The glucocorticoid dexamethasone programs human dendritic cells for enhanced phagocytosis of apoptotic neutrophils and inflammatory response

by Judit Hodrea

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The Examination takes place at the Department of Biochemistry and Molecular Biology, Medical and Health Science Center, University of Debrecen, at 11:00 a.m. on 29th of November, 2011.

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The Ph.D. defense takes place at the Lecture Hall of the 1st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen, at 1:00 p.m. on 29th of November, 2011
1. Introduction

The immunology research field is still growing fast and attracts many investigators to provide novel information leading to understand more the molecular mechanism of diseases and develop new and efficient therapies. This is well reflected in the decision that the Nobel Prize in Physiology or Medicine in 2011 was awarded to scientists working in the frontline of immunology; Bruce A. Beutler and Jules A. Hoffmann "for their discoveries concerning the activation of innate immunity" and Ralph M. Steinman "for his discovery of the dendritic cell and its role in adaptive immunity".

Being key mediators of immune responses, dendritic cells (DCs) have deserved this special attention. They were first described in 1868, in the epidermal layer of the skin by Paul Langerhans, while he was a medical student, who thought the cells were part of the nervous system. But the name “dendritic cells” was coined by Ralph Steinman and Zanvil Cohn in 1973, who discovered that these cells are new types of white blood cells with particular features and functions, so opening new perspectives in research of the immune system. By now these cells have become targets of vaccines or are applied as immuno-therapies. Who could have thought that the knowledge about these cells would grow so fast that the discoverer himself would take part in “dendritic-cell based immunotherapy of his own design”?

1.1. Dendritic cells and their function

DCs are derived from hematopoietic progenitors. They are professional phagocytes and the most potent antigen-presenting cells found in most tissues, both lymphoid and non-lymphoid organs. They got their names because of the dendrites that they grow, being tree-like cells (Greek, dendron, tree) possessing very large contact surface to the environment compared to the volume of the cell.

What helps DCs to be specialized in the detection of antigens is the wide range of pattern recognition receptors that they express (Toll-like, RIG-I like and NOD-like receptors) hence conserved molecular components of the pathogens can be recognized. Besides this, they also express cytokine and chemokine receptors as well as the C-type lectin receptor family, which was also proposed as a major sensor of pathogens. Prior interaction with the antigens, dendritic cells are in an “immature” state, characterized by high phagocytic capacity, low expression of MHC complex and co-stimulatory molecules (e.g. CD80, CD86). The uptake of the antigens takes place by phagocytosis, macropinocytosis or via receptor mediated endocytosis. Phagocytosis of apoptotic cells
is restricted to the immature state of DCs and mediated by $\alpha_v \beta_5$ integrin, the scavenger receptor CD36 and the MFGE8.

The uptake of the antigen is followed by the processing and peptide presentation through the cells surface associated MHC class I or II molecules. Dendritic cells getting the maturation inducing signals, coming directly from the pathogens or via inflammatory stimuli, change their chemokine receptor expression pattern and migrate from the peripheral tissues to the draining lymphoid organs. On reaching these organs DCs undergo the “maturation” process which is associated with phenotypic changes, including down-regulation of phagocytic capacity, up-regulation of MHC and co-stimulatory molecules, and secretion of cytokines; all these changes make them functional antigen-presenting cells. Antigen loaded DCs induce the antigen-specific immunity leading to the proliferation of T cells and differentiation into helper and effector cells that have unique cytokine profile and function.

DCs possess the unique capacity to prime naive T cells and induce not only T cell immunity but also T cell tolerance. The outcome of DC mediated immune response (stimulation or tolerance) depends on the type of the pathogens or other antigens and the co-stimulatory molecules and cytokines that instruct the polarization of T-cells. Antigens from apoptotic cells can be effectively presented to CD8$^+$ T cells via MHC class I molecule, a process known as cross-presentation. This is important in eliciting cytotoxic T cells in responses against viruses and tumors in humans.

DCs are able to induce humoral immunity as well, as a result of their interaction with B cells. Plasmacytoid DCs induce memory B cell differentiation into effector plasma cell via type I interferon and IL-6, and type I interferon activates myeloid DCs that regulate B cell priming and acquisition of memory phenotype via IL-12, IL-6 and Blys/Baff, B cell activating factor.

In vitro, human dendritic cells can be generated from CD34$^+$ progenitors in the presence of granulocyte-macrophage colony-stimulatory factor (GM-CSF) and tumor necrosis factor alpha or IL-13. Large number of DCs can be generated using GM-CSF and interleukin-4 (IL-4). This is a generally used protocol to generate in vitro monocyte-derived DCs which resemble immature tissue DCs, a heterogeneous population containing both CD1a$^-$ and CD1a$^+$. CD1a belongs to CD1 membrane proteins, that present a variety of microbial and self-lipids to T cells and it is widely used as human DC marker.

Due to the fundamental role of dendritic cells in initiation of T cell mediated immunity they are in the focus of current immunotherapeutic research and vaccine development strategies.
1.2. Clearance of apoptotic cells

As a result of normal processes of the body, billions of cells die every day which include cells that are generated in excess in tissues as part of the normal development, aged or damaged cells coming from infections or disease. Under physiological conditions many of these events are planned in time and space, hence it is called programmed cell death.

Apoptosis is the major programmed cell death type essential for development and for the maintenance of tissue homeostasis. During development it has a role in formation of the tissue structures and the removal of excess cells. The senescent or damaged cells die through apoptosis before they become harmful to the host. Morphologically apoptosis is characterized by: rounding-up of the cell, nuclear condensation and fragmentation, plasma membrane blebbing but maintenance of its integrity, reduction of cellular and nuclear volume, retraction of pseudopodes and minor modification of cytoplasmic organelles.

The rapid and efficient phagocytic clearance of apoptotic cells ensures the tissue homeostasis and resolution of inflammation by preventing uncontrolled release of harmful intracellular content. Both professional phagocytes like macrophages and dendritic cells and nonprofessional phagocytes (neighboring cells such as endothelial and epithelial cells and fibroblasts) take part in engulfment of apoptotic cells, but the previous ones have a higher rate and capacity for phagocytosis. The very complex process of apoptotic cell removal is orchestrated by several molecules and signaling pathways and it can be divided into four major steps.

