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DENDRITIC CELLS

Adaptation to replating of dendritic cells synergizes with Toll-like receptor stimuli and enhances the pro-inflammatory cytokine profile

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Abstract

Background. As initiators of the adaptive immune response, dendritic cells (DCs) can be used for anti-cancer immunotherapy. On addition of proper maturation stimuli DCs mature and produce pro-inflammatory cytokines that skew T cells in the direction needed for anti-cancer therapy. Further optimization of DC maturation might improve the efficacy of DCs for clinical application. We describe that replating and a subsequent resting period enhance the inflammatory properties of the DCs. *Methods.* Cultured immature monocyte-derived DCs were harvested and, after replating, were stimulated immediately or after 2 h of rest. Cytokine production was assessed using enzyme-linked immunosorbent assay (ELISA). Dynamics of mitogen-activated protein kinase (MAPK) and nuclear factor kappa b (NFκB) activation in DCs was analyzed using flow cytometry and imaging flow cytometry. *Results.* Resting immature DCs after replating, before addition of Toll-like receptor (TLR) ligands, increased the production of pro-inflammatory but not anti-inflammatory cytokines. In addition, the speed of MAPK phosphorylation and nuclear translocation of NFκB was increased when DCs were allowed to rest before TLR stimulation. The effect was imprinted, transient and did not reflect a temporary loss of responsiveness, indicating that signaling induced by culture adaptation of DCs synergizes with TLR signals to increase cytokine production. DCs rested before TLR stimulation induced more interferon (IFN)-γ production in CD4-positive and CD8-positive T cells. *Conclusion.* Introduction of a resting step in the DC maturation method, which is cheap and easy to implement, will improve the generation of pro-inflammatory DCs for cancer immunotherapy. These DCs enhanced Th1 polarization and IFN-γ production by CD8 T cells, both important hallmarks for the induction of efficient anti-cancer immunity.

Key Words: *pro-inflammatory cytokines, synergy, Toll-like receptor 4*

Introduction

Dendritic cells (DCs) are professional antigen-presenting cells that function at the basis of the adaptive immune response. DCs reside in peripheral tissues and function as sensors for various danger signals by continuously sampling the environment by endocytosis, pinocytosis and using both intracellular and cell-surface receptors. Danger signals that can activate DCs include pathogen-associated molecular patterns (PAMPs), which are recognized by pattern recognition receptors (PRRs) on the DCs [1], such as Toll-like receptors (TLRs) [2].

After ligand recognition, immature DCs (iDCs) undergo a process called maturation, during which DCs migrate toward the lymph node. There, DCs activate T cells by giving three signals: antigen presentation, co-stimulation and cytokine production. The type of cytokines produced directs the differentiation route of the T cells into different effector T-cell types [3].

On TLR ligand recognition, signal transduction occurs via adaptors MyD88-adapter-like (Mal) and myeloid differentiation factor 88 (MyD88), or adaptors Toll-Interleukin receptor (TIR)-domain–containing adapter-inducing interferon-β (TRIF)-related adaptor

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molecule (TRAM) and TRIF. Signaling via the MyD88-dependent pathway induces phosphorylation of mitogen-activated protease (MAP) kinases ERK, p38 and JNK leading to nuclear translocation of transcription factors AP1 and nuclear factor kappa b (NFκB) and ultimately to transcription of pro-inflammatory cytokines [2]. Signaling via the TRIF-dependent pathway can cross-talk into the MyD88-dependent pathway [4]. Second, it induces nuclear translocation of transcription factor IRF3, leading to up-regulation of co-stimulatory molecules and production of type I interferons [5,6].

As antigen-presenting cells DCs help shape the adaptive immune response. DCs are being extensively assessed for their clinical potential as an anti-cancer immunotherapeutic cell product to induce and/or enhance tumor-specific immune response. Desirable properties of DCs used as cancer immunotherapy are their ability to migrate toward lymph nodes, their capacity to induce cytotoxic T lymphocytes and the ability to polarize CD4 T cells toward Th1 cells that are needed to support anti-cancer CD8 T-cell function.

