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Ursolic acid isolated from *Uncaria rhynchophylla* activates human dendritic cells via TLR2 and/or TLR4 and induces the production of IFN-γ by CD4+ naïve T cells

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ABSTRACT

Ursolic acid is triterpene isolated from *Uncaria rhynchophylla* and is a pharmacologically active substance. The induction of dendritic cell maturation is critical for the induction of Ag-specific T-lymphocyte response and may be essential for the development of human vaccine relying on T cell immunity. In this study, we investigated that the effect of Ursolic acid on the phenotypic and functional maturation of human monocyte-derived dendritic cells *in vitro*. Dendritic cells harvested on day 8 were examined using functional assay. The expression levels of CD1a, CD80, CD83, CD86, HLA-DR and CCR7 on Ursolic acid-primed dendritic cells was slightly enhanced. Ursolic acid dose-dependently enhanced the T cell stimulatory capacity in an allogeneic mixed lymphocyte reaction, as measured by T cell proliferation. The production of IL-12p70 induced by Ursolic acid-primed dendritic cells was inhibited by the anti-Toll-like receptor-2 (TLR2) mAb and anti-TLR4 mAb. Moreover, Ursolic acid-primed dendritic cells expressed levels of mRNA coding for both TLR2 and TLR4. The majority of cells produced considerable interferon-gamma (IFN-γ), but also small amounts of interleukin (IL-4)-4. Ursolic acid-primed dendritic cells have an intermediate migratory capacity towards CCL19 and CCL21. These results suggest that Ursolic acid modulates human dendritic cells function in a fashion that favors Th1 polarization via the activation of IL-12p70 dependent on TLR2 and/or TLR4, and may be used on dendritic cells-based vaccines for cancer immunotherapy.

1. Introduction

The immune system is confronted with antigens and proteins that have not been encountered previously encountered by the body. Dendritic cells are professional antigen-presenting cells and play a key role in the induction of these immune responses (Banchereau and Steinman, 1998; Lanzavecchia and Sallustoa, 2001; Mellman and Steinman, 2001). Dendritic cells orchestrate a variety of immune responses by stimulating the differentiation of naïve CD4+ T cells into helper T effectors such as Th1, Th2, Treg cells and Th17 cells and several factors determine the direction of T cell polarization (Romagnani, 1994; Kuchroo et al., 1995; Lederer et al., 1996; Tao et al., 1997; Forster et al., 1999; Lezz et al., 1999; Tanaka et al., 2000; O'garra, 2001; Steinman and Dhodapkar, 2001). The cytokine profile present during an immune reaction is an important element in directing the response to T cell polarization. A maturation process, IL-12 production, the up-regulation of MHC and costimulatory molecules, is critical for initiation of primary T cell response. Th1 responses predominate in organ-specific autoimmune disorders, acute allograft rejection and in some chronic inflammatory disorders (Trinchieri and Scott, 1994). Although different dendritic cells subsets may have some intrinsic potential to preferentially induce Th1, Th2, Treg cells or Th17 cells, dendritic cells also display considerable functional plasticity in response to signals from microbes and the local microenvironment (Steinman, 2007). Numerous stimuli can mediate dendritic cells maturation, the best characterized being TLR ligands and signals such as CD40L delivered by T cells and innate lymphocytes (Hermann et al., 1998). In addition to their essential role in T cell priming, dendritic cells are also involved in innate immunity through the production of cytokines and the activation of NK or NKT cells. Thus, dendritic cells play a pivotal role in orchestrating the immune response.

