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Research paper

Application of six-color flow cytometry for the assessment of dendritic cell responses in whole blood assays

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ABSTRACT

Analysis of peripheral blood dendritic cells (PBDCs) is increasingly reaching clinical relevance in a wide range of pathologies, in which investigating the capacity of DC subsets to respond adequately to specific stimuli may aid the comprehension of underlying immunopathologic mechanisms. The evaluation of PBDC responses directly challenged in whole blood (WB) samples offers many advantages over other methods that require DC isolation and culture, but it is limited in multiparametric analysis, currently based on 3- or 4-color assays. Therefore, in this study we developed a 6-color assay dedicated to the analysis of PBDC responses upon WB stimulation. We incubated WB samples with ligands to toll-like receptors (TLRs) with a clear-cut distribution on myeloid DCs (mDCs) or plasmacytoid (pDCs) and analyzed DC responses in terms of upregulation of activation/maturation markers, as well as production of a wide range of regulatory cytokines. Four colors were used to gate on mDCs and pDCs that were identified as lineage-/HLA-DR+/CD11c+ and lineage-/HLA-DR+/CD123+, respectively, and two further colors were used to analyze either the surface expression of CD80, CD86, CD40 or CD83, or the intracellular accumulation of IL-12, tumor-necrosis factor (TNF)-α, interferon (IFN)-α, IL-6, IL-10 or IL-4. With this method, we could directly compare in the same flow cytometric tube the responses of mDCs and pDCs to TLR stimulation, and investigate the reciprocal coexpression of distinct activation markers or regulatory cytokines. We suggest that the 6-color WB assay presented here may represent a novel tool for investigating the complex biology of DCs.

1. Introduction

Analysis of peripheral blood dendritic cells (PBDCs) is increasingly reaching clinical relevance in a wide range of pathologies, including infectious, neoplastic and autoimmune diseases, due to the identification of DCs as key antigen-presenting cells that prime, polarize and regulate adaptive immune responses. Therefore, possible changes in the number and function of DCs may be relevant to the comprehension of the pathogenesis and the monitoring of a wide range of diseases.

The capacity of DCs to activate potent immune responses qualitatively matched to the disease-causing agent relies on many factors, which include the ability of these cells to capture, process and present antigens in peptide–MHC complexes that interact with different classes of lymphocytes; their cell surface expression of costimulatory molecules that represent a necessary signal for the activation of lymphocytes; their ability to produce regulatory cytokines that play a critical role in the regulation and polarization of adaptive immune responses. All these properties of DCs are differentially regulated by different pathogenic stimuli that induce

Abbreviations: PBDC, peripheral blood dendritic cell;WB, whole blood; TLR, toll-like receptor;mDC, myeloid DC;pDC, plasmacytoid DC;TNF, tumor-necrosis factor;IFN, interferon;PBMCs, peripheral blood mononuclear cells; poly(I:C), polyribosinoinic–polyribocytidylic acid;LPS, lipopolysaccharide;IQ, imiquimod;ODN, oligodeoxynucleotide;FMO, fluorescence minus one.

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DCs to mature in distinct ways, thereby activating alternative host immune responses. They also partially depend on the ontogeny of DCs: myeloid DCs (mDCs) are prone to assume a wide range of markers and functions when localized in different tissues, while plasmacytoid DCs (pDCs) are mainly involved in tolerance maintenance in their immature state, although upon activation they play a crucial role in viral immunity through robust production of interferon-α (IFN-α) (Steinman and Banchereau, 2007; Merad et al., 2007; Liu, 2005).

DCs sense the pathogenic stimuli through engagement of pattern-recognition receptors that are involved in the response to microbial molecular structures as well as to injury and damaged tissues. Among these receptors, toll-like receptors (TLRs) represent an important family of sensors constituted of 11 functional different receptors, each recognizing distinct molecular patterns derived from various microorganisms, including bacteria, viruses, fungi and protozoa (Kawai and Akira, 2007). Microbial ligands to TLRs comprise a wide range of molecules with strong adjuvant activity that activate DCs by promoting their upregulation of costimulatory molecules and the production of cytokines aimed to allow the effective presentation of microbial antigens to cells of the adaptive immune system. TLR-induced polarization towards immune responses adequate to the disease-causing agent mainly depends on the cytokines released by DCs that in turn are conditioned by the specific TLR engaged and its distribution on distinct DC subsets (Kawai and Akira, 2007; Akira et al., 2006; Reis e Sousa, 2004). Investigating the capacity of DC subsets to adequately respond to specific stimuli may be very important for understanding the immune responses occurring in many physiologic and pathologic conditions.

