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The novel differentiation of human blood mononuclear cells into CD1a-negative dendritic cells is stimulated in the absence of exogenous cytokines by an extract prepared from pinecones

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Abstract

The production of dendritic cells, both in-vivo and in-vitro, has become the intense focus of research activities. Common to many of these production protocols is the use of cytokines, typically granulocyte-monocyte colony stimulating factor and either interleukin 4 or tumor necrosis factor alpha or a combination of all three. Herein, we report our findings that a proprietary pinecone extract is capable of in a dose-dependent manner, and in the absence of exogenous cytokines, the rapid differentiation from peripheral blood mononuclear cells of mature CD1a-negative dendritic cells.

Keywords: Dendritic cells; Peripheral blood mononuclear cells; Pinecone extract

1. Introduction

Dendritic cells (DC) are recognized as the most potent antigen presenting cells of the immune system [1,2]. Their ability to uptake, process, and present antigen to T cells is central to the development of immune responses against bacterial- and viral-infected cells, cancer cells, and against vaccine antigens. Recently, dendritic cells have been used in the creation of dendritic cell vaccines for the purpose of treating cancer [3]. The DC vaccine is generated in-vitro from a patient's peripheral blood mononuclear cell (PBMC) derived DC which are loaded with antigen derived from the patient's tumor. The antigen-loaded DC are then reintroduced into the patient where they present tumor antigen and stimulate the production of cytotoxic T lymphocytes (CTL). If effective, this strategy results in the generation of a CTL response powerful enough to produce tumor stasis, regression, or elimination. Strategies have also been developed in an attempt to stimulate the generation of DC in-vivo at the site of a tumor [4], at the site of a vaccine

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inoculation [5], or systemically [6]. Common to these strategies is the delivery of granulocyte colony stimulating factor (GM-CSF), either as the recombinant protein or as a DNA vector expressing the gene for GM-CSF.

In vitro, addition of GM-CSF to either CD34+ hematopoeitic stem cells or CD14+ monocytes isolated from peripheral blood stimulates differentiation only to macrophage. Differentiation to DC has been found to require the addition of interleukin 4 (IL-4) or tumor necrosis factor alpha (TNF-α) to cultures containing GM-CSF [1,2,7,8]. DC derived from these methods develop from the myeloid lineage and express the cluster determinant antigen CD11c and HLA-DR but not CD14 or CD123. If cultured in GM-CSF and IL-3, DC that are CD11c negative, CD14 negative, CD123 (IL-3 receptor) and HLA-DR positive can be detected. These cells are believed to be derived from a lymphoid lineage and can be detected circulating in the peripheral blood [9].

Dendritic cells, whether derived from myeloid or lymphoid lineages, circulate in low numbers in the blood and are estimated to represent less than 0.1% of PBMC [10]. This low frequency of circulating DC significantly restricts their harvest directly from peripheral blood for use as a source of DC for therapeutic use. Preparation of large numbers of DC for use in therapeutic protocols has required exogenously supplied cytokines (GM-CSF, IL-4, TNF-a, etc.) for the differentiation and expansion of DC cultured in-vitro [11].

Herein, we describe our findings that the in-vitro treatment of human peripheral blood mononuclear cells with a standardized pinecone extract (PCE, TB10900) rapidly leads to the production of dendritic cells in the absence of exogenously supplied cytokines while oral delivery of the extract significantly boosts the IgG2a response to a model protein vaccine.

2. Methods

2.1. Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from Florida Blood Services (St. Petersburg, FL) using density gradient centrifugation (Histopaque 1.077; Sigma, St. Louis, MO or Nycoprep 1.077; Gibco BRL, Grand Island, NY). The resulting PBMC were suspended in RPMI 1640 with GlutaMax containing 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME at 2×10^6 cells/ml. $CD14^+$ and $CD3^+$ cells were isolated from PBMC using magnetic microbeads from Miltenyi Biotech (Auburn, CA). These cells were consistently >95% $CD14^+$ or $CD3^+$ as determined by immunofluorescent staining.

2.2. Murine splenocyte cultures

Female Balb/cJ and C3H/HeJ mice (5–6 weeks of age) that had been purchased from Jackson Laboratories (Bar Harbor, ME) were anesthetized by injection of 0.25 ml of Avertin and then euthanized by cervical dislocation as approved by the institutional animal care and use committee. Using sterile techniques the spleens were placed in a Stomacher bag containing 5 ml of complete media. The bag was placed in the Stomacher for 60 s on medium setting to generate a single cell suspension. The single cell suspension was filtered through a 40-μm screen into a 50-ml conical tube. Then, 10 ml of 1X RBC lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) was added to the cell suspension. The splenocytes were immediately pelleted at $500 \times g$ for 5 min and then suspended in complete media to a concentration of 2×10^6/ml.

2.3. Pinecone extract

Extracts of pinecones (PCE, TB10900) were prepared using proprietary methods (US Patent 4,985,249 and patent pending). Shreds of pinecones were washed successively in water and then 95% ethanol and then rinsed with water. The shreds were dried and ground to a particle size between 80 and 120 mesh. The grounds were mixed with a 1% aqueous alkaline solution and extracted at 121 °C for 1 h in an autoclave. The resulting mixture was cleared of particulates by high-speed centrifugation, neutralized with HCl and then autoclaved. The extract was standardized to the concentration of polyphenyls.

2.4. Cell cultures

One hundred microliters of human PBMC or murine splenocytes were added to wells in 96 well