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HLA-DR$^+$ Immature Cells Exhibit Reduced Antigen-Presenting Cell Function But Respond to CD40 Stimulation[1]*

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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^{hi}) 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.

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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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(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) provided buffy 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 (IFN-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 *PfCS* 327–335 peptide and the *PfCS* 282–383 polypeptide were kindly provided by Dr. Giampietro Corradin (Institut de Biochimie, Lausanne, Switzerland). Sheep red blood cells were obtained from Equicell (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 *X-VIVO* [15] 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/Schering/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 E_2 (PGE_2; 1 μg/ml; Sigma). The CpG oligodeoxynucleotide 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 ($Lin^-HLA\text{-}DR^+CD11c^+CD123^+$) and DR^+IC ($Lin^-HLA\text{-}DR^+CD11c^-CD123^-$) 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 (10^7 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 PGE_2; 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 (10^7 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 BFA. 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, 5×10^5 to 10×10^5 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 ($\geq$ 90% $CD3^+$ cells). Alternatively, $CD4^+$ T-cells were purified by positive selection with anti-CD4 microbeads ($\geq$ 90% $CD4^+$; Miltneyi 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

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