HLA-DR⁺ Immature Cells Exhibit Reduced Antigen-Presenting Cell Function But Respond to CD40 Stimulation¹*

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Abstract

Dendritic cells (DC) have been implicated in the defective function of the immune system during cancer progression. We have demonstrated that patients with cancer have fewer myeloid (CD11c⁺) and plasmacytoid (CD123⁺) DC and a concurrent accumulation of CD11c⁺CD123⁻ immature cells expressing HLA-DR (DR⁺IC). Notably, DR⁺IC from cancer patients have a reduced capacity to stimulate allogeneic T-cells. DR⁺IC are also present in healthy donors, albeit in smaller numbers. In this study, we assessed whether DR⁺IC could have an impact on the immune response by comparing their function with DC counterparts. For this purpose, DR⁺IC and DC were purified and tested in the presentation of antigens through major histocompatibility complex (MHC) II and MHC-I molecules. DR⁺IC were less efficient than DC at presenting antigens to T-cells. DR⁺IC induced a limited activation of T-cells, eliciting poor T-helper (Th) 1 and preferentially inducing Th2-biased responses. Importantly, despite DR⁺IC’s poor responsiveness to inflammatory factors, in samples from healthy volunteers and breast cancer patients, CD40 ligation induced phenotypic maturation and interleukin 12 secretion, in turn generating more efficient T-cell responses. These data underscore the importance of inefficient antigen presentation as a mechanism for tumor evasion and suggest an approach to improve the efficacy of DC-based immunotherapy for cancer.

Keywords: Antigen-presenting cell, dendritic cell subsets, CD40 ligand, apoptosis, immune dysfunction.

Introduction

A growing body of evidence has demonstrated that dendritic cells (DC) play a crucial role in the induction of antitumor immune responses. DC are capable of recognizing, processing, and presenting tumor antigens to T-cells, thereby initiating tumor-specific immune responses [1,2]. Numerous clinical observations suggest that DC infiltration of tumors correlates with better prognosis [3–5]. These data imply that DC play a beneficial role for patients with regard to antitumor responses. However, tumors employ a variety of mechanisms to evade elimination by the immune system, and DC appear particularly susceptible to tumor-mediated suppression [6–9].

The production of immunosuppressive factors is a crucial mechanism by which tumors evade immune surveillance [10]. Among these factors are cytokines, arachidonic acid metabolites, glycosphingolipids, or polyamines—all modulators of immune function produced by malignant cells [11–15]. However, most studies addressing the nature of tumor–DC interactions have utilized in vitro–derived DC models, which may not reflect the natural biology of immune responses occurring in vivo [11,13,14].

We have reported in A Population of HLA-DR⁺ Immature Cells Accumulates in the Blood Dendritic Cell Compartment of Patients with Different Types of Cancer (accompanying paper) that the blood DC compartment (Lin⁻HLA-DR⁺ cells) in patients with breast and prostate cancers, as well as malignant glioma, has fewer myeloid (CD11c⁺DC) and plasmacytoid (CD123⁺DC) DC, and a significant accumulation of a population of HLA-DR⁺CD11c⁻CD123⁻ immature cells (DR⁺IC). This increase correlates with stage of disease and tumor size, thus indicating a clear association with tumor progression. Notably, DR⁺IC from cancer patients are deficient in their capacity to stimulate allogeneic T-cells. This population is also present in healthy donors, although here it represents a significantly lower proportion of the blood DC compartment.

In this study, to characterize the possible role of these cells in immunity, DR⁺IC from healthy donors were purified, and their functionality was evaluated by comparison with DC. We report that DR⁺IC have a limited response to “danger signals” and poor antigen-presenting cell (APC) function. Importantly, in DR⁺IC from healthy volunteers and breast cancer patients, CD40 ligation induces phenotypic maturation as well as interleukin

Abbreviations: DC, dendritic cells; DR⁺IC, HLA-DR⁺ immature cells; APC, antigen-presenting cell; Lin, lineage marker; CTL, cytotoxic T-lymphocyte; Th, T-helper

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¹This work was funded by the National Breast Cancer Foundation, Australia. A.P.C. was supported by the University of Queensland International Postgraduate Research and the Paul Mackay Bolton Cancer Research Scholarships.

*This article refers to supplementary material, which is designated by “W” (i.e., Table W1, Figure W1) and is available online at www.bcdecker.com.

Received 30 September 2005; Revised 39 September 2005; Accepted 3 October 2005.

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DOI 10.1593/neo.05448
(IL) 12 secretion, confirming that this approach can be used to improve the function of circulating APC (DC and DR*IC) in patients with cancer.

Materials and Methods

Patients, Donors, and Blood Products

A total of 46 healthy donors (26 females and 20 males, 25–80 years of age) volunteered for the study. The Australian Red Cross Blood Service (Brisbane, Australia) provideduffy coats. In addition, six female patients 42 to 68 years of age with histologically confirmed breast adenocarcinoma were enrolled in the study. These patients presented with early disease (stages I and II) and had received no prior cancer treatment. Staging was performed according to the International Union Against Cancer UICC TNM Classification. Ethical approval for all forms of collection was obtained from the research ethics committee of both clinical (Wesley Medical Centre) and scientific (Queensland Institute of Medical Research) institutions. Fifty or 500 ml of venous blood was collected and processed immediately for all experiments involving direct culture or flow cytometric purification, respectively.