The hooks of *Uncaria* sp. are contained in Choto-san as the main component herb. Choto-san has been used for hypertension and dementia, and well used as an important of many Chinese prescriptions in China, Korea and Japan. Ursolic acid is isolated from *Uncaria rhynchophylla* and phytochemically classified as triterpene. Ursolic

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acid augments the inhibitory effects of anti-cancer drugs on growth human tumor cells and triggers apoptosis in cancer cells. Triterpene have been identified as a unique class of natural products possessing diverse biological activities. Terpenes contain pharmacologically active substance. We have reported that numerous terpenes induce the differentiation of dendritic cells from human monocytes, and drive Th1 and Th2 (Takei et al., 2005, 2007, 2008). For immunotherapeutic applications, it appears crucial to identify factors that might affect the differentiation and function of dendritic cells. Although various terpene compounds have pharmacological activity, relatively little is known in regards to the influences Ursolic acid exerts on the initiation of specific immune response at the level of dendritic cells. Therefore, to further understand the cellular basis of immunological abnormalities associated with Ursolic acid exposure, we investigated ability of Ursolic acid on human dendritic cells differentiation and function in detail.

2. Materials and methods

2.1. Culture medium, reagents and monoclonal antibodies

The culture medium used in this study was RPMI 1640 (Gibco-BRL) supplemented with 10% FBS (Hyclone, Logan, UT) and 1% penicillin–streptomycin (Gico-BRL). Recombinant human IL-4 (IL-4), recombinant human granulocyte-macrophage colony-stimulation factor (GM-CSF), tumor necrosis factor-alpha (TNF-α) purchased from R&D systems (Minneapolis, MN). Lipopolysaccharide (LPS) from *Esherichia coli* was purchased from Sigma-Aldrich (St. Louis, MO). For flow cytometry, monoclonal antibodies (mAbs) toward the following antigens were purchased from Becton-Dickinson (San Jose, CA): anti-CD14-FITC (fluorescent isothiocyanate), anti-CD1a-PE (phycoerthrin), anti-CD38-PE, anti-80-PE, anti-CD83-PE, anti-CD86-PE, anti-HLA-DR-FITC, and anti-CCR7-FITC. Endotoxin levels in all agents were below 1.0 EU/ml.

2.2. Isolation of Ursolic acid from U. rhynchophylla

Ursolic acid was prepared as previously described (Lee et al., 2000). The purity of Ursolic acid was >99%. Ursolic acid was dissolved in dimethyl sulfoxide. The concentration of dimethyl sulfoxide in the culture medium was 0.1%, which had no effect upon the culture and the production of cytokines under the conditions used in this study. The endotoxin in Ursolic acid was removed using End Trap 5/1 (Profos AG, Regensburg, Germany). Endotoxin levels in Ursolic acid were below 0.05 EU/ml.

2.3. Generation of monocyte-derived dendritic cells

All cell subsets were isolated from human peripheral blood of normal healthy donors. Peripheral blood mononuclear cells were first isolated from heparinized whole blood by Ficoll/Isopaque/1.077 g/ml (Pharmacia, Freiburg, Germany) density gradient centrifugation (465 *g*, 30 min, 22 °C) as previously described (Grage-Griebenow et al., 1993). Peripheral blood mononuclear cells were further separated into CD14+ monocytes and lymphocytes using the magnetic activated cell sorter (MACS) according to the manufacturer's instructions (Miltenyi Biotec; Auburn, CA) (Takei et al., 2005). The purity of CD14+ monocytes was always more than 90%. Monocytes were cultured with GM-CSF (50 ng/ml) and IL-4 (25 ng/ml) in RPMI 1640 supplemented with 10% FBS and 1% penicillin–streptomycin for 6 days. Dendritic cells were generated by stimulating immature dendritic cells in RPMI 1640 supplemented with 10% FBS and 1% penicillin–streptomycin containing GM-CSF and IL-4 for additional 2 days with various concentrations of Ursolic acid, but with LPS (100 ng/ml) or TNF-α (25 ng/ml). All subsequent tests were performed after harvesting the cells at day 8 and after removing GM-CSF, IL-4, Ursolic acid, LPS or TNF-α by extensive washing. The medium was replenished with cytokines every 2 days. Some cultures were supplemented with anti-TLR2 mAb (10 μg/ml, R&D Systems) or anti-TLR4 mAb (10 μg/ml, R&D Systems). To determine the production of IL-6, IL-10 and IL-12p70 by Ursolic acid-, LPS- or TNF-α-primed dendritic cells, dendritic cells (2×10^4 cell/well) were stimulated with CD40-L-transfected J558 cells (5×10^4 cell/well, kindly provided by Prof. Kalinski at University of Pittsburgh, PA) for 24 h. The cell-free supernatants were collected and frozen at −20 °C until measurement of cytokines using enzyme-linked immunosorbent assay (ELISA).

