THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

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

by

Judit Hodrea

Supervisor: Prof. Dr. László Fésüs, M.D., M.H.A.Sc.

UNIVERSITY OF DEBRECEN

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Abbreviations

APC	- antigen presenting cells
2-MEA	- β-mercaptoethylamine
ATP	- adenosine-5'-triphosphate
B cells	- B lymphocytes
Bai 1	- brain-specific angiogenesis inhibitor 1
Blys/Baff	- B-cell activating factor
BSA	- bovine serum albumin
CD	- celiac disease
cDCs	- classic dendritic cells
CFDA-SE	- carboxyfluorescein diacetate succinimidyl ester
CTL	- cytotoxic lymphocytes
DCs	- dendritic cells
DMSO	- dimethyl sulfoxide
DNA	- deoxyribonucleic acid
DNase	- deoxyribonuclease
ECM	- extracellular matrix
EDTA	- ethylenediaminetetraacetic acid
ELISA	- enzyme-linked immunosorbent assay
ELISPOT	- enzyme-linked immunosorbent spot
ER	- endoplasmic reticulum
FBS	- fetal bovine serum
FITC	- fluorescein-5-isothiocyanate
flt-3L	- Fms-related tyrosine kinase 3 ligand
G proteins	- guanine nucleotide-binding proteins
GAPDH	- glyceraldehyde 3-phosphate dehydrogenase
GCs	- glucocorticoids
G-CSF	- granulocyte colony-stimulating factor
GDP	- guanosine diphosphate
GM-CSF	- granulocyte-macrophage colony-stimulatory factor
GR	- glucocorticoid receptor
GTP	- guanosine-5'-triphosphate
HEPES	- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	- human leukocyte antigen
HPA	- hypothalamus-pituitary-adrenal
HRP	- horseradish peroxidase
iDCs	- immature dendritic cells
IgE/G/	- immunoglobulin E/G
IL-	- interleukin
INF	- interferon
KO	- knockout
LPC	- lysophosphatidylcholine
LPS	- lipopolysaccharides
LRP	- lipoprotein receptor-related proteins
M-CSF	- macrophage colony-stimulating factor
mDCs	- mature dendritic cells
MERTK	- c-mer proto-oncogene tyrosine kinase

MFGE8	- milk fat globule-EGF-factor 8
MHC	- major histocompatibility complex
Mo-DCs	- monocyte-derived dendritic cells
NOD	- nucleotide-binding oligomerization domain
PBMCs	- peripheral blood mononuclear cells
PBS	- phosphate buffered saline
PDCs	- plasmocytoid DCs
PE	- phycoerythrin
PFA	- paraformaldehyde
PGE_2	- prostaglandin E_2
PI_3P	- phosphatidylinositol 3-phosphate
PMN	- polymorphonuclear leukocytes
preDC	- pre-dendritic cells
PRR	- pattern recognition receptors
PS	- phosphatidylserine
PVDF	- polyvinylidene fluoride
RNA	- ribonucleic acid
S1P	- sphingosine-1-phosphate
SDS	- sodium dodecyl sulfate
SLE	- systemic lupus erythematosus
T	- T lymphocytes
Tfh	- follicular helper T lymphocytes
TGFβ	- transforming growth factor beta
TGM2	- tissue transglutaminase
Th	- helper T lymphocytes
TIM-4/1/3	- T-cell immunoglobulin mucin- 4/1/3
TLDA	- TaqMan low-density array
TLR7/9	- Toll-like receptors 7/9
TNFα	- tumor necrosis alpha
T_{reg}	- regulatory T lymphocytes
UTP	- uridine-5'-triphosphate
VLDLR	- very-low-density-lipoprotein receptor
WT	- wild type

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Supplementary 1

Judit Hodrea, Gyöngyike Majai, Zoltán Doró, Gábor Zahuczky, Attila Pap, Éva Rajnavölgyi and László Fésüs: The glucocorticoid dexamethasone programs human dendritic cells for enhanced phagocytosis of apoptotic neutrophils and inflammatory response.
J Leukoc Biol."Accepted for publication"

Supplementary 2

Judit Hodrea, Máté Á. Demény, Gyöngyike Majai, Zsolt Sarang, Ilma Rita Korponay-Szabó and László Fésüs: Transglutaminase 2 is expressed and active on the surface of human monocyte-derived dendritic cells and macrophages.
Immunol. Lett. 4; 130(1-2): 74-81, 2010

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 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"?

Dendritic cells function as sentinels in the organism being able to detect and capture various kinds of antigens, including pathogens (bacteria, virus, toxins), dead cells from tumors, transplants or tissues under microbial or autoimmune attack as well as apoptotic cells formed under normal conditions, the efficient and rapid clearance of which is crucial in maintaining tissue homeostasis. After processing antigens, they expose the molecules on their surfaces and alert the other cells of the immune system to respond in an antigen-specific manner. These cells are able to induce immune tolerance as well, a function equally important as the induction of the immune response. Failure in silencing the immune system can lead to autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, SLE, etc.