For this application, patient-derived monocytes are harvested and differentiated *in vitro* to iDCs. Subsequently iDCs are matured by the addition of TLR ligands or other stimuli in the presence of tumor antigens, which programs the DCs to induce an anti-cancer immune response when transfused back into the patient. Immunotherapy using DCs has been tested in numerous clinical trials since the mid-1990s, however, clinical responses using DCs as anti-cancer therapy vary [7,8]. Further optimization of DC maturation might improve the efficacy of DCs. Different maturation strategies have been published [8–17], although, as yet, no optimal strategy for maturing DCs for immunotherapy has been determined.

Cross-talk between TLRs and other signal transduction routes has been described abundantly and has even been proposed to function in conferring specificity to TLR signaling [18–20]. After harvesting of DCs and subsequent replating, adaptation to changed culture conditions by DCs may occur, leading to signal transduction that can cross-talk with signal transduction induced by DC maturation stimuli such as lipopolysaccharide (LPS). Here we describe that when DCs are stimulated after a period of resting the speed of LPS-induced signal transduction and the pro-inflammatory cytokine profile of the DCs are enhanced. The synergized cytokine production is imprinted and is still present on CD40 ligand re-stimulation of the DCs. Moreover, co-culture with T cells showed that both CD4-positive and CD8-positive T cells produced enhanced levels of interferon (IFN)-γ, important for an enhanced anti-cancer immune response.

Resting DCs is a low-cost method that is easy to implement in good manufacturing practice DC maturation protocols and enhances the inflammatory cytokine response by a wide range of maturation stimuli, including the Th1-inducing clinically applicable monophosphoryl lipid A (MPLA)/IFN-γ maturation.

Methods

Generation of monocyte-derived DCs

On informed consent monocytes were isolated from fresh apheresis material of healthy volunteers (Sanquin Blood Supply) using the Elutra cell separation system (Gambro), as described previously [4,11]. Twenty million monocytes were cultured in Cellgro DC serum-free culture medium (Cellgenix) supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) (1000 IU/mL), interleukin (IL)-4 (800 IU/mL; Cellgenix), penicillin (100 U/mL) and streptomycin (100 µg/mL; Invitrogen Breda) in 80 cm^2 culture flasks (Nunc). After 6 days of culture iDCs were harvested by incubation with 0.38% trisodiumcitrate (Merck) for 5 min at 37°C and replated in fresh Cellgro DC culture medium before maturation. Yield of iDCs was approximately 50%.

Stimulation of iDCs

iDCs were harvested and seeded in 96-well plates (1×10^5 cell/well (Nunc) in Cellgro supplemented with 1% fetal calf serum (FCS) (Bodinco). Cells were stimulated with TLR ligands LPS from *Escherichia coli O111:B4* (50 ng/mL, Invivogen), R848 (5 µg/mL, Invivogen), Pam3Csk4 (5 µg/mL, Invivogen) or cytokine maturation cocktail (10 ng/mL IL-1β [Cellgenix], 10 ng/mL tumor necrosis factor [TNF]-α [Cellgenix] and 1 µg/mL prostaglandin E_2 (PGE_2) [Sigma Aldrich]) immediately after seeding or, alternatively, cells were incubated at 37°C/5% CO_2 for several hours before addition of stimuli. Stimulation using LPS or MPLA was performed in presence or absence of IFN-γ (1000 IU/mL, Immukine; Boehringer Ingelheim). Unlike LPS, stimulation using MPLA does not require presence of serum factor LPS-binding protein (LBP) [10,11,21]. Therefore stimulation using MPLA from *Salmonella minnesota re595* (2.5 µg/mL, Invivogen) was performed as described above, except that after harvest the cells were resuspended in Cellgro without addition of FCS. After stimulation DCs were incubated for 20 h at 37°C/5% CO_2, after which culture supernatant was harvested. Alternatively, DCs were stimulated as described above using LPS in the presence of 12.5 ng/mL purified human LBP (Hycult Biotech) instead of 1% FCS. Yield of mature DCs was approximately 50% of iDCs, for both rested and non-rested DCs.