Antibodies, Reagents, and Cytokines

The following monoclonal antibodies (mAb) were used: IL-2, IL-4, tumor necrosis factor α (TNF-α), interferon γ (INF-γ), CD25, CD69, CD8, CD27, CD3, CD14, CD19, CD20, CD56, CD34, CD123, CD80, CD86, and IgG1, IgG2a, and IgG2b isotype controls from BD Pharmingen (BD Biosciences, San Jose, CA); CD4, HLA-DR, CD40, CD83, CD19, and IgG1 isotype controls from Beckman Coulter (Fullerton, CA); and IL-10 and IL-12 from Caltag Laboratories (Burlingame, CA). All antibodies were used as fluorescein isothiocyanate (FITC)—, PE—, biotin—, APC—, or PE-Cy5—conjugated mAb. The synthetic PICS 327–335 peptide and the PICS 282–383 polypeptide were kindly provided by Dr. Giampietro Corradin (Institut de Biochimie, Lausanne, Switzerland). Sheep red blood cells were obtained from Equine (Melbourne, Victoria, Australia). The complete medium was Roswell Park Memorial Institute 1640 (RPMI 1640), supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), HEPES (25 mM), and nonessential amino acids (all purchased from Gibco Life Technologies, Gaithersburg, MD). Where indicated, RPMI 1640 supplemented with pooled human AB serum (Red Cross Blood Service) or serum-free medium (Biowhittaker, Walkersville, MD) was used for culture. Brefeldin-A (BFA; 10 μg/ml), phorbol myristate acetate (PMA) (0.025 mg/ml), and ionomycin (1 μg/ml) were obtained from Sigma (St. Louis, MO). Granulocyte—macrophage colony-stimulating factor (GM-CSF; 1000 U/ml) and IL-4 (1000 U/ml; SANDOZ/Scheri/Plough) or all-trans-retinoic acid (ATRA; 10−4–10−9 M; Sigma) were used at the indicated concentrations. The combination of proinflammatory cytokines [16] consisted of IL-1β (10 ng/ml), IL-6 (10 ng/ml), TNF-α (10 ng/ml) (R&D Systems, Minneapolis, MN) plus prostaglandin E2 (PGE2; 1 μg/ml; Sigma). The CpG oligo(den)ucleotide 2216 (CpG ODN; 3 μg/ml) [17] was acquired from Geneworks (Melbourne, Victoria, Australia). Lipopolysaccharide (LPS; 50 ng/ml) and double-stranded RNA (poly I:C; 50 μg/ml) [18] were purchased from Sigma. IFN-γ (10 ng/ml; Mabtech, Stockholm, Sweden) or soluble human recombinant CD40 ligand (CD40L; 2 μg/ml; kindly provided by Amgen, Seattle, WA) was used at the indicated concentration.

Cell Purification

Following isolation of peripheral blood mononuclear cells (PBMC) by density gradient centrifugation, cells were stained with the lineage mixture (CD3, CD14, CD19, CD20, and CD56) and CD34 (all FITC), HLA-DR (PE), CD11c (APC), and biotinylated CD123, followed by streptavidin (APC), and were sorted. CD34 was added to the lineage marker (Lin) to exclude circulating hematopoietic stem cells, and 7-AAD was used as a viability indicator. Viable DC (Lin− HLA-DR+C11c+CD123+) and DR*IC (Lin− HLA-DR−CD11c−CD123−) were sorted in parallel (99% purity) using MoFlo Sorter (Dako Cytomation, Fort Collins, CO) and immediately resuspended in complete medium.

Maturation and Cytokine Secretion

Four-color flow cytometry was used to analyze the phenotype and cytokine secretion of DC and DR*IC. For assessment of phenotypic maturation, PBMC were cultured (106 cells/ml) in six-well plates for 18 to 36 hours in complete medium in the presence of inflammatory cytokines (IL-1β, IL-6, TNF-α plus PGE2; CC), LPS, poly I:C, CpG ODN, or CD40L and subsequently stained for flow cytometric analysis. Doses and incubation times were optimized in preliminary experiments. For cytokine secretion, PBMC were cultured (107 cells/ml) in six-well plates for 18 to 36 hours in complete medium in the presence of CC, poly I:C, or CD40L (in addition to IFN-γ and IL-1β) and in the presence of LPS. Cytokine secretion was assessed by intracellular staining. Cells were stained for surface markers, fixed with 1% paraformaldehyde, and stained with PE-conjugated cytokine-specific mAb (TNF-α, IL-10, and IL-12) in 0.2% saponin/phosphate-buffered saline (PBS) at 4°C overnight. In all experiments, 5 × 105 to 10 × 105 events were collected within the mononuclear cell gate. Data were acquired on a FACs Calibur flow cytometer and analyzed using CellQuest 3.1 (BD Biosciences), FloJo (TreeStar, San Carlos, CA), or Summit (Dako Cytomation) software. Where indicated, culture supernatants were collected and assayed using an IL-12p70 ELISA kit (Mabtech), according to the manufacturer’s instructions.