In the clinical practice glucocorticoids are very commonly used to treat autoimmune and inflammatory diseases as well as to prevent allograft rejection, because of the immunomodulatory effect of the drug. Although many researchers work to shed light on the mechanism of their action, many questions remain to be answered. In our work we investigated the effect of the glucocorticoid dexamethasone on functions of dendritic cells, including regulation of apopto-phagocytic genes, clearance of apoptotic cells and T cell activation.

2. Theoretical background

2.1. Dendritic cells and their function

Dendritic cells (DCs) are derived from hematopoietic progenitors. They are professional phagocytes and the most potent antigen-presenting cells (APC) found in most tissues, both lymphoid and non-lymphoid organs (Steinman 1991). 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. The research field on characterization dendritic cell function and molecular markers is very active; therefore different nomenclatures have been developed.

The older criteria to identify DCs is based on the morphology, capacity of phagocytosis, scavenging and microbial killing, expression of cell surface markers, antigen processing and presentation ability and initiation of the immune system profile (Cao *et al.* 2010; Crozat *et al.* 2010; Swiecki *et al.* 2010; Ueno *et al.* 2010). The newer criteria of classification are linked to the functions in immune tolerance, the specific hematopoietins (G-CSF, M-CSF, flt-3L, GM-CSF) and their receptors for monocyte-independent and -dependent DC formation *in vivo*, the committed precursor cells (monocyte and dendritic cell progenitor, common dendritic cell progenitor, preDC) and linked to the transcriptional programs and controls. The criteria mentioned above are reviewed by Steinman et al. (Steinman *et al.* 2010). The newest classification given by Idoyaga *et al.* describes five types of DCs: plasmocytoid DCs (PDCs), classic or tissue resident DCs (cDCs), migratory DCs, monocyte-derived DCs (Mo-DCs) and Langerhans cells (Idoyaga *et al.* 2011). PDCs are known to produce large amount of type 1 INF through their nucleic acid sensors (TLR7 and TLR9) in response to viruses, the cDCs are found in lymphoid organs such as spleen and lymph node, while the migratory DCs can be found in non-lymphoid organs (skin, lung, intestine) – these cells function as sentinels in peripheral tissue and migrate through the lymphatics to the lymph node, bearing antigens from the periphery where they present them to T-cells inducing activation or tolerance of the immune system. DCs differentiated from monocytes (monocyte-derived DCs) in various tissues by microenvironmental factors as a result of inflammation or metabolic changes (Gordon *et al.* 2005; Leon *et al.* 2007), are still being defined. The Langerhans cells are found in the epidermis and other squamous epithelia. These types of DCs can be further categorized in different subtypes which differ in qualitative and quantitative expression of surface receptors but all subsets link innate and adaptive immunity.

What helps DCs to be specialized in the detection of antigens is the wide range of pattern recognition receptors (PRR) that they express, hence conserved molecular components of the pathogens can be recognized. Among these PRR the Toll-like receptors, RIG-I like receptors and NOD-like receptors (Manicassamy *et al.* 2009; Takeuchi *et al.* 2010) have to be mentioned. Besides these, they also express cytokine and chemokine receptors (Sallusto *et al.* 1994; Marsland *et al.* 2005) as well as the C-type lectin receptor family which was also proposed as a major sensor of pathogens (Brown *et al.* 2001; Geijtenbeek *et al.* 2009). Prior the interaction with the antigens, dendritic cells are in "immature" state (immature dendritic cells), not fully-functional antigen-resenting cells yet. In this stage they are 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 (Sallusto *et al.* 1995; Albert *et al.* 1998). 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 receptor (Albert *et al.* 1998; Miyasaka *et al.* 2004).

The uptake of the antigen is followed then by processing and peptide presentation through the cells surface associated MHC class I or II molecules. Dendritic cells receiving the maturation inducing signals, coming directly from the pathogens or via inflammatory stimuli, change their chemokine receptor expression pattern so they can migrate from the peripheral tissues to the draining lymphoid organs (Rescigno *et al.* 1999). On reaching these organs DCs undergo the "maturation" process which is associated with phenotypic changes, including down-regulation of phagocytic capacity, up-regulation of co-stimulatory molecules (such as CD83, CD86), MHC and secretion of cytokines- all these changes making 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.