As a first step apoptotic cells release soluble chemoattractants, so called “find-me” signals, which help to attract the phagocytes to the site of death. The specific recognition of the apoptotic cells is a second step of the engulfment process, and depends on the “eat-me” signals, exposed on the apoptotic cells and on the engulfment receptors on the phagocytes, which can recognize the “eat-me” molecules. The most investigated eat-me marker is the exposure of phosphatidylserine on the surface of apoptotic cells (present exclusively on the inner leaflet of the plasma membrane in healthy cells). Several molecules have been shown to contribute to the specific recognition of the apoptotic cells leading to induction of different signaling pathways resulted in the engulfment of the apoptotic cells, as the third step. The internalized cell components are degraded into amino acids, nucleotides, fatty acids and monosaccharides in the lysosomes which will be used to make new macromolecules.

Besides the clearance of apoptotic cells the production of anti-inflammatory mediators by phagocytes as well as the inhibition of proinflammatory cytokine production results in suppression of inflammation and keeps the immune system “silent”. If the apoptotic cells are not cleared efficiently...
or on time, they lose the membrane integrity while they undergo to secondary necrosis and the cell content is released in the extracellular space. The outcome of the immune response is the main difference between engulfment of apoptotic or necrotic cell the first one being anti-inflammatory the latter one proinflammatory.

There are strong evidences that molecular defects in the uptake mechanism of apoptotic bodies lead to altered immune tolerance and autoimmune diseases in mammalian organisms. Impaired cell clearance has been associated with SLE (Systemic lupus erythematosus), atherosclerosis, respiratory diseases, neurological diseases as well as tumorigenesis.

1.3. Glucocorticoids

Glucocorticoids (GCs) are small lipophilic steroid hormones exerting their effect by binding to the intracellular glucocorticoid receptor, a member of the nuclear hormone receptor family, which resides in the cytoplasm in the absence of ligands in complex with chaperonic molecules and immunophilins. After ligand binding the glucocorticoid receptor (GR) translocates to the nucleus, and directly or indirectly regulates the transcription of target genes.

Endogenous GC is synthesized as cortisol in the adrenal cortex in response to excitation of the hypothalamus-pituitary-adrenal axis upon different stimuli, including infection, inflammation, pain and stress. Besides the metabolic effects of cortisol (increase of blood glucose level, stimulation of gluconeogenesis, mobilization of amino acids and fatty acids), endogenous GCs also exert a wide range of immunomodulatory activities.

GCs are widely used as therapeutic agent to treat autoimmune, chronic inflammatory and allergic diseases, lymphomas and leukemias and to prevent allograft rejection after transplantation although they have severe side effect such as osteoporosis, muscle weakness, atrophy of the skin or potentially insulin resistance in diabetes. The therapeutic effects of the glucocorticoids were initially attributed only to their inhibitory effect on T cell immunity. In T cells GR affects the immune functions through a variety of mechanisms: GCs can induce T cell apoptosis, can inhibit the transcription of many T cell derived cytokines and enhances T\textsubscript{reg} subpopulations in vitro and in mice.

Several studies, however, have demonstrated that GCs have impact on other immune cells as well, in a cell type specific manner. In human monocytes GCs have anti-apoptotic effect, induce an anti-inflammatory phenotype and augment phagocytosis of apoptotic cells by macrophages, cells with a central role in innate immunity and in the initiation of adaptive immunity. In dendritic cells GCs were reported to induce a tolerogenic phenotype and suppress their activation. In vivo it was shown that their migration towards the lymph nodes is inhibited. The differentiation and maturation of dendritic cells was also influenced and the mannose receptor-mediated endocytosis was up-
regulated by GCs. However, the effect of glucocorticoids on the phagocytosis of apoptotic cells by dendritic cells has not been investigated yet. Furthermore, knowing from our previous results that long-term interaction of apoptotic neutrophils with DCs renders them prone to proinflammatory cytokine responses and based on reports in the literature that GCs induce a tolerogenic phenotype of DC, we found it important to investigate whether dexamethasone (Dex) can suppress the proinflammatory effect of apoptotic neutrophil.

Although most of the studies give report about the immune suppressive effect of GCs, there are some discrepancies since *in vivo* and *in vitro* studies have suggested both suppressive and enhancing effect of GCs on inflammatory and immune response.

### 1.4. Tissue transglutaminase

Tissue transglutaminase (TGM2) is a multifunctional enzyme belonging to a large calcium-dependent transamidating acyltransferase protein family, which involves blood coagulation FXIII, 6 other enzymes (TG1, 3-7) and protein 4.2 without transglutaminase activity.

Although TGM2 is located mainly in the cytoplasm, it is also found in the mitochondria, in the nucleus and outside of the cells. It is not secreted through the classical ER-Golgi pathway; the cytoplasmic TGM2 is delivered to perinuclear recycling endosomes, interacts inside the endosome with internalized β1 integrins and is secreted as a complex with them. Outside the cells either it stays on the cell surface or is deposited in the extracellular matrix, where it crosslinks matrix components promoting tissue stability, cell adhesion and cell migration. Under continuous tissue injury, higher expression and elevated extracellular trafficking TGM2 can participate in the pathogenesis of tissue scarring.

In addition to its catalytic activity toward protein-bound specific glutamine residues and leading to either deamidation or formation of ε(γ-glutamyl)lysine crosslinks between proteins, TGM2 serves as a G protein in transmembrane signaling, has protein disulphide isomerase and protein kinase activity, binds integrins and fibronectin on the cell surface. On the surface of mouse macrophages it interacts with both integrin β3 and its phagocytosis ligand MFGE8. In fibroblasts its externalization depends on syndecan-4 and its surface localization requires an intact fibronectin binding motif. On the cell surface it is suspected to be present as a peripheral membrane protein tethered to the cell through interactions with integral membrane components. It’s binding to integrins and heparan sulphate proteoglycans has been documented. Recently it was shown that TGM2 is internalized from the cell surface and undergoes lysosomal degradation.