Antigen Presentation to T-cells

Allogeneic T-cells were obtained from buffy coats by rosetting PBMC with neuraminidase-treated sheep red blood cells (> 90% CD3+ cells). Alternately, CD4+ T-cells were purified by positive selection with anti-CD4 microbeads (> 90% CD4+; Miltenyi Biotec, Bergisch Gladbach, Germany). Tetanus toxoid (TT)—specific T-cell lines were generated from healthy donors vaccinated within the past 3 years. PBMC were cultured in complete medium in the presence of 0.1 μg/ml TT and IL-2 (1 U/ml) added on day 7. Cells were expanded for a
further 7 days before freezing. TT-specific cells (> 90% CD3+ cells) were thawed and cultured for 4 to 5 days in a medium with IL-2 before use in antigen presentation assays. DC and DR*IC were purified from healthy donors using MoFlo Sorter, as described above. Varying numbers of DC and DR*IC were cultured with 1 × 10^5 allogeneic T-cells for 5 days in complete medium. Sixteen hours prior to harvesting, 1 μCi/well [3H]-thymidine was added to each well. [3H]-thymidine incorporation was measured in a β-scintillation counter (MicroBeta Trilux Scintillation Counter; Wallac, Turku, Finland). For elicitation of cytotoxic T-lymphocyte (CTL) activity, T-cells were primed with allogeneic DC or DR*IC (test) or syngeneic T-cell−depleted mononuclear cells (control). After 5 days in culture, alloreactive T-cells were collected and washed, and CTL activity was determined in a standard chromium release assay against appropriate allogeneic target cells (lymphoblastoid cell lines, LCL). For major histocompatibility complex (MHC) II presentation, 5 × 10^5 DC or DR*IC was cultured with 1 × 10^5 autologous TT-specific CD4+ T-cells in the presence or absence of antigen (TT: 1.0 μg/ml) in 5% AB pooled human serum (ABS)/RPMI 1640. [3H]-thymidine incorporation was measured after 5 days in culture. For MHC-I presentation, 2 × 10^4 DC or DR*IC from HLA-A201* donors was pulsed with decreasing concentrations (10−6–10−12M) of a 9-mer CD8+ T-cell epitope (PICS327–335) for 2 hours at 37°C in X-VIVO[15]. Cells were washed, resuspended in 5% ABS/RPMI 1640, and cultured (4 hours at 37°C) with the antigen-specific HLA-A201−restricted CD8+ T-cell clone [19] (APC/effecter ratio of 1:1) in the presence of BFA. Cells were harvested; stained with anti-CD3 (FITC), anti-CD4 (PE-Cy5), and anti-CD8 (APC); washed; and fixed with 1% paraformaldehyde before staining with IFN-γ (PE) in 0.2% saponin/PBS at 4°C overnight. IFN-γ synthesis was analyzed using flow cytometry. For cross-presentation, 7 × 10^5 DC and DR*IC from HLA-A201* donors was pulsed with a 102-mer polypeptide (PICS282–383; 100 μg/ml) for 2 hours in X-VIVO[15] at 37°C. Cells were washed, resuspended in 5% FCS/RPMI 1640, and cultured with 3.5 × 10^5 T-cells specific for the 9-mer epitope [19] (PICS327–335). Supernatants were collected and assayed using an IFN-γ ELISA kit (Mabtech) according to the manufacturer’s instructions. As an internal control, T2 cells were unable to cross-present (IFN-γ < 100 pg/ml).

**Phenotypic Analysis of T-cells**

The phenotype of purified T-cells was determined before and after allogeneic priming. Briefly, 5 × 10^5 sorted DC or DR*IC was cultured with 10^5 allogeneic T-cells in complete medium. After 5 days, T-cells were harvested, stained, and analyzed by flow cytometry. For cytokine secretion, 5 × 10^5 DC or DR*IC was incubated with 10^5 alloreactive T-cells. Alternatively, DC or DR*IC were incubated in the presence of CD40L for 36 hours prior to addition of T-cells. After 5 days in culture, supernatants were collected, and IFN-γ production was assessed by ELISA. Where indicated, T-cells were restimulated with PMA and ionomycin in the presence of BFA for 4 hours at 37°C, stained for surface markers, and fixed with 1% paraformaldehyde before incubation with PE-conjugated cytokine-specific mAb (IFN-γ, TNF-α, IL-2, and IL-4) in 0.2% saponin/PBS at 4°C overnight.

**Cell Migration Experiments**

Chemotaxis assays were performed as previously reported [20] using a 3-μm pore transwell system (Costar, Corning, NY), with some modifications. Briefly, 1 × 10^5 PBMC in 100 μl of complete medium were added to upper inserts, which were transferred to wells containing 500 μl of medium, with or without chemokines. The chemokines tested were CCL21 (300 ng/ml) and CXCL12 (75 ng/ml; PeproTech, Rocky Hill, NJ). Cells were allowed to migrate for 1 hour at 37°C, collected, and stained. For cell number quantitation, 45,000 fluorescent beads were added to each sample before acquisition, and samples were run until 10,000 beads had been counted. During analysis, samples were continuously mixed to prevent sedimentation of the beads. The proportion of CD11c+DC, CD123+DC, and DR*IC was determined by flow cytometry, and absolute cell numbers in each sample were estimated, as previously reported [21]. Briefly, with reference to Figure 3A, cell numbers were calculated as: 

\[ \text{cell numbers} = \frac{\text{(number of beads added/beads acquired in R1)} \times \text{(number of PBMC in R2)}}{\text{((percentage of DC or DR*IC in R3, R4, or R5))}} \]

Cell numbers were determined before (input) and after migration. Migratory capacity was expressed as the percentage of migrated cells with regard to input, as previously reported [20].

**Tumor-Derived Supernatant (TDSN) Experiments**

Breast cancer cell lines MDA-MB231, MA11, MB435, SKBR3, and MCF-7, as well as colorectal cancer cell line LOVO, were a generous gift of Dr. M. McGuckin (Mater Medical Research Institute, South Brisbane, Australia). TDSNs were prepared by seeding 10^7 tumor cells in 20 ml of complete medium and incubating for 72 hours. Before passage, culture supernatants were collected, centrifuged to remove cells, dialyzed against a fresh medium (24–48 hours at 4°C in the dark) to replenish nutrients, and stored at −20°C. Dialysis membranes (membra cell; Polylabo, Strasbourg, France) with a Mw cutoff of under 10,000 to 14,000 were used. For each experiment, PBMC were cultured (10^5 cells/ml) for 24 hours in the presence of 50% (vol/vol) PBMC-conditioned medium (control) or TDSN. Cells were harvested, washed, and stained for apoptosis using the Annexin V detection Kit (BD Biosciences) or the terminal deoxynucleotidyl transferase−mediated dUTP-biotin end labeling (TUNEL) Apo−BrDU−Red DNA fragmentation kit (Biovision, Mountain View, CA), following the manufacturer’s instructions.

**Statistical Analysis**

Comparisons of samples to establish statistical significance were determined by two-tailed Student’s t test for independent samples. Results were considered statistically significant when P < .05.