2.4. Immunophenotype studies

Dual-Color immunofluorescence flow cytometry was performed using the following panel of monoclonal antibodies: PE-conjugated antihuman CD1a, FITC-conjugated antihuman CD14, PE-antihuman CD38, PE-antihuman CD80, PE-antihuman CD83, PE-antihuman CD86, FITC-antihuman-HLA-DR and FITC-antihuman-CCR7. Negative controls were isotype-matched with irrelevant monoclonal antibodies (Becton-Dickinson). Cells were re-suspended in staining medium containing PBS and 0.1% NaN_3 and then fixed with 1.0% paraformaldehyde. Isotype controls were run in parallel. Cell debris was eliminated from the analysis by forward and side scatter gating. The samples were analyzed on FACSCalibur (Becton-Dickinson) with CellQuest software (Becton-Dickinson). Ten thousand cells were analyzed per sample. The results were expressed as MFI.

2.5. Allogeneic mixed lymphocyte reaction

$CD4^+$ naïve T cells for the allogeneic mixed lymphocyte reaction assay were obtained from allogeneic peripheral blood mononuclear cells using magnetic cell sorting and separation of biomolecules (MACS) beads (Miltenyi Biotec). The purity of isolated cells was >95% of $CD4^+$ naïve T cells as determined by flow cytometry using FACSCalibure. Allogeneic $CD4^+$ naïve T cells (5×10^4 cell/well) were co-cultured in 96-well round-bottomed culture plates with graded doses (2×10^2 to 5×10^4) of irradiated (30 Gy) dendritic cells. After 5 days, cells were pulsed with 1 μCi [^{3}H]-methylthymidine per well for 16 h, then harvested and analyzed in a liquid scintillation counter.

2.6. Determination of naïve T cell polarization by dendritic cells

Determination of naïve T cell polarization by dendritic cells was carried out as previously described (Takei et al., 2005). Dendritic cells were co-cultured with naïve T cells (2.5×10^5 cells/200 μl) at 1:5 dendritic cells/T cells ratio in 96-well U-bottomed tissue culture plates (Costar, Cambridge, MA). Briefly, naïve $CD4^+CD45RA^+CD45RO^-$T cells were isolated from allogeneic peripheral blood mononuclear cells using MACS beads (Miltenyi Biotec). The purity of isolated cells was >95% for naïve $CD4^+CD45RA^+CD45RO^-$T cells as determined by flow cytometry using FACS Calibur. Some cultures were supplemented with neutralizing Abs to block endogenous cytokines: anti-IL-12 mAb (10 μg/ml, R&D Systems). On day 5, cells were washed out completely and expanded with fresh medium containing 10 U/ml recombinant human IL-2 (IL-2) (R&D Systems). One hundred microliters of culture supernatant was replaced by fresh medium of the same concentration every 3 days. On day 14, cells were washed, counted and T cells (10^6/ml) were re-stimulated with Dynabeads CD3/CD28 (Invitrogen) for 24 h. The cell-free supernatants were collected and frozen at −20 until measurement of cytokines using ELISA.

2.7. Intracellular cytokine staining

The intracellular cytokine concentrations of the harvested T cells were measured by FACS analysis as previously described (Takei et al., 2005). Briefly, T cells (10^6/ml) were stimulated with phorbol