Many studies report about the involvement of TGM2 in different biological functions such as cytoskeletal rearrangements, cell death, signaling, stabilization of extracellular matrix and
Introduction

Phagocytosis. Disregulations of the enzyme function can lead to different inflammatory diseases including diabetes, neurodegenerative diseases, sclerosis, rheumatoid arthritis and celiac disease.

Celiac disease (CD) is an autoimmune disorder characterized by nutrient-induced small intestinal inflammation, diarrhoea, malabsorption, failure to thrive and a spectrum of extraintestinal symptoms. The consumption of wheat, barley or rye (ingestion of gluten) leads to appearance of the symptoms, while dietary exclusion of these cereals results in complete remission and constitutes the single major therapeutical measure in this disease. CD is characterized by villous atrophy, crypt hyperplasia and lymphocytic infiltration, these hallmarks of celiac bowel lesions develop after exposure to gluten peptides at DQ2 and DQ8 HLA settings and are driven by gluten-reactive intestinal CD4\(^+\) T cells. These T cells recognize gluten peptides in which glutamines are deamidated to glutamate by TGM2 leading to negatively charged residues preferentially bound to and presented by DQ2- or DQ8 HLA molecules. TGM2 is the major autoantigen in celiac disease with a significant pathogenic role and it has been suggested that the cell surface-associated TGM2 on APCs can be involved in gluten uptake and the two are processed in a way that autoantibodies appear against both. According to the prevailing pathomechanistic theory TGM2-mediated deamidation of gluten is important for effective gluten presentation while cross-presentation of gluten cross-linked to TGM2 itself gives rise to autoantibody production.

When membrane-bound TGM2 is endocytosed in dendritic cells gluten peptides that are bound to the TGM2 active site and free gluten peptides co-internalized with TGM2 could undergo TGM2-mediated deamidation before the peptides bind to HLA-DQ2 or DQ8 in endosomes. Therefore, it is an important issue whether TGM2 can be found or not on the surface of antigen presenting cells and whether it can work there as a transglutaminase.
2. Aim of the studies

- To examine whether in human monocyte-derived immature dendritic cells dexamethasone has any effect on the phagocytosis of apoptotic neutrophils and which apopto-phagocytic genes are involved in this phenomenon;

- To investigate how dexamethasone influences the proinflammatory response to allogeneic apoptotic neutrophils in human dendritic cells;

- To learn whether dexamethasone can alter the process of T cell activation by DCs occurring after phagocytosis of apoptotic neutrophils;

- To check whether dendritic cells express TGM2 at the cell surface and to clarify whether it is active.
3. Materials and Methods

**Cell culture and reagents**

Human peripheral blood mononuclear cells were isolated from “buffy coats” of healthy blood donors by density gradient centrifugation through Ficoll–Paque Plus. CD14+ cells were separated by magnetic sorting with MACS, followed by washing with PBS-0.5% BSA-2mM EDTA. To generate iDCs, monocytes were plated into 6-well culture dishes at a density of $2 \times 10^6$ cells/ml and cultured for 2 or 5 days in AIM-V medium containing 800 U/ml GM-CSF and 500 U/ml IL-4. Medium was supplemented with IL-4 and GM-CSF at day 0 and day 3. For glucocorticoid treated samples dexamethasone was added to the cell culture medium from day 0 of differentiation.

To generate mDCs, cells were activated on day 5 of culture with 100 ng/mL LPS and 10 ng/ml INFγ for a period of 16h. For LPS stimulated iDCs on the fifth day the medium was supplemented with cytokines and 100 ng/ml LPS was added for additional 16h.

Allogeneic neutrophils were isolated from peripheral blood of healthy volunteers by density gradient centrifugation using Histopaque 1119 and Histopaque 1077 and cells were cultured for 16h in IMDM supplemented with 10% human AB serum while they underwent spontaneous apoptosis. Autologous lymphocytes were kept at −70°C in cell-freezing medium (FBS: DMSO – 9:1) until they were used for the T cell activation assay.

**Phagocytosis assays**

DCs were stained with CMTMR and the freshly isolated neutrophils were labeled with the yellow-green fluorescent dye CFDA-SE, and stainings were done according to the manufacturer’s protocol. Before the assay the labeled apoptotic neutrophils were washed 3 times with PBS and dendritic cells were counted and replated in fresh medium. DCs and apoptotic neutrophils were co-cultured for 8h at a ratio of 1:5 at 37°C and 5% CO₂ atmosphere, for each sample in duplicates. In case of Dex treated cells the glucocorticoid was not present during the co-incubation. Cells were collected by trypsinization, washed with PBS, fixed with 1% PFA in PBS and analyzed by FACS Calibur. DCs were gated on the basis of forward scatter and side scatter properties and the percentage of cells positive both for CMTMR and CFDA-SE was determined.

For the blocking experiments, prior to co-incubation with apoptotic neutrophils, the replated dendritic cells were pre-incubated with 10 µg/ml anti-MERTK (clone 125508) or anti CD14 antibodies for 15 min at 37°C and 5% CO₂ atmosphere. The antibodies were present during the phagocytosis assay as well. For testing the effect of ADORA3 antagonist on phagocytosis DCs were
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treated with the selective adenosine A3 receptor antagonist MRS1220 for a period of 1h, 37°C and 5% CO₂ atmosphere and the antagonist was present throughout the phagocytosis period.

Cell surface labeling

Prior to each labeling, dendritic cells were incubated in 50% human AB serum for 30 min at 37°C to block the unspecific binding sites, then washed with PBS-1% BSA and stained for 30 min on ice with the different antibodies respectively with the matching control antibodies. Effectivity of the monocyte to dendritic cell differentiation was controlled by the phenotypic analysis of untreated and GC treated cells using PE-conjugated anti-CD209, anti-CD14, anti-CD1a and anti-CD40 antibodies respectively their isotype control antibodies IgG2b, IgG2a and IgG1, all used at a ratio of 1:50, as indicated from the manufacturer.