**Results**

**Allogeneic and Antigen-Specific Presentation**

In an accompanying paper (A Population of HLA-DR* Immature Cells Accumulates in the Blood Dendritic Cell Compartment of Patients with Different Types of Cancer),
we described a population of DR\textsuperscript{+} immature cells (DR\textsuperscript{IC}) that was overrepresented in patients with advanced cancers. These cells are also present in healthy individuals, although at lower numbers. We confirmed that DR\textsuperscript{IC} isolated from healthy donors have phenotype, morphology, and function similar to those of DR\textsuperscript{IC} studied in patients with cancer (data available as supplementary data; Figure W1 and Table W1). Considering the limitation in obtaining sufficient DR\textsuperscript{IC} from patients with cancer, we proceed to a further detailed functional evaluation of these cells, which are present in healthy individuals.

We showed that DR\textsuperscript{IC} express HLA-DR and moderate amounts of CD40 and CD86, whereas CD80 and CD83 are absent. To investigate whether incomplete expression of co-stimulatory molecules could impact on the functionality of DR\textsuperscript{IC}, we set out to evaluate their capacity to present antigens to T-cells. Given their elevated expression of HLA-DR (MHC-II molecules), we first assessed their ability to present a soluble antigen (TT) to a CD4\textsuperscript{+} T-cell line. Autologous DC pulsed with TT efficiently stimulated antigen-specific CD4\textsuperscript{+} T-cells to proliferate. In contrast, DR\textsuperscript{IC} had a significantly lower ability to induce CD4\textsuperscript{+} T-cell proliferation (Figure 1A). We also assessed the capacity of DR\textsuperscript{IC} to present antigens in the context of MHC-I molecules. For this purpose, DR\textsuperscript{IC} were pulsed with decreasing concentrations of a 9-mer peptide (PICS 327–335), and recognition was tested by coculture with an antigen-specific HLA-A201\textsuperscript{+} restricted CD8\textsuperscript{+} T-cell clone [19]. We found that DC and DR\textsuperscript{IC} from HLA-A201\textsuperscript{+} donors could similarly present the peptide at a molar range between 10\textsuperscript{6} and 10\textsuperscript{–6} (Figure 1B). Interestingly, despite an equally efficient presentation of this epitope when added as an exogenous polypeptide (Figure 1B), DR\textsuperscript{IC} were significantly less efficient than DC in their ability to process the parent 102-mer polypeptide (PICS 282–383) and subsequently to cross-present the naturally processed 9-mer epitope (PICS 327–335) to the CD8\textsuperscript{+} T-cell clone [19] (Figure 1C). These results indicated that DR\textsuperscript{IC} were inefficient at cross-presenting an exogenous antigen in the context of MHC-I molecules. Thus, we assessed their ability to induce CTL activity in an allogeneic system. We found that effector T-cells induced by DR\textsuperscript{IC} exhibited discretely lower levels of CTL activity than those induced by DC (Figure 1D). Put together, these results are consistent with reduced ability to process exogenous antigen for presentation on MHC-I or MHC-II. Finally, we set out to investigate whether DR\textsuperscript{IC} could impair T-cell responses generated by competent DC. Two different experimental systems were used. First, allogeneic T-cells were cultured with DC and increasing numbers of DR\textsuperscript{IC} (Figure 1E). Second, TT-specific CD4\textsuperscript{+} T-cells were cultured with DC and increasing numbers of DR\textsuperscript{IC} in the presence of antigen (Figure 1F). In both systems, DR\textsuperscript{IC} appeared not to have an inhibitory effect on T-cell responses. Instead, addition of increasing numbers of DR\textsuperscript{IC} enhanced the proliferation of T-cells.

Figure 1. Antigen presentation to T-cells. (A) For MHC-II presentation, 5 \times 10\textsuperscript{5} purified DC or DR\textsuperscript{IC} were cultured with 10\textsuperscript{5} autologous TT-specific CD4\textsuperscript{+} T-cells in the presence or absence of antigen (TT, 1.0 \mu g/ml). Proliferation was measured after 5 days in culture. Data shown represent the average of five independent experiments. (B) For MHC-I presentation, 2 \times 10\textsuperscript{5} purified DC (●) or DR\textsuperscript{IC} (○) were pulsed with decreasing concentrations (10\textsuperscript{−6} – 10\textsuperscript{−12} M) of a 9-mer CD8\textsuperscript{+} T-cell epitope (PICS 327–335), washed, and cultured with the antigen-specific CD8\textsuperscript{+} T-cell clone (APC/effector ratio of 1:1). IFN-γ secretion by the T-cell clone was determined by intracellular staining. Data shown represent the average of three donors assessed, each in triplicate. (C) For cross-presentation, 7 \times 10\textsuperscript{4} purified DC or DR\textsuperscript{IC} were pulsed with a 102-mer polypeptide (PP, PIKS 282–383). Cells were washed and cultured with 3.5 \times 10\textsuperscript{5} T-cells specific for the corresponding CTL epitope (PICS 327–335) (APC/effector ratio of 30:1). Supernatants were assayed for IFN-γ content by ELISA. Data shown represent the average of three donors assessed, each in triplicate. (D) For elicitation of CTL, 10\textsuperscript{5} allogeneic DC (●), allogeneic DR\textsuperscript{IC} (○), or syngeneic T-cell–depleted mononuclear cells (□) (control). After 5 days in culture, CTL activity was determined by culturing 10\textsuperscript{5} alloreactive T-cells with decreasing numbers of chromium-loaded allogeneic targets (LCL from appropriate donors). Data shown represent the average of three donors assessed, each in triplicate. (E and F) For assessment of the effect of DR\textsuperscript{IC} on T-cell activation elicited by competent DC, two systems were tested. (E) First, 10\textsuperscript{5} allogeneic T-cells were primed with 5 \times 10\textsuperscript{5} DC and increasing numbers of DR\textsuperscript{IC}. (F) Second, 10\textsuperscript{5} TT-specific T-cells were restimulated with 5 \times 10\textsuperscript{5} DC in the presence of antigen and increasing numbers of DR\textsuperscript{IC}. Proliferation was measured after 5 days in culture. Data shown represent the average ± SEM of three donors assessed, each in triplicate. Statistically significant differences between DC and DR\textsuperscript{IC} are indicated (∗P < .05, **P < .01).
Migratory capacity. (A) Flow cytometric analysis for evaluation of T-cells were analyzed for CCR7 production. Data shown represent the average of five donors assessed. (B) For cytokine secretion by T-cells, 5 × 10^5 purified DC or DR^+^IC were cultured with 10^5 allotypic T-cells. After 5 days, cells were harvested and CD3^+^, CD69, CD27, or CCR7 expression. Data shown represent the average ± SEM of five donors assessed. Statistically significant differences are indicated (*P < .05). For cytokine secretion, that of CD27 and CCR7 decreased [22].

