One representative out of two experiments including six mice per group is shown.
prostaglandin $\text{E}_2$ (Fig. 2). These unpulsed DC had tumoricidal ac-
tivity against three different target murine cell lines capable of
forming tumors in syngeneic C57BL/6 mice. We analyzed the
mechanism of tumor rejection in one of the three targets and
showed that the intratumoral lymphocyte infiltration comprised a
mixture of T cells and NK cells.

Remarking, removal of CD11c$^{+}$B220$^-$NK1.1$^+$CD49$^+$ IKDC
cells from the DC bulk culture caused virtually complete loss of the
tumoricidal effect. This was unlikely to result from artifact
caused by FACS sorting because back-of-the-sorted BM-IKDC
fraction was capable of restoring killing function to a level greater
than that which had been induced by unsorted DC, and remarkably
few (5 \times 10^5) BM-IKDC were capable of inducing antitumor re-
spONSE. The BM-IKDC population is shown to have TRAIL-
and NKG2D-dependent NK-type cytotoxicity in vitro. Ab-blocking
experiments on splenocytes from unpulsed DC-treated mice show
induction of cytotoxic activity of both CD8$^+$T cells and
NK1.1$^+$positive cells. Moreover, in the tumor regression we have
observed induced by purified BM-IKDC, there is infiltration of
tumors by activated T cells and accumulation of Ag-specific activat-
ted T cells in spleen, showing in vivo Ag presentation to have
occurred. However, it is not clear whether the host or the adop-
tively transferred DC (or both) are responsible for the presentation.
One model is that direct cytotoxicity by IKDC allows for recruit-
ment of host DC, which take up tumor Ag and stimulate host
Ag-specific T cells or NK cells. An alternate model is that IKDC
perform both killing and presentation of Ags from the killed tumor
cells. These different models need to be formally assessed, for
example in animals lacking host Ag presentation function. More-
over, very high degrees of purity of FACS-sorted IKDC are needed
to exclude Ag presentation by contaminating conventional DC for
both in vitro and in vivo assessments of IKDC Ag presentation
function. Similarly, experiments using unpulsed DC and BM-
IKDC from perforin-deficient hosts would be one way to delineate
whether direct target cell killing is an essential component of the
tumoricidal effect. Our data showing absence of tumoricidal effect
of IKDC in hosts lacking T cells and NK cells suggests that the
BM-IKDC-mediated direct tumor cytotoxicity observed in vitro is
insufficient to inhibit tumor growth in vivo following administra-
tion of BM-IKDC at a distant site.

A surprising finding is that as well as regression of established
tumors, the unpulsed DC also inhibited outgrowth of subsequently
challenged tumor (protocol 2 in Fig. 2) by stimulating the mixed T
cell and NK cell response. This suggests that the adoptively trans-
ferred DC survive in vivo until the time of the tumor challenge 1
wk later and then are able to stimulate an mixed adaptive and
innate immune response on encounter with tumor. This contention
will need formal testing in experiments with marked DC, both
conventional DC and IKDC.

Zitvogel and coworkers have previously demonstrated the ca-
pacity of IKDC derived from spleens of mice treated with IL2
and imatinib to induce the regression of B16 tumors in immuno-
deficient mice following intratumoral injection (20). In com-
mon with our findings, in the absence of host immune effector
cells there was no effect on growth of a contra舌尖al tumor.
This suggests that in vivo cytotoxic effects of IKDC on tumor
growth require direct inoculation into tumor. Alternatively, the
lack of BM-IKDC effect on tumor growth in immunodeficient
mice in our hands could be due to different migratory or func-
tional NK activity of BM-IKDC compared with splenic IKDC
from IL-2- and imatinib-treated mice.