Cell surface expression of MERTK and CD14 was detected with 10 µg/ml unlabeled monoclonal anti-MERTK and anti-CD14, respectively IgG1 (isotype control) antibodies followed by FITC-conjugated anti-mouse antibody which was used at a ratio of 1:50. For CD14 direct labeling with PE-CD14 and PE-IgG2a antibodies were used at a ratio of 1:50.

For surface TGM2 labeling monoclonal anti-TGM2 antibodies were used TG100 and CUB7402, 4G3 supernatant H23, G92, pab0062 respectively mouse IgG1 isotype control antibody at (1µg/10⁶ cells). The secondary antibody was FITC-conjugated anti-mouse antibody used at a ratio of 1:50.

In each case the labeling was performed on ice for a period of 30 min and 10⁶ cells/sample were used. As the last step, the cells were washed with PBS-1% BSA, fixed with 1% PFA in PBS and then the stained cells were detected by flow-cytometry.

Immunoblotting

Monocytes and dendritic cells differentiated in the presence or absence of Dex were collected and washed with PBS followed by their lysis in 50 mM Tris–HCl containing 0.1% Triton X-100, 1 mM EDTA, 15 mM 2-MEA and proteinase inhibitors. Insoluble cellular material was removed by centrifugation and the lysates were mixed with 5× Laemmli loading buffer, boiled for 10 min and 15 µg protein of each sample were loaded onto a 10% SDS polyacrylamide gel. Proteins were transferred onto PVDF membranes followed by blocking with 5% skimmed milk. Membranes were probed by monoclonal anti-TGM2 antibody either 4G3 or CUB7402 followed by incubation with HRP-conjugated anti-mouse antibody for 1h at room temperature. For loading control, mouse monoclonal antibodies 6C5 to GAPDH or β-actin were used overnight at 4°C. Immunoblots were developed with Immobilon Western chemiluminescent substrate.
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**Cell surface TGM2 activity measurement**

On the fifth day of their differentiation immature dendritic cells were collected, washed and replated in 24 well culture dishes in 300 µl of fresh RPMI serum-free medium at a density of 2 x 10^6 cells/well. Except for controls, cells were incubated with 25 µg/ml 5-BP, casein (2 mg/ml) and either CaCl₂ (2mM) or EDTA (4 mM). Incubation with 1 µg anti-TGM2 antibody (TG100 and CUB7402) was for 30 min at 37 °C before adding the indicated components to the cells. Cell-free supernatants were collected, centrifuged and prepared with 5x LB for Western blot analysis. 15 µl of each sample were loaded onto 12% SDS polyacrylamide gel. Proteins were transferred onto PVDF membranes followed by blocking with 5% skimmed milk. Membranes were probed by HRP-conjugated streptavidin, monoclonal anti-TGM2 antibody (CUB7402) and GAPDH. Immunoblots were developed with Immobilon Western chemiluminescent substrate.

**Determination of TNFα secretion**

Differentiated but not stained monocyte-derived DCs were co-cultured with unstained apoptotic neutrophils for 8h, then stimulated with 0.1 µg/ml LPS and 10 ng/ml IFNγ for an additional 16h. The culture supernatants were harvested and stored for cytokine measurements at -20 °C. The concentrations of released TNFα were measured by the human TNFα duo set ELISA kit according to the manufacturer’s specifications. The measurement was performed in each case in triplicates.

**Human IFNγ ELISPOT assay**

Dendritic cells differentiated for 5 days in the presence or absence of different concentration of Dex were co-cultured with non-labeled allogeneic apoptotic neutrophils for 8h, then autologous lymphocytes were added at a ratio of 1:25 for 5 days at 37°C in a 5% CO₂ atmosphere. Cells were collected on day 5 and subjected to anti-human IFNγ Ready-Set-Go ELISPOT assay on MultiScreen-HTS PVDF plates. After 48h at 37°C, the cells were removed, and the plates were washed with PBS. The cytokine spots were detected by biotinylated anti-IFNγ antibody followed by avidin-HRP conjugated antibody. The reaction was stopped by washing with tap water and the air-dried plates were analyzed by a computer-assisted ELISPOT image analyzer. Secreted IFNγ was measured by the OptiEIA system.

**RNA preparation and TaqMan real-time RT-PCR**

Total RNA was isolated from untreated and dexamethasone treated human monocytes and monocyte-derived dendritic cells using TRIzol Reagent. To determine the expression level of apoptophagocytic genes, a 384-well TaqMan Low Density Array (TLDA), custom made for the Apoptophagocytosis research group in our Department was used, with two replicates per target gene and
three biological parallels. 18S rRNA was used as endogenous control and gene expression values were calculated based on the $\Delta\Delta C_t$ method. Relative expression were determined using the equation where relative quantity equals $2^{-\Delta\Delta C_t}$. Fold changes represent either the ratio of relative gene expressions (average of three donors) of differentiated dendritic cells and monocytes referred to as “Differentiation” or the ratio of dexamethasone treated and non treated referred to as “Differentiation and Dex”.

**Animal experiments**

**Animals**

Experiments were performed according to local ethical guidelines and approved by the Animal Experimental Committee of University of Debrecen: 8-12 weeks old C57BL/6J and ADORA3 KO mice were kept in SPF conditions and were killed after isoflurane narcosis by cervical dislocation.

**Isolation and differentiation of mouse bone marrow derived DCs**

Bone marrow cells were isolated from the femur of mice using 26G needle and saline. The bone marrow cells were washed in saline, then resuspended and cultured in 25 mM HEPES modified RPMI-1640 medium supplemented with 10% FBS, 2mM glutamine, penicillin-streptomycin. Bone marrow cells were plated in 6-well culture plates (1.5x10^6 cells/ml) and were differentiated to DCs by mouse GM-CSF (20 ng/ml) and mouse IL-4 (20 ng/ml) for 9 days. Every third day half of the old medium were changed to fresh medium containing cytokines. Cells were treated with dexamethasone from day 0 of differentiation.