Figure 2. Phenotype of primed T-cells. (A) For surface phenotype, 5 × 10^3 sorted DC or DR^+^IC were cultured with 1 × 10^6 allogeneic T-cells in complete medium. After 5 days, cells were harvested and CD3^+^ T-cells were analyzed for CCR7. CD69, CD27, or CCR7 expression. Data shown represent the average of five donors assessed. (B and C) For cytokine secretion by T-cells, 5 × 10^5 purified DC or DR^+^IC were cultured with 10^5 allotypic T-cells. After 5 days in culture, (B) supernatants were collected and IFN-γ production was assessed by ELISA, or (C) cells were collected and CD3^+^ cells were stained with cytokine-specific mAb (IFN-γ, TNF-α, IL-2, and IL-4). Percentage of positive T-cells for each cytokine was determined, and type 1/type 2 ratio was calculated. Data shown correspond to individual donors, each assessed in triplicate. Means are shown as horizontal lines, and error bars indicate SEM.

Statistically significant differences between DC and DR^+^IC are indicated (*P < .05, **P < .01).

Surface and Cytokine Phenotype of Primed T-cells

The above data demonstrated that, although DR^+^IC were not suppressive, they had a reduced capacity to present exogenous antigens to T-cells. Next, we sought to determine whether such reduction in antigen-presenting capacity could affect the nature of the T-cell responses generated. For this purpose, the phenotype and cytokine profile of T-cells primed with DC or DR^+^IC were determined. First, we analyzed the expression of markers CD25, CD69, CD27, and CCR7 on CD3^+^ T-cells. Although expression of CD25 and CD69 increased following in vitro stimulation, that of CD27 and CCR7 decreased [22]. In our experiments with freshly purified cells, a minimal proportion of T-cells expressed the activation markers CD25 (2.2 ± 0.8) and CD69 (2.9 ± 1.0). In addition, a constant proportion of T-cells expressed CD27 (62.0 ± 4.0) and CCR7 (28.5 ± 6.5). Following stimulation, cultures primed with DC had a higher proportion of T-cells positive for CD25 and CD69, but a lower proportion of CCR7^+^ and CD27^+^ T-cells than cultures stimulated with DR^+^IC (Figure 2A). We also assessed the ability of DR^+^IC to stimulate IFN-γ secretion in T-cells. As shown in Figure 2B, supernatants taken from allogeneic T-cells cultured with DR^+^IC contained significantly lower levels of IFN-γ than those cultured with DC. Given that the balance between type 1 [T-helper (Th) 1] and type 2 (Th2) cytokines is more relevant than absolute values of single cytokines in driving immune responses, ratios between cytokine-expressing T-cells were determined by measuring the intracellular expression of IFN-γ, TNF-α, IL-2 (Th1), and IL-4 (Th2). As shown in Figure 2C, Th1/Th2 ratios were significantly higher (P < .05) in cultures primed with DC, indicating a strong Th1 response. In contrast, in cultures primed with DR^+^IC, a higher percentage of T-cells expressed IL-4 (DC: 5.1 ± 1.8% vs DR^+^IC: 8.2 ± 2.4%), indicating that a Th2 response was prevalent.

Migratory Capacity of DR^+^IC

Having assessed DR^+^IC’s function as APC, we sought to determine their migratory capacity. Migration of APC toward lymphoid organs is critical for the initiation of T-cell immunity and requires APC to respond to lymph node–directing chemokines such as CCL21 (secondary plasmacytoid tissue chemokine, SLC) and CXCL12 (stromal-derived cell factor). Here, we evaluated the migratory capacity of DR^+^IC and DC in response to these chemokines. Given that cell migration can be hampered by poor survival and manipulation of isolated cells [20], we performed migration assays with whole PBMC, taking advantage of quantitative flow cytometry (Figure 3A). Our experiments confirmed that CD11c^+^DC are a highly motile population responding vigorously to CCL21 and CXCL12 [20]. In keeping with previous reports, CD123^+^DC had a reduced capacity to migrate when compared to CD11c^+^DC [20], demonstrating marginal chemotaxis to CXCL12 and moderate chemotaxis to CCL21. Interestingly, DR^+^IC displayed a stronger chemotaxis than CD123^+^DC. In particular, they were significantly responsive to CCL21, with at least 60% of the starting population migrating across transwells (Figure 3B; P < .05). These results indicated that whereas DR^+^IC displayed limited APC capacity when compared to DC, they exhibited considerable migratory capacity to a lymph node–directing chemokine.

