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Niflumic acid renders dendritic cells tolerogenic and up-regulates inhibitory molecules ILT3 and ILT4

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Abstract

Niflumic acid is a member of non-steroidal anti-inflammatory agents, from which aspirin was recently shown to inhibit maturation of human-monocyte derived dendritic cells (DCs). DCs are crucial regulators of the immune response, capable of inducing immunity as well as tolerance. In our *in vitro* study we showed a tolerogenic effect of NFA on phenotype and function of LPS-matured monocyte-derived DCs. Different drug concentrations dose-dependently down-regulated the expression of co-stimulatory molecules, particularly CD80 and lowered the expression of dendritic cell marker CD1a. Opposingly, the expressions of two inhibitory surface molecules, associated with tolerogenic DCs, immunoglobulin-like transcripts (ILT)3 and ILT4 were induced in treated DCs. The levels of TNFα production by NFA-treated DCs did not change significantly compared to controls, whereas the IL-12p70 and IL-10 production was completely abrogated at higher drug concentrations. However, at lower drug concentrations, the production of IL-12p70 was increased. There were no significant differences in the uptake of FITC labeled dextran by treated DCs compared to untreated cells. In allogeneic cultures with whole $CD4^+$ T cells, dendritic cells differentiated in the presence of NFA appeared poor stimulators of $CD4^+$ T-cell proliferation, even compared to immature DCs (iDCs). These results indicate the immunosuppressive properties of NFA, which may be therapeutically useful in controlling chronic immune and/or inflammatory diseases, by modulating DC characteristics towards tolerogenic DCs.

1. Introduction

Niflumic acid (NFA) is a drug belonging to a class of non-steroidal anti-inflammatory agents (NSAIDs). Its use is not as wide-spread as that of e.g. salicylates, although it is still used today due to its anti-pyretic and analgesic properties. NFA exerts its anti-inflammatory actions mainly through selective inhibition of cyclooxygenase-2 (COX-2) [1]. There

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have been several studies conducted in the seventies regarding the clinical use of NFA for the treatment of rheumatoid arthritis and osteoarthritis [2–5]. It is also known as a chloride channel blocker [6,7], affecting such physiological factors as mucin production [8]. There are also reports on inhibition of catecholamine uptake by NFA [9].

Dendritic cells (DCs) include a heterogeneous family of professional antigen presenting cells involved in initiation of immunity and in immunologic tolerance. DCs initiate immunity by the activation of naive B and T cells into effector cells of the adaptive immune system, while on the other side contribute to immune tolerance by mechanisms that include deletion of effector and induction of regulatory T cells [10].

Immature DCs are very efficient at taking up antigens by constitutive macropinocytosis, receptor-mediated endocytosis and phagocytosis, which is pivotal for their sentinel function in immunity. After internalization, most exogenous antigens are processed through an endosomal and lysosomal pathway in which proteins are cleaved into peptides and loaded onto major histocompatibility complex (MHC) class II molecules [11]. Some antigens are released into the cytosol, allowing them access to the proteasome, which in the end loads generated peptides onto MHC class I molecules [12].

After maturation DCs become professional antigen presenting cells. The maturation of dendritic cells is triggered by numerous stimuli such as endogenous factors that are released by necrotic cells (for example, heat-shock proteins), inflammatory cytokines, such as tumor necrosis factor α (TNF-α), microbial components, such as lipopolysaccharide (LPS), which bind to toll-like receptors (TLRs) or by activated T cells which express CD40L, a ligand for co-stimulatory molecules on dendritic cells [10]. Mature DCs down-regulate their endocytic capacity, up-regulate co-stimulatory molecules CD40, CD80 and CD86, as well as MHC molecules. The production of bioactive IL-12 and TNF-α increases. The maturing DCs migrate to lymphoid organs, where they stimulate naive T cells by three concurrent signals: recognition of a MHC-peptide complex by the T-cell receptor (TCR), followed by engagement of co-stimulatory signals and the para-autocrine production of cytokines [13].