**Isolation of neutrophil granulocytes and phagocytosis assay**

Neutrophil granulocytes were isolated by density gradient centrifugation from mouse bone-marrow (C57BL/6J) using Histopaque 1119 and Histopaque 1077. To generate apoptotic neutrophils the cells were cultured for 24h in IMDM supplemented with 10% mouse serum and stained with CFDA-SE. The purity of the neutrophils was controlled by May-Grünwald/Giemsa staining. On the 9th day of differentiation mouse dendritic cells were counted and replated in serum-free medium and co-incubated with washed apoptotic mouse neutrophils for a period of 8h, at a ratio of 1:5 at 37°C and 5% CO_2 atmosphere. Samples were collected by trypsinization, washed with PBS, fixed with 1% PFA and analyzed by FACS Calibur.

**Statistical analysis**

Statistical analysis was performed by paired Student’s t-test and the value of p<0.05 was used to determine statistical significance.
4. Results

4.1. Dendritic cells differentiated in the presence of dexamethasone have increased phagocytic capacity

Human monocyte-derived macrophages have a high capacity to engulf apoptotic neutrophils and the presence of dexamethasone during the differentiation process can increase the uptake of apoptotic cells. In our experiments even after several hours of co-incubation the percentage of dendritic cells with engulfed apoptotic neutrophils was 9-25% varying among donors. We found that similarly to macrophages, the presence of dexamethasone during the 5 days of differentiation of monocytes to dendritic cells, leaded to increased phagocytosis. This Dex-mediated effect showed donor-dependent variability but did not correlate with the concentration of Dex - although it was observed that the smallest concentration of Dex (10 nM) was the most effective in the majority of donors.

4.2. The presence of GC allows but skews DCs differentiation to a CD1a⁺CD14⁻ subtype

To see the effect of Dex on the differentiation of the cells in a time-dependent manner, the expression of surface markers was detected at an earlier stage of differentiation as well. While the surface expression of CD14 should decrease with differentiation, treatment with glucocorticoid prevented this decline. We also observed that the CD1a⁺ population of DCs varied among the donors, but the dexamethasone treatment decreased the DC1a positivity of the cells, even when applied in the lowest concentration and from the early time point of differentiation. Control experiment revealed that surface expression of DC-SIGN (CD209), expressed by both CD1a⁻ and CD1a⁺ cells did not change by the glucocorticoid treatment.

4.3. Gene expression pattern of monocyte-derived dendritic cells differentiated in the absence or presence of dexamethasone

To learn the molecular mechanism of the elevated phagocytosis in dendritic cells upon glucocorticoid treatment we studied by TLDA the expression pattern of a previously designed panel of 95 apopto-phagocytic genes. After evaluating the gene expression pattern of control and Dex treated DCs we focused on up-regulated genes.
4.3.1. Apopto-phagocytic genes up-regulated during the differentiation of immature dendritic cells and by glucocorticoid treatment

Samples with the most efficient Dex treatment enhancing phagocytosis in iDCs of three donors were selected to analyze the apopto-phagocytic gene expression pattern by TLDA. Genes were normalized to the level of 18S rRNA and changes in gene expression during differentiation and Dex treatment were determined based on the average relative expressions of 3 donors. Comparing iDCs to monocytes 17 genes were up-regulated during the 5 days of differentiation. Among these genes there are 6 which show more than 10 times increase in their relative gene expression compared to the monocytes: DOCK1, FCGR2B, GAS6, IRF4, PROS1 and PPARG.

Comparing the average relative expressions of iDCs differentiated in the presence of Dex versus non treated cells, 10 apopto-phagocytic genes were up-regulated more than two times. Among these genes there were 4 which were already up-regulated during the 5 days of differentiation so they were further increased by the glucocorticoid. Namely, these 4 genes were the cell surface molecule ADORA3 and FCGR2B, the bridging molecules C1QA and C2.

4.3.2. Genes up-regulated by dexamethasone in all donors

The relative gene expressions of the apopto-phagocytic genes up-regulated by the glucocorticoid treatment were analyzed separately in each donor as well and the highly up-regulated ones in all donors are: the adenosine receptor ADORA3, the phagocytosis receptors MERTK and CD14, C1QA (which can form a bridge between dying cells and phagocytes), and the digestive enzyme DNASE2.

4.3.2.1 Dexamethasone is already effective from day 2 of differentiation

Monocytes of the same donors were differentiated to dendritic cells but the cells were cultured after 2 days and phagocytosis assay with apoptotic neutrophils was performed. Enhanced engulfment of apoptotic cells was observed even in this early stage of differentiation however the most effective Dex concentrations are not the same as in iDCs. The up-regulation of the same apopto-phagocytic genes by Dex (ADORA3, MERTK, CD14 and C1QA) observed in iDCs (after 5 day of differentiation) could be detected at the early stage of differentiation as well.
4.4. Investigation of the role of MERTK in Dex mediated increase in the phagocytosis of apoptotic neutrophils

4.4.1. Dex treatment induces expression of MERTK on the surface of dendritic cells

TLDA analysis showed that MERTK was highly up-regulated by the glucocorticoid treatment so the cell surface expression of the protein was also investigated. Flow-cytometry data show that it can be detected only on dexamethasone treated iDCs.

4.4.2. Effect of MERTK blocking antibody on the increased phagocytosis of apoptotic neutrophils induced by Dex

As we were able to detect this protein on the Dex treated samples, we wanted to see whether the antibody against MERTK, used successfully for phagocytosis blocking in case of human macrophages, could block the dexamethasone induced enhancement of phagocytosis. We performed phagocytosis assay with control and Dex treated immature dendritic cells after pre-incubation with the antibody but there was no inhibition of apoptotic cell uptake.

4.5. Investigation of the role of CD14 in Dex mediated increase in the phagocytosis of apoptotic neutrophils

4.5.1 Cell surface expression of CD14 is induced by Dex in iDCs

Similar to MERTK, CD14 was also up-regulated in iDCs upon glucocorticoid treatment. The increased expression at the cell surface of the protein could be detected as well with both direct and indirect labeling.