Figure 3. Migratory capacity. (A) Flow cytometric analysis for evaluation of the migration of DC and DR^+^IC in unseparated PBMC. For cell number estimation, a fixed number of fluorescent beads (R1) were added to each PBMC preparation (R2). Subsequently, Lin^−^HLA-DR^+^ cells (R3) were divided into CD11c^+^DC (R4), CD123^+^DC (R5), and DR^+^IC (R6), and their frequency was estimated before (input) and after (migrated) migration. Absolute numbers for each subpopulation were calculated as described in Materials and Methods section. Numbers shown indicate the percentage of total cells within the corresponding gate. (B) Summary of chemotaxis data in response to CCL21 and CXCL12. Migration is shown as the percentage of migrated cells with regard to its input. Data shown represent the average ± SEM of five donors assessed. Statistically significant differences are indicated (*P < .05).
Resistance to Tumor-Induced Apoptosis

Next, we set out to obtain insights into possible mechanisms that could be responsible for the decrease in DC and the accumulation of DR\(^+\)IC that has been documented in cancer patients. Tumor-derived factors have been reported to induce apoptosis in APC, including DC [23]. Thus, we hypothesized that accumulation of DR\(^+\)IC could represent the selective survival of cells resistant to tumor-induced apoptosis. We investigated this hypothesis by measuring apoptosis in DC and DR\(^+\)IC following culture in the presence of TDSNs. TDSNs were filtered and dialyzed against a fresh medium prior to use, excluding the possibility of apoptosis induced by nutrient depletion. As shown in Figure 4A, assessment of Annexin V binding revealed minimal apoptosis on fresh samples or following culture with PBMC-conditioned medium (control). However, following a 24-hour incubation time, all TDSN induced apoptosis in DC to a significantly greater extent than in DR\(^+\)IC (Figure 4, A and B). These results were consistently observed in the presence of all TDSN tested, including breast (MB231, MA11, MB435, SKBR3, and MCF7) and colon (LOVO) cancer cell lines. Comparable results were obtained by TUNEL assays. Indeed, a significantly higher proportion of TUNEL\(^+\) DC compared to DR\(^+\)IC was observed in cultures incubated with all TDSN tested (MA11, MB435, MCF7 and SKBR3).

Phenotypic Maturation in Response to CD40 Ligation

Given the immaturity and abundance of DR\(^+\)IC in cancer patients, it was of interest to determine whether these cells could be induced to 1) differentiate or 2) perform as competent APC. Several cytokines and growth factors previously reported to induce maturation of immature cells [24] were tested, including GM-CSF (1000 U/ml), IL-4 (1000 U/ml), TNF-\(\alpha\) (10 ng/ml), and ATRA (10\(^{-8}\) to 10\(^{-9}\) M). These maturation factors failed to induce DR\(^+\)IC differentiation in vitro. Less than 10% of the cells survived, and only GM-CSF appeared to maintain viability (> 60%) beyond 72 hours, as assessed by trypan blue exclusion (data not shown). Our initial experiments also indicated that DR\(^+\)IC responded weakly to pro-inflammatory cytokines and various pathogen-derived factors. Because 40% to 50% of DR\(^+\)IC expressed the CD40 antigen, we evaluated whether CD40 ligation could induce a more robust response. To limit donor-to-donor variation, we analyzed the maturation of cells purified from the same individual donors and compared CD40 stimulation with poly I:C, CC, and the combination of CD40L plus poly I:C. The latter was included to examine the potential for synergism between CD40 ligation and TLR signalling on the functional profile of DR\(^+\)IC. As shown in Figure 5A, DC responded vigorously to all proinflammatory factors, with CD40 ligation inducing the most robust upregulation in the expression of CD86 and HLA-DR.

The combination of CD40L plus poly I:C did not induce a stronger response. Notably, although DR\(^+\)IC failed to respond to inflammatory factors, CD40 ligation induced a significant upregulation of HLA-DR and CD86. As with DC, combination of CD40L plus poly I:C did not induce increased maturation compared to CD40L alone (Figure 5B). To confirm these results, electron microscopy was performed on sort-purified DC and DR\(^+\)IC. Compared to untreated DC, CD40-stimulated DC were larger (10–12 \(\mu\)m) and had more cytoplasmic inclusions and dendrites, as well as reduced amounts of condensed chromatin in the nucleus (Figure 5C). However, CD40-stimulated DR\(^+\)IC were a little different with respect to cytoplasmic organelles and inclusions. However, they were dramatically increased in size (7–8 \(\mu\)m) and had less condensed chromatin in the nucleus (Figure 5D).

Cytokine Secretion in Response to CD40 Ligation

To determine whether phenotypic maturation was paralleled by functional improvement, we analyzed the secretion

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**Figure 4. Resistance to apoptosis.** (A) A representative flow cytometric analysis of apoptosis of DC and DR\(^+\)IC. PBMC were incubated in the presence of 50% (vol/vol) PBMC-conditioned medium (control) or TDSNs (MB231, MA11, MB435, SKBR3, and LOVO) for 24 hours. Subsequently, Lin\(^-\) HLA-DR\(^+\) cells were subdivided into DC (CD11c\(^+\)/CD123\(^+\)) or DR\(^+\)IC (CD11c\(^-\)/CD123\(^-\)) based on their expression of CD11c and CD123 (y axis). Apoptosis was determined by the detection of exposed phosphatidylserine residues with Annexin V (x axis). Numbers indicate the percentage of cells that are positive for Annexin V. (B) Summary of apoptosis data estimated with Annexin V and TUNEL assays. Results shown represent the average \(\pm\) SEM of five donors assessed. Statistically significant differences between DC and DR\(^+\)IC are indicated (*P < .05, **P < .01; nd, not done).
of IL-12, IL-10, and TNF-α in DC and DR1IC. Previous reports indicated that blood DC are poor producers of cytokines following CD40 stimulation [25]. Our initial experiments confirmed these results and indicated that DC required the addition of IFN-γ and IL-1β for cytokine secretion following CD40 ligation (data not shown). Thus, we compared proinflammatory (CC) and pathogen-mimic factors (poly I:C) with CD40 ligation under these conditions. As previously reported [16,25], CC induced minimal synthesis of IL-12, and CD40 ligation induced robust production of this cytokine by DC (Figure 6A). Notably, CD40L also induced IL-12 secretion by DR1IC. The combination of CD40L plus poly I:C induced a profile comparable to CD40L alone (Figure 6B). Next, we assessed whether CD40 ligation also improved the ability of DR1IC to induce IFN-γ secretion in T-cells. For this purpose, DC and DR1IC were stimulated with CD40L, washed, and cultured with T-cells. We found that untreated DC were strong stimulators of IFN-γ secretion, and this ability was enhanced on stimulation with CD40L (Figure 6C). Interestingly, although DR1IC were less efficient inducers of IFN-γ secretion, CD40-stimulated DR1IC were significantly better (Figure 6D). Indeed, following CD40 ligation, the capacity of DR1IC to induce IFN-γ secretion was increased by 11.1 ± 2.4 times compared to 7.6 ± 1.5 for DC. Similarly, the capacity of DR1IC to induce proliferation was enhanced, on average, by 1.7 ± 0.5 times compared to 1.0 ± 0.2 for DC, suggesting that DR1IC were more sensitive to CD40 stimulation than DC.