Until recently, the main separation of DCs based on their maturation state was that of immature and mature DCs, where immature DCs would be the main inducers of T-cell anergy or regulatory T cells and mature DCs the inducers of immunity [13–16]. However, recent findings suggest that tolerance is observed when partial- or semi-maturation of DCs occurs [17,18]. Such cells were designated with various characteristics, with the most established being low co-stimulatory molecule expression, accompanied with low or absent production of pro-inflammatory cytokines and high expression of inhibitory molecules. Considering the molecular characteristics of tolerogenic DCs, the immunoglobulin-like transcripts (ILT)3 and ILT4, were recently found to be expressed on tolerogenic DCs and have been recognized crucial for the tolerogenic capacity acquired by DCs [19].

Pharmacological induction of tolerogenic, partially mature DCs has lately been achieved with numbers of agents (15-deoxyspergualin, vitamin D3, aspirin, etc.) of which many, including salicylates, were shown to interfere with Nf-κB transcription factor [20].

Using human-monocyte derived DCs, we have investigated the effects of NFA on DC phenotype and function. We measured the expression of co-stimulatory molecules in comparison to inhibitory molecules. In context of function we studied the levels of cytokine production, antigen uptake and the capacity to stimulate proliferation of allogeneic $CD4^+$ T cells.

2. Materials and methods

2.1. Materials

NFA, as well as selective COX inhibitors SC560 and CAY10404 were purchased from Cayman chemicals (MI, USA). The chemicals were dissolved in DMSO prior to each experiment in a way that the final DMSO content in culture media did not exceed 0.1%.

2.2. Dendritic cell preparation and culture

Buffy coats from the venous blood of normal healthy volunteers were obtained by the Blood Transfusion Centre of Slovenia, according to institutional guidelines. Peripheral blood mononuclear cells (PBMCs) were isolated using Lympholyte®-H (Cedarlane laboratories, Ontario, Canada). The cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS), counted, and used as a source for immunomagnetic isolation of CD14-positive cells (CD14 Microbeads, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of the $CD14^+$ cells was always greater than 95% as determined by flow cytometry. CD14-positive cells were cultured in RPMI 1640 (Cambrex) medium, supplemented with 10% fetal bovine serum (FBS), gentamicin (50 μg/ml; Gibco, Paisley, UK), 500 U/ml of rhGM-CSF and 400 U/ml of rhIL-4 (both Gentaur, Paris, France). On day 2, half of the medium was exchanged with the addition of starting quantities of rhGM-CSF and rhIL-4. After 5 days, non-adherent, immature DCs were harvested and characterized by flow cytometry as $CD1a^{hi}$, $CD80^-$, $CD83^-$, $CD86^{low}$ and $HLA\text{-}DR^{low}$. Cells were counted and resuspended in the medium containing 500 U/ml of rhGM-CSF and 20 ng/ml LPS and matured for 2 further days. On days 2 and 5, NFA was added to the cultures in concentrations 0.02 mM, 0.04 mM, 0.08 mM and 0.16 mM. In some cases immature DCs were incubated with 0.16 mM of NFA only on day 5 for 30 min before the addition of LPS, to measure its effect solely on the maturation process of DCs.

2.3. Purification of T cells from PBMCs

T cells were purified from human buffy coats. $CD4^+$ T cells were obtained by positive selection using CD4 microbeads (Milteny Biotec GmbH, Bergisch Gladbach, Germany). The purity of the cells was always greater than 95% as determined by flow cytometry.

2.4. Allogeneic T-cell proliferation

DCs obtained after 7 days of culture were washed twice in RPMI 1640 medium containing gentamicin and treated with mitomycin C (Sigma) to block their proliferation. When using immature DCs, the cells were frozen in RPMI 1640 containing 10% DMSO and 10% FBS, and thawed after two days to conduct parallel proliferation experiments. Purified whole $CD4^+$ T cells were used as responders. The assay was carried out in 96-well V-bottomed plates, with a total volume per well of 200 μl. Numbers of cells used were 1×10^4 and 1×10^5 for DCs and responder T cells, respectively. On day five of culture the wells were pulsed with 1 μCi/well [^{3}H]thymidine (Perkin Elmer, Boston, USA) and proliferation was measured by [^{3}H] thymidine incorporation after 18 h by liquid scintillation counting.

2.5. Endocytosis assay

To analyze the endocytosis of DCs, 1×10^5 cells were incubated at 37 °C for 1 h with 1 mg/ml of FITC labeled dextran (Sigma). After