4.5.2 Effect of anti-CD14 antibody on the increased phagocytosis of apoptotic neutrophils induced by Dex

We wanted to see whether the anti-CD14 antibody used for indirect surface CD14 labeling could inhibit the phagocytosis enhancing effect of the glucocorticoid. iDCs were pre-incubated with the antibody and then loaded with apoptotic neutrophils, but no inhibition of Dex effect could be observed.
4.6. Role of ADORA3 in dexamethasone induced increase of apoptotic neutrophils phagocytosis

The relative expression of adenosine receptor A3 (ADORA3) in iDCs differentiated in the presence of glucocorticoid showed an almost 7 fold increase compared to the non-treated cells. To test the effect of ADORA3 on Dex mediated enhancement of apoptotic neutrophils uptake we used the highly selective ADORA3 antagonist MRS1220. iDCs were pre-incubated with the antagonist and then loaded with apoptotic neutrophils. MRS could decrease the Dex-induced enhancement of phagocytosis in 3 of the donors and one donor did not show any changes in apoptotic cell uptake in response to the antagonist pre-treatment. The glucocorticoid concentration where we could see the phagocytosis decreasing effect also varied among the donors.

The involvement of ADORA3 in the Dex effect was tested in a murine system as well. Dendritic cells were differentiated from bone marrow progenitors in the absence or presence of Dex of wild type and ADORA3 knockout mice for a period of 9 days. Then phagocytosis assays were performed with murine apoptotic neutrophils. In case of wild type dendritic cells Dex increased the uptake of apoptotic cells while the lack of ADORA3 resulted in the unresponsiveness of dendritic cells to Dex treatment and there was even less phagocytosis by them upon Dex treatment.

4.7. Enhanced apoptotic cell phagocytosis by dexamethasone treated dendritic cells leads to proinflammatory cytokine secretion and T lymphocyte activation

To test the effects of Dex on DC-mediated inflammatory responses iDCs were generated in the presence or absence of Dex and then loaded with human allogeneic apoptotic neutrophils. After 8h co-incubation the cells were stimulated with LPS and IFNγ for additional 16h before measuring the concentration of secreted TNFα in the culture supernatants. Surprisingly, iDCs differentiated in the presence of Dex responded with increased TNFα secretion and when these cells were fed by apoptotic neutrophils there was even higher cytokine release as compared to controls. The most effective Dex concentration leading to this response varied among individuals.

To assess the T cell polarizing potential of apoptotic cell-loaded DCs of the same donors, cells were co-cultured the with autologous T lymphocytes for an additional 5 days and the IFNγ-secreting T cells were detected by ELISPOT assay. Despite the high variability of the cellular response among donors, we could detect higher numbers of IFNγ-secreting T cells in all cases when Dex-treated DCs were exposed to apoptotic neutrophils. Dex by itself was able to potentiate iDCs for T cell activation, but this effect was more pronounced in neutrophil-loaded DCs. Again, the most effective Dex concentrations were donor-dependent.
As differences in the internalizing capacity, cytokine and chemokine profile and T cell polarizing potential were shown between the CD1a⁺ and CD1a⁻ DCs, cells of the same donors were stained for CD1a. iDCs were CD1a positive (40-60%), and the glucocorticoid treatment, similar to the previous results, skews the DC differentiation to a CD1α negative subtype.

4.8. Apopto-phagocytic genes down-regulated in immature dendritic cells during differentiation and by the glucocorticoid treatment

Comparing the average relative expressions of dendritic cells derived from 3 donors to monocytes we found 41 apopto-phagocytic genes with at least 2 fold decrease in their relative gene expression level. Among these genes, there are 8, which are in common with the genes down-regulated by Dex. Namely these genes were: the surface molecule adenosine A2a receptor, the phagocytosis receptors as AXL, ITGAX, ITGB, LRP1, the bridging molecules like complement component 3 and ICAM3 and the interferon regulatory factor 1. We also could see that in line with the cell surface labeling data the expression of CD14 at mRNA level is decreased during the 5 days of differentiation to immature dendritic cells (FC = 0.005).

Comparing the average relative expression of iDCs differentiated in the presence of the glucocorticoid to non treated cells we found 22 apopto-phagocytic genes which were down-regulated. Among the most down-regulated apopto-phagocytic genes we further investigated TGM2 which was down-regulated both in mRNA and protein level from early time points of differentiation.

4.9. Expression of TGM2 on the surface of human dendritic cells

4.9.1. TGM2 can be detected on the surface of monocyte-derived dendritic cells

It was suggested that cell-surface TGM2 on antigen presenting cells can be directly involved in gluten-reactive T-cell activation. We used a battery of commercially available and proprietary antibodies (CUB7402, TG100, 4G3 supernatant, H23, G92, pab0062) in indirect immunofluorescent labeling-coupled flow-cytometry to test if TGM2 was present on the surface of these cells. While most antibodies (4G3, CUB7402, H23, G92, pab0062) gave negative results, the TG100 monoclonal antibody bound iDCs efficiently. The increasing amounts of TGM2 in the course of differentiation detected on Western blots were paralleled by similarly elevating signal intensities of the cell surface labeling on consecutive days.
4.9.2. TGM2 is active on the surface of iDCs

With regard to the potential involvement of transglutaminase activity in the processing of gliadin peptides to the antigenic deamidated forms, we sought to determine if the cell surface enzyme could be active. To this end we incubated dendritic cells with N-N dimethyl casein and N-(5 aminopentyl)biotinamide (5-BP). Incorporation of the biotinyl residue into casein was detected by Western blots of cell-free supernatants of the reactions with streptavidin–horseradish-peroxidase. To investigate whether the cells were injured during the assay and TGM2 could leak out of the cells, we also looked for the presence of GAPDH, a glycolytic enzyme abundant in the cytoplasm of most cells, and TGM2 itself in the cell free supernatant. GAPDH appeared in the supernatants, but TGM2 could hardly be detected. Furthermore, there was no significant difference in cell surface TGM2 activity when separately obtained donor cells were compared, though the amount of TGM2 in the supernatant was almost undetectable in one of these, suggesting that most of the demonstrated activity originated from cell surface TGM2. In order to block/inhibit the TGM2 activity on the cell surface and in the liquid phase we pre-incubated the cells with TG100 and CUB7402, then added casein and 5-BP to them. No inhibition of cell surface TGM2 could be observed thought it is known that both antibodies inhibit TGM2 activity in solution.