**Maturation and Cytokine Secretion in Samples from Breast Cancer Patients**

Having determined that DR1IC from healthy donors responded vigorously to CD40 stimulation, we sought to determine whether a comparable response was observed in samples from cancer patients. First, we analyzed phenotypic maturation in DC and DR1IC from breast cancer patients by comparing CD40L, poly I:C, CC, and CD40L plus poly I:C. As shown in Figure 7A, DC responded to CC and poly I:C with moderate upregulation of the expression of HLA-DR and CD86. Stimulation of DC with CD40L induced the strongest increase in CD86 and HLA-DR expression. CD40L plus poly I:C did not exhibit improved maturational capacity of DC compared to CD40L alone. Importantly, CD40L induced the most significant upregulation of costimulatory expression in DR1IC. As with DC, CD40L plus poly I:C did not induce increased maturation in DR1IC compared to CD40L alone. Next, we analyzed the secretion of IL-12, IL-10, and TNF-α in DC and DR1IC by intracellular staining. These experiments demonstrated that, whereas CC induced minimal cytokine production, CD40L induced robust IL-12 secretion in DC and DR1IC (Figure 7B). In keeping with the data described above, addition of CD40L plus poly I:C did not demonstrate a synergistic effect on cytokine production (Figure 7B). Finally, we compared the capacity of CC, CD40L, or poly I:C-stimulated PBMC from breast cancer patients (blood DC compartment distribution of CD11c+DC: 51.7 ± 9.0%, CD123+DC: 18.0 ± 3.6, and DR1IC: 29.8 ± 6.2%).

**Figure 5.** Maturation in response to CD40 stimulation. (A and B) PBMC were cultured for 18 to 36 hours in the presence of CC, poly I:C, CD40L, or CD40L plus poly I:C and upregulation in the expression of HLA-DR, CD40, CD80, CD83, or CD86, as assessed by flow cytometry for (A) DC and (B) DR1IC. Phenotypic maturation is expressed as ΔMFI between unstimulated and stimulated cultures. Results shown represent the average ± SEM of five donors assessed. Statistically significant differences between CD40L and CC or poly I:C are indicated (*P < .05, **P < .01, ***P < .001). (C and D) PBMC from one healthy donor were isolated, and DC (C) or DR1IC (D) were sorted-purified for EM. Cells were analyzed immediately after isolation (left panel) or following stimulation with CD40L (right panel). Size bars represent 1 μm. Results shown are representative of three donors examined.

**Figure 6.** Cytokine secretion in response to CD40 stimulation. (A and B) Secretion of TNF-α, IL-10, and IL-12 was assessed by intracellular staining. PBMC were cultured for 18 to 36 hours in the presence of CC, poly I:C, CD40L, or CD40L plus poly I:C in the presence of BFA, as described in Materials and Methods section. Cytokine secretion is expressed as ΔMFI between unstimulated and stimulated cultures. (C and D) For determination of secretion of bioactive IL-12, 5 × 10⁶ purified (C) DC or (D) DR1IC were stimulated with CD40L, washed extensively, and cocultured with 10⁶ allogeneic T-cells. After 5 days in culture, supernatants were collected, and IFN-γ production was assessed by ELISA. Results shown represent the average ± SEM of five donors assessed. Statistically significant differences between CD40L and CC or poly I:C are indicated (*P < .05, **P < .01, ***P < .001).
to produce IL-12 as well as to induce IFN-γ secretion and proliferation by allogeneic T-cells. As shown in Figure 7, C–E, CD40L induced the strongest IL-12 secretion, as well as IFN-γ production and proliferation by allogeneic T-cells.

Discussion
DC have been implicated in the defective function of the immune system during cancer progression [26]. Several reports suggest that that the effect of tumors on DC function is a generalized process with clinical relevance [9,27]. Despite this, few studies have focused on the systemic effects of cancer on circulating DC [6,28,29] as opposed to in vitro–derived DC [11,13–15]. Blood DC arguably represent the most relevant DC population given that they directly reflect the natural biology of immune responses occurring in vivo. The blood DC compartment (Lin−HLA-DR+ cells) [30] is comprised of myeloid (CD11c+DC) and plasmacytoid (CD123+DC) subsets [31]. In an accompanying paper, we show that the blood DC population in patients with cancer displays an alteration in the distribution of the CD11c+DC and CD123+DC subsets, exhibiting a significant accumulation of a heterogeneous population encompassing immature myeloid cells (DR+IC). This population of immature cells is also found in healthy donors, although here it represents a small proportion of the circulating DC compartment.

Morphologic and phenotypic analyses of DR+IC purified from healthy volunteers demonstrated characteristics similar to those of DR+IC isolated from cancer patients (supplementary data; Figure W1 and Table W1). Therefore, in this study, we sort-purified DR+IC from healthy volunteers and thoroughly evaluated their functionality to determine whether these immature cells could have an impact on immune response. First, we defined their capacity to present antigens to T-cells. We found that DR+IC were less efficient than DC in several systems, including MHC-II–restricted antigen presentation and MHC-I–restricted cross-presentation. In the peripheral blood of cancer patients, the abundance of immature cells with a limited ability to present antigens may be responsible, at least in part, for the failure of blood DC to generate adequate T-cell responses [6,9,32]. Alternatively, this could be due to direct suppression of T-cell function exerted by immature cells, as has been reported in patients with head and neck, lung, and breast cancers [24,33]. Interestingly, although DR+IC had a reduced capacity to stimulate T-cells, they did not inhibit T-cell proliferation in either an allogeneic or an antigen-specific manner when competent DC were also present. Differences in the lineage composition of DR+IC (a heterogeneous population encompassing immature myeloid and lymphoid subpopulations) and expression of molecules associated with antigen presentation (HLA-DR, CD40, CD86, and CD1c) could account for the differing observations.