Methods have been described by us and other Authors that allow the evaluation of PBDC responses to TLR ligands directly in whole blood (WB) samples (Della Bella et al., 2003, 2006, 2007a,b; Ida et al., 2006; Bueno et al., 2001). Compared with other methods that analyse DCs from blood samples after multistep purification procedures or in vitro culture, this type of analysis offers key advantages consisting in providing results that more directly reflect the in vivo situation, as well as need for very small blood samples and rapidity of execution. However, because the identification of PBDCs and their subsets in WB samples requires a combination of parameters due to the lack of unique markers on these cells, currently available methods present some limitations in multiparametric analysis because they are based on 3- or 4-color assays.

In order to allow multiparametric analysis of PBDC responses in WB assay, in this study we developed a 6-color assay dedicated to the analysis of DC maturation and intracellular cytokine expression in response to WB stimulation. The new assay was based on a 6-color strategy that we recently optimized to enumerate and characterize unstimulated PBDCs (Giannelli et al., in press). We incubated WB samples with microbial ligands to TLRs with a clear-cut distribution on mDCs or pDCs and analyzed DC responses in terms of upregulation of costimulatory molecules and maturation markers, as well as production of a wide range of regulatory cytokines. We demonstrate that the application of 6-color flow cytometry to this experimental model allows to investigate the expression and coexpression of different parameters directly compared between subsets.

2. Methods

2.1. Subjects

Peripheral blood was obtained by venipuncture from 8 healthy volunteers (5 females, 3 males, mean age 26.4 years, range 22–42) and anti-coagulated with sodium heparin. A signed informed consent was obtained from all participants.

2.2. Sample stimulation with TLR ligands

WB samples diluted v/v in RPMI 1640 medium (Euroclone, Wetherby, West York, UK) were incubated in the absence or presence of either TLR3 ligand polyriboinosinic–polyribocytidylic acid [poly(I:C); 25 μg/ml; Sigma Chemicals Co., St. Louis, MO], or TLR4 ligand lipopolysaccharide (LPS serotype 055:B5; 100 ng/ml; Sigma Chemicals Co.), or TLR7 ligand imiquimod (IQ; 10 μg/ml; InvivoGen, San Diego, CA), or TLR9 ligand CpG (CpG-oligodeoxynucleotide type A, ODN2216; 30 μg/ml; InvivoGen). Individual ligands were prepared according to the manufacturer's recommendations. ODN2216 was chosen as the best stimulator of IFN-α production among other ODNs (Martinson et al., 2006). WB samples were incubated at 37 °C in a 5% CO_2 humidified atmosphere for 3, 5 or 20 h after addition of stimuli, as indicated. The concentrations of TLR ligands as well as the duration of cultures were established based on previous studies (Della Bella et al., 2003, 2006, 2007a,b; Ida et al., 2006; Rodrigue-Gervais et al., 2007; Bueno et al., 2001; Testerman et al., 1995; Megyeri et al., 1995; Piccioli et al., 2005; Pichyangkul et al., 2001) and preliminary experiments. The protein transport inhibitor brefeldin A (BFA; 10 μg/ml; Sigma Chemicals) was added to samples destined for cytokine analysis after 1–5 h from the addition of stimuli, as indicated, to allow intracellular accumulation of cytokines.

2.3. Sample staining

A complete list of the monoclonal antibodies (mAbs) used in this study is reported in Table 1. mAbs specific for lineage markers, HLA-DR, CD11c and CD123 were used in all samples to gate on mDCs and pDCs, while the other mAbs (FITC- and PE-conjugated) were used in varying combinations. For cell surface markers, WB samples (100 μl) were incubated at the end of the culture with mAbs for 20 min; erythrocytes were lysed by incubation with ammonium chloride for 10 min. For cytokine analysis, samples were fixed, permeabilised and stained with cytokine-directed mAbs, using the Leucoperm Reagent (AbD Serotec, Oxford, UK), according to the manufacturer's instructions. Staining conditions for each mAb were preliminarily determined in titration assays. All operations were done at 4 °C in the dark.

2.4. Flow cytometric analysis

Data acquisition and analysis were performed on a FACSCanto flow cytometer (Becton Dickinson) equipped with two lasers (Argon 488 and HeNe 633) and capabilities