4.9.3. LPS-treated monocyte-derived iDC have increased expression of TGM2

It has been suggested that in individuals genetically predisposed to CD an unspecific inflammatory process in the gut can be responsible for the accumulation and activation of TGM2. We used bacterial lipopolysaccharide to simulate the activation of and in case of 16h of LPS stimulation and mature dendritic cells we found an increased surface expression of TGM2 detected by TG100.
5. Discussion

Glucocorticoids are stress hormones naturally released during the course of an immune response and they represent an essential link between the central nervous system and the regulation of the innate immune system.

Endogenous glucocorticoids exist in two different forms, being either active (cortisol) or inactive (cortisone). Exogenous (therapeutic) GCs are powerful anti-inflammatory and immunosuppressive agents widely used in the treatment of many autoimmune, allergic and chronic inflammatory diseases and organ transplant rejection. The immunosuppressive and anti-inflammatory property of GCs cannot be attributed only to their well-known effect on T cells, but also specific impact on nearly every cell type of the immune system, particularly on macrophages and dendritic cells.

GCs may act at the very first step of the immune response by modulating DC differentiation, maturation and function, freezing the cells at an immature stage and enhancing both mannose receptor-mediated and fluid-phase endocytosis. When we tested the effect of glucocorticoids on human immature monocyte-derived dendritic cells, which resemble tissue resident migratory DC, we also observed the morphological changes (less adherent cells with abundant cytoplasm) reported earlier. Phagocytosis of apoptotic cells by these cells, investigated with an in vitro phagocytic system established previously to mimic events at in vivo inflammation sites, was increased in GC treated DCs. The dosage of Dex required for and the extent of the enhancement of phagocytosis varied among individual donors. The varying sensitivity to Dex treatment is very likely due to the molecular heterogeneity and polymorphism of the glucocorticoid receptor generating diversity in the sensitivity of GC response.

The TaqMan Low Density Array used to study the mechanism behind the GC induced increase in phagocytosis in human dendritic cells, contained genes involved in the apopto-phagocytic process that can be grouped in the following categories: receptors (integrins, scavenger receptors, adenosine receptors, tyrosine kinases, etc.), bridging molecules, signal generators, effectors, cytokines, nuclear receptors, engulfment genes, autophagy genes, interferon regulatory family genes. The 10 genes found up-regulated by dexamethasone during the differentiation to immature dendritic cells are members of cell surface molecules (ADOR3, FCGR2B), bridging molecules (C1QA, C2), phagocytosis-tethering/tickling-receptors (CD14, MERTK, SCARB1), effectors (DNASE2), cytokines (IL10) and inflammatory regulators (NLRP12). From these genes ADORA3, MERTK, and CD14 were further investigated. There was a significant reprogramming of phagocytic function related gene expression by Dex since the 5 days differentiation process itself in the absence of Dex
results in the induction of 17 apopto-phagocytic genes and only 4 of them overlapped with the Dex up-regulated genes, namely ADORA3, C1QA, C2 and FCGR2B.

In monocyte-derived dendritic cells Dex up-regulates the expression of MERTK not only at mRNA but at cell surface expression as well, although by applying blocking antibodies against MERTK we could not diminish the GC induced enhancement of phagocytosis by DCs differentiated either for 2 or 5 days. A similar result was obtained with the cell surface molecule CD14 which is also a receptor involved in the recognition and phagocytosis of cells undergoing apoptosis. GC treated DCs are positive for surface CD14 staining as we also could observe, but there was no decrease in apoptotic cell engulfment of GC-treated DCs in the presence of the anti-CD14 antibody. It cannot be excluded that the bindings of the anti-MERTK and anti-CD14 antibodies was not strong enough to block the phagocytosis, or the role of these surface molecules are not pronounced enough in mediating the enhancement of phagocytosis by Dex that their blocking would make a significant difference in phagocytosis efficiency. CD14 can be internalized and it also has a soluble form shedded from the cell surface, so this could also interfere with the blocking effect of the anti-CD14 antibody.

Adenosine A1 and A3 receptors are expressed on immature myeloid and plasmocytoid dendritic cells and their activation induces chemotaxis as a result of mobilization of intracellular calcium and reorganization of the cytoskeleton in immature DCs. In human monocytes ADORA3 was found to be up-regulated by GC. Our results show that also in human monocyte-derived dendritic cells Dex up-regulates the expression of ADORA3. Using a potent and highly selective antagonist of the human A3 adenosine receptor (MRS1220) we could attenuate the dexamethasone induced enhancement phagocytosis of apoptotic neutrophils. To investigate further the role of ADORA3 we tested the effect of Dex in dendritic cells of ADORA3 KO mice and observed that it was ineffective to increase phagocytosis of apoptotic neutrophils. Thus, adenosine seems to be a soluble mediator acting via ADORA3 in glucocorticoid treated dendritic cells and by its cAMP lowering effect can possibly contribute to the increased phagocytosis of apoptotic cells.

We showed previously that in contrary to human macrophages DCs respond to apoptotic allogeneic neutrophils in an unexpected way by secreting proinflammatory cytokines and T cell activation. In Dex treated DCs we could detect even higher number of IFNγ producing T cells under such conditions which may be attributed to the increased phagocytosis of apoptotic neutrophils. We even detected elevated IFNγ secretion in some samples treated with different concentration of Dex but not loaded with the dying neutrophils. Following stimulation with LPS+ IFNγ high level of the proinflammatory cytokine TNFα was measured form the supernatant of the DCs exposed to apoptotic cells, which was further enhanced by the Dex. Furthermore, Dex itself could increase TNFα production of DCs which possibly could be attributed to the up-regulation of Toll-like receptors.
(TLR-4 and TLR-2) by GCs as it was shown in PBMCs. These results demonstrate that the GC effect is more complex than it has been described so far and may drive inflammatory and immune suppressive responses in a context-dependent manner. It was also shown in endothelial cells that the presence of dexamethasone enhances inflammation initiated by ATP, which is another example when GCs are not uniformly immunosuppressive and their effect depends on cell type, dose and timing of exposure.