The expression of HLA-DR and costimulatory molecules, together with poor APC function, indicated that inefficient antigen presentation, rather than direct suppression, was the major mechanism by which DR+IC could affect immune competence in cancer. Indeed, immature APC with deficient expression of costimulatory molecules and poor capacity to
stimulate T-cell responses have been reported to present antigens inadequately and to induce tolerance in cancer patients [7]. Our results demonstrated that, in contrast to DC, in cultures primed with DR^{+}IC, a smaller proportion of T-cells expressed high levels of antigens upregulated following adequate T-cell activation. Furthermore, T-cells primed with DR^{+}IC showed Th2 bias in cytokine secretion, suggesting that DR^{+}IC provided a different type of “stimulation” to T-cells when compared to DC. This is important because Th1 cytokines are necessary for the induction and maintenance of antitumor cytotoxic responses in vivo, whereas Th2 responses have been shown to subvert Th1-mediated immunity [34]. In fact, a clear correlation between Th2 prevalence and cancer progression has been demonstrated in patients with renal cell carcinoma and melanoma [35], suggesting that Th2 bias could provide a microenvironment that favors tumor survival and disease progression. Our data demonstrate that DR^{+}IC can induce Th2 bias in T-cells and migrate in response to the lymph node–driven chemokine CCL21. Based on the above data, it is interesting to speculate that if DR^{+}IC are capable of migration to lymphoid organs, then the effect of Th2 cytokines induced through inadequate presentation could suppress Th1 immunity and impair antitumor responses.

We also investigated possible mechanisms that are responsible for the significant accumulation of DR^{+}IC in cancer. Increased recruitment or altered survival of immature cells could be responsible for this phenomenon. In fact, tumor cells have been reported to secrete factors that recruit immature cells [36], or express proapoptotic factors (such as nitric oxide, TGF-β, IL-10, or gangliosides) that affect the survival of IC. Further investigations into the mechanism underlying TDSN-induced apoptosis have indicated a modulation of antiapoptotic molecule expression (i.e., Bcl-2; Pinzon-Charry et al., unpublished observations), in keeping with reports that CD40 ligation protects DC from tumor-induced apoptosis through increased expression of Bcl-2 [37]. Interestingly, expression of functional CD40 ligand has been described in various tumor cell types as an autocrine antiapoptotic mechanism [38], possibly providing minimal CD40 stimulation to bystander immune cells. Given the elevated expression of CD40 and the exquisite sensitivity to signalling through this pathway, this mechanism could play a relevant role in resistance to tumor-induced apoptosis by DR^{+}IC. In this context, increased expression of CD40 could represent a mechanism for resistance to apoptosis developed by cells accumulated under the influence of tumor products.

Our results are also relevant because blood DC have been proposed for use in DC-based immunotherapy protocols in cancer, but have thus far produced limited response rates [39]. Blood DC offer the theoretical advantage of being in their natural state of differentiation and are presumably capable of stimulating immune responses in a more physiological manner. DR^{+}IC represent a significant proportion of the blood DC compartment in cancer patients, and this may explain, at least in part, the failure of blood DC from cancer patients to generate adequate T-cell responses [6,9,32]. For this reason, identification of factors that enhance the function of APC that are increased in cancer patients and are intrinsically resistant to tumor-induced apoptosis could potentially improve the efficacy of blood DC immunotherapeutic approaches for cancer.

Importantly, data from healthy volunteers and patients with breast cancer showed that CD40 stimulation induced robust phenotypic maturation, upregulation of antigen-presenting machinery, and secretion of IL-12 by DR^{+}IC. Although the interaction between CD40 and its ligand has been recognized as an important element for conditioning DC to prime and expand tumor-specific cytotoxic responses [40,41], at present, no studies have been reported on CD40L-conditioned DC for immunotherapy in patients with cancer. Nevertheless, the potential of CD40L as an efficient stimulator for another type of professional APC (B-cells) under clinically applicable conditions has been demonstrated [42], and two cancer vaccine trials with CD40L-conditioned DC are currently listed in the Physician Data Query web site of the National Cancer Institute [43]. Our findings confirm that the help signal provided by CD40 engagement is one of the most potent stimuli for the maturation of DC and immature APC (DR^{+}IC), which are abundant in cancer patients, and also indicate that this approach for DC-based cancer immunotherapy warrants further consideration.

In conclusion, we document that the immature population of DR^{+}IC that accumulate within the blood DC compartment of patients with cancer could contribute to immune suppression by means of inefficient APC function, displacement of DC, and generation of inadequate immune responses. These data underscore the importance of thoroughly evaluating endogenous APC function in patients with cancer. More importantly, our finding that CD40 ligation boosts the APC function of DR^{+}IC supports the use of exogenously “trained” APC to correct the unbalanced immunologic performance in cancer and may prove to be crucial in improving the efficacy of DC-based immunotherapies for cancer.

Acknowledgements

The authors thank Grace Chojinowski, Paula Hall, and Clay Winterford for MoFlo sorting and technical assistance with electron microscopy. We also thank Greg Bryson, Michael McGuckin, and Giampietro Corradin for providing antibodies, tumor cell lines, and synthetic polypeptides, as well as Geoff Hill for critically reviewing the manuscript. We are grateful to the Australian Red Cross Blood Service and Sullivan Nicolaides Pathology Laboratories for blood samples, to Amgen for kindly providing soluble CD40L, and to our patients and volunteers without whom this study would have not been possible.

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