Therefore, we have identified BM-IKDC as the primary cell
type involved in the initiation of tumor growth inhibition induced
by unpulsed bone marrow-derived DC adoptive transfer in a mu-
nine cancer model. However, some controversy has arisen regard-
ing the identity and function of the IKDC population, which shares
the same phenotype as the CD11c$^{+}$B220$^-$NK1.1$^+$CD49$^+$ popu-
lation described in this study (23–25, 28). We have elected to call
these cells BM-IKDC but stress that our data, as far as they go, do
not exclude the possibility that the BM-IKDC may represent ac-
tivated and highly potent NK cells capable of inducing regression
of established tumor through induction of secondary immune re-
ponses, but without having Ag presentation capacity in their own
right. It would be important in future experiments to perform a
direct comparison in vivo of the tumor inhibitory properties of
BM-IKDC and conventional NK cells. The lack of tumor regres-
sion in hosts lacking functional B cells, T cells, or NK cells con-
firms that BM-IKDC, although cytotoxic, rely on cells of the host
immune system for the majority of their tumoricidal activity in
vivo. Conventional NK cells are also known to activate host APC
and so this direct comparison would provide interesting data on
relative tumoricidal potency.

The relationship of IKDC to NK cells and DC has been ques-
tioned in terms of ontogeny as well as function (23–25). For
example, both NK and IKDC depend primarily on IL-15 and com-
mon $\gamma$-chain signaling for development (23, 25) and both lack
expression of PU.1 (23). Analysis of published gene expression
profiles suggests that splenic IKDC share a transcriptional signa-
ture with NK cells but have relatively little overlap with DC sub-
sets (29). However, it has recently been shown that IKDC arise
from progenitors of the lymphocytic lineage distinct from pDC or
NK progenitors (28). It will be important to determine whether
BM-IKDC within bulk DC preparations also appear most closely
derived from NK cells and whether BM-IKDC from IL-15 knock-
out mice have similar tumoricidal activity.

Further work is needed to elucidate the mechanism of tumor
regression induced by BM-IKDC. We have unequivocally dem-
onstrated cytotoxic activity of these cells in vitro; however, the

<table>
<thead>
<tr>
<th>Day and Analysis Conditions</th>
<th>% of Viable Cells That Are CD11c$^+$</th>
<th>% of IKDC in the Viable Cell Population</th>
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<tbody>
<tr>
<td>Day 1</td>
<td>25 ± 3.50</td>
<td>3.5 ± 1.25</td>
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<tr>
<td>Day 1, CD49b enrichment</td>
<td>15 ± 5.25</td>
<td>2.6 ± 0.55</td>
</tr>
<tr>
<td>Day 3</td>
<td>47.5 ± 4.5</td>
<td>3.25 ± 1.55</td>
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<tr>
<td>Day 3, CD49b enrichment</td>
<td>72.25</td>
<td>9 ± 3.25</td>
</tr>
<tr>
<td>Day 7, LPS activation</td>
<td>78.25 ± 2.75</td>
<td>5 ± 1.00</td>
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<tr>
<td>Day 7, prostaglandin $\text{E}_2$ activation</td>
<td>70.83 ± 4.23</td>
<td>4.75 ± 1.23</td>
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</tbody>
</table>

* DC bone marrow-derived progenitors (10^6) were cultured in medium supplemented with rmGM-CSF and rmIL-4 as described in Materials and Methods. Bulk DC cultures were FACs analyzed for CD11c expression at different indicated days. The percentage of IKDC was determined using triple staining for CD11c$^{+}$, B220, and NK1.1$^+$ cells before or after CD49b beads selection (days 1 and 3). The data are expressed as means ± SD of five independent experiments.
ability to present Ag in vitro and in vivo needs formal demonstration, especially as others have failed to demonstrate any Ag presentation function in IKDC derived from splenocytes (23). Moreover, the ability of as few as 5 × 10⁴ purified BM-IKDC to inhibit growth of established tumors in immunocompetent mice suggests a remarkable potency in terms of induction of antitumor immunity, as well as obvious potential clinical applications. It will be of great interest to determine whether an analogous population exists in human bone marrow- or monocyte-derived DC populations and to assess their Ag presentation and NK functions.

Disclosures
The authors have no financial conflicts of interest.
References