The extensive change in the gene expression pattern is also manifested in the large number of down-regulated apopto-phagocytic genes. One of the down-regulated genes is transglutaminase 2, which was proposed to takes part in the formation of phagocytic portals by interacting with both integrin β₃ and MFGE8. It was also showed that the loss of TGM2 leads to delayed phagocytosis of apoptotic cells by macrophages and to development of autoimmunity in mice. In human dendritic cells TGM2 is highly induced during the 5 days of differentiation, even from day 2, at mRNA and protein levels as well. The relevance of studying the expression of TGM2 in the professional antigen-presenting cells, the immature dendritic cells, is linked to celiac disease.

Duodenal dendritic cells can be directly involved in gluten-reactive T cell activation in celiac disease but in the lack of a reliable anti-TGM2 antibody, the question whether these APCs express on the cell surface TGM2 and whether that is active, was opened. TGM2-specific antibodies and celiac disease associated anti-TGM2 auto-antibodies, which react with the purified enzyme in solution or recognize intracellular TGM2 in immunohistochemistry and immunofluorescence, show context dependence in recognizing cell surface csTGM2.

We also tried several TGM2 specific antibodies to detect the cell surface protein, but only the labeling with TG100 proved to be successful. Our results also show that TGM2 is active on iDCs meaning that its conformation and interacting partners leave keep or make it transamidation capable. This finding is significant in connection to the theory of the role of TGM2 in celiac disease: TGM2-mediated deamidation of gluten is thought to be important for effective gluten presentation whereas cross-presentation of gluten cross-linked to TGM2 itself gives rise to autoantibody production. When membrane-bound TGM2 is endocytosed in dendritic cells gluten peptides that are bound to the TGM2 active site and free gluten peptides co-internalized with TGM2 can undergo TGM2-mediated deamidation before the peptides bind to HLA-DQ2 or DQ8 in endosomes. Our observation that LPS-stimulated dendritic cells have increased csTGM2 supports the suggestion that an initial inflammatory response to gluten can accelerate this process. Inhibition of TGM2 with antibodies or selective inhibitors, therefore, can be a valid approach to palliate celiac disease.

Although TGM2 is strongly down-regulated by Dex, immature dendritic cells have increased phagocytic capacity. This means that alternative pathways up-regulated by Dex can replace the apopto-phagocytic action of TGM2 while other functions of this protein, which might not be
Discussion

compatible with GC-mediated response of DCs - e.g. facilitation of cellular attachment, signaling as a G protein, protein cross-linking– are not manifested.

Our result, that the immunosuppressant and anti-inflammatory drug, dexamethasone, can reprogram professional antigen-presenting cells in vitro for inflammatory response, agrees with data of investigators, who showed that glucocorticoids regulate the immune system in a complex way and that their effects are context dependent.

The interaction of the host’s DC subpopulations with allogeneic apoptotic neutrophils may happen in the transplanted organ as a result of early tissue injury causing infiltration of donor neutrophil granulocytes. The strong proinflammatory effect of these allogeneic apoptotic neutrophils is one of the possible reasons of allograft rejection. Based on the results presented here it should be strongly considered that Dex, which is often used as immunosuppressive agent to prevent transplantation rejection, may have an undesired effect promoting the activation of autologous T lymphocytes toward Th1 effector cells via dendritic cells engulfing an increased number of apoptotic neutrophils, thereby facilitating rather than delaying immune-rejection. It should be looked into how this effect compares to the many other immunosuppressive effects of steroids in magnitude.
6. Summary

Clearance of apoptotic cells is crucial in maintaining tissue homeostasis. It is performed mainly by professional phagocytes including dendritic cells, the key-players of the immune system. In our work we investigated the role of glucocorticoid dexamethasone on the functions of monocyte-derived human dendritic cells.

Dexamethasone increased the phagocytosis of apoptotic neutrophils by immature dendritic cells. DCs develop their capacity to engulf apoptotic cells by up-regulating a set of apopto-phagocytic genes. This gene expression pattern was reprogrammed when differentiation took place in the presence of the synthetic glucocorticoid dexamethasone, which increased the expression of phagocytosis receptors MERTK and CD14, the bridging molecule C1QA, DNASE2 and the adenosine A3 receptor (ADORa3). The increased phagocytosis was attenuated by the addition of ADORA3 antagonist and could not be observed when bone marrow derived dendritic cells of ADORA3 knockout mice were treated with Dex. Although MERTK is up-regulated at cell surface level as well, specific antibodies could not inhibit or block the Dex induced increase of apoptotic cell uptake.

We could detect release of the inflammatory cytokine TNFα in the supernatants of Dex treated human dendritic cells, loaded with allogeneic apoptotic neutrophils, and stimulated with LPS and IFNγ. Furthermore, upon Dex treatment iDCs could activate autologous T lymphocytes toward Th1 effector cells and this was enhanced by their exposure to allogeneic apoptotic neutrophils.

Several apopto-phagocytosis genes were down-regulated by the glucocorticoid in iDCs, among them TGM2, which was showed previously to be essential in apoptotic cell uptake. TGM2 is also the major auto-antigen in celiac disease, and it has been suggested that its expression on the surface of APC, can be involved in gluten uptake and in the appearance of auto-antibodies. We showed that monocyte-derived iDCs express large amount of TGM2, and this could be detected on the cell surface. We also showed that the cell surface TGM2 is catalytically active and upon LPS stimulation the surface expression level increased, supporting the hypothesis that an unspecific inflammatory process in the gut may expose more transglutaminase activity. The fact that TGM2 is down-regulated by the glucocorticoid treatment while the phagocytic capacity is increased during the differentiation shows that alternative pathways up-regulated by Dex can replace the apopto-phagocytic action of TGM2.
7. Publications

Publication list prepared by the Kenézy Life Sciences Library

List of publications related to the dissertation

   IF:4.626 (2010)

   DOI: http://dx.doi.org/10.1016/j.imlet.2009.12.010
   IF:2.511

List of other publications

   DOI: http://dx.doi.org/10.4161/auto.7.3.14583

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Publications

Oral presentations


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8. Keywords

Dendritic cells, dexamethasone, phagocytosis, apopto-phagocytic genes, apoptotic neutrophils, T cell activation, inflammation, tissue transglutaminase
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