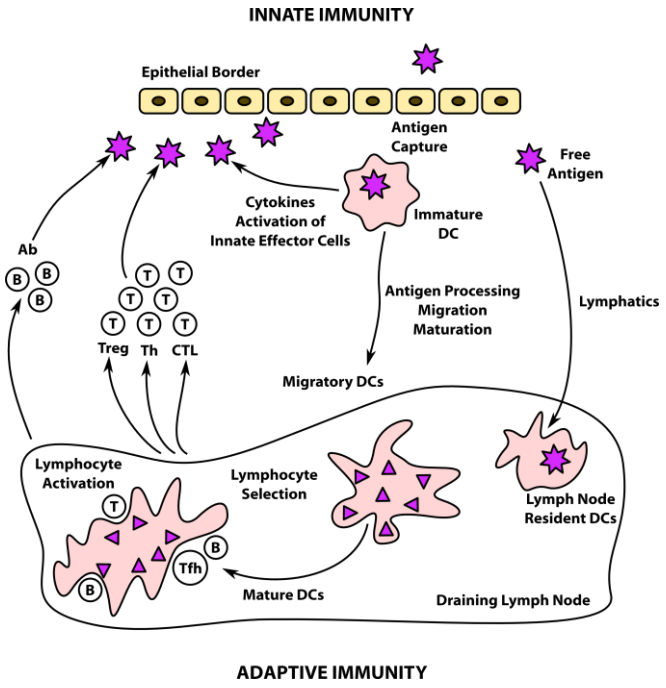


Figure 1. DCs link innate and adaptive immunity. *Immature DCs capture the antigens in the peripheral tissues followed by the antigen presentation in the draining lymph node while they undergo maturation. The soluble antigens reaching the lymph nodes through the lymphatic, are captured, processed and presented by the resident DCs. Lymphocytes (T, B, NK, and NKT cells) and their products are under the control of DCs.*

Figure adapted from (Palucka et al. 2010)

Dendritic cells 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 pathogens or other antigens and the co-stimulatory molecules and cytokines that instruct the polarization of T-cells (Kalinski *et al.* 1999; van Beelen *et al.* 2007; Wilson *et al.* 2007; Joffre *et al.* 2009). For example, production of IL-12p70 by DCs polarizes the Th cells towards INFγ secreting Th1 cells (Kapsenberg 2003), but also induces the generation of follicular helper cells (Tfh) which are able to prime B cell antibody responses (Schmitt *et al.* 2009). Production of IL-23 together with IL-1β leads to the Th17 cell development (Wilson *et al.* 2007). Particularly in mice, IL-10 secretion of DCs generates Th2 type of T cells (Dillon *et al.* 2006). Secretion of IL-10 along with low co-stimulation can result in the induction of T_{reg} cells (Zhou *et al.* 2009), suppressors of immune responses (Rescigno 2010).

In addition, antigens from apoptotic cells can be effectively presented to $CD8^+$ T cells via MHC class I molecule, a process known as cross-presentation (Burgdorf *et al.* 2008; Amigorena *et al.* 2010). This is important in eliciting cytotoxic T cells (CTL) in responses against viruses and tumors in humans (Blachere *et al.* 2005). Autophagy has been identified as a route by which cytoplasmic and nuclear antigens are delivered to MHC class II molecules for presentation to $CD4^+$ T cells and it has also recently been implicated in MHC class I cross-presentation of tumor antigens and the activation of $CD8^+$ T cells (Crotzer *et al.* 2009).

Dendritic cells are able to induce humoral immunity as well, as a result of their interaction with B cells (Batista *et al.* 2009). 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 (Jego *et al.* 2005; Batista *et al.* 2009).

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 alpha (TNFα) (Caux *et al.* 1992) or IL-13 (Piemonti *et al.* 1995; Morse *et al.* 1999; Sato *et al.* 1999). Large number of DCs can be generated using GM-CSF and interleukin-4 (IL-4) (Romani *et al.* 1994; Sallusto *et al.* 1994; Chapuis *et al.* 1997). This is a generally used protocol to generate *in vitro* monocyte-derived DCs which resemble immature tissue DCs (Grassi *et al.* 1998), 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 (Brigl *et al.* 2004) and it is widely used as human DC marker.

Due to the fundamental role of dendritic cells in initiation of T cell mediated immunity DCs are in the focus of current immunotherapeutic research and vaccine development strategies (Palucka *et al.* 2005; Benko *et al.* 2008).

2.2. 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 (PCD) (Lockshin *et al.* 2001). In the last few years the research field on cell death has been expanded and several new types of death have been described. For this reason recently with the recommendation of the Nomenclature Committee on Cell Death unified criteria was proposed for the definition of different cell death categories such as apoptosis, autophagic death, necrosis, cornification and others based on morphological and biochemical processes and definitions for atypical cell death modalities have been given as well (Kroemer *et al.* 2009).

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 also die through apoptosis before they become harmful to the host. Morphologically apoptosis is characterized by: rounding-up of the cell (*oncosis*), nuclear condensation and fragmentation (*karyopycnosis* and *karyorrhexis*), plasma membrane blebbing (*zeiosis*) but maintenance of its integrity, reduction of cellular and nuclear volume, retraction of pseudopodes and minor modification of cytoplasmic organelles (Kerr *et al.* 1972; Kroemer *et al.* 2009).

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 (Parnaik *et al.* 2000). 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 it is presented in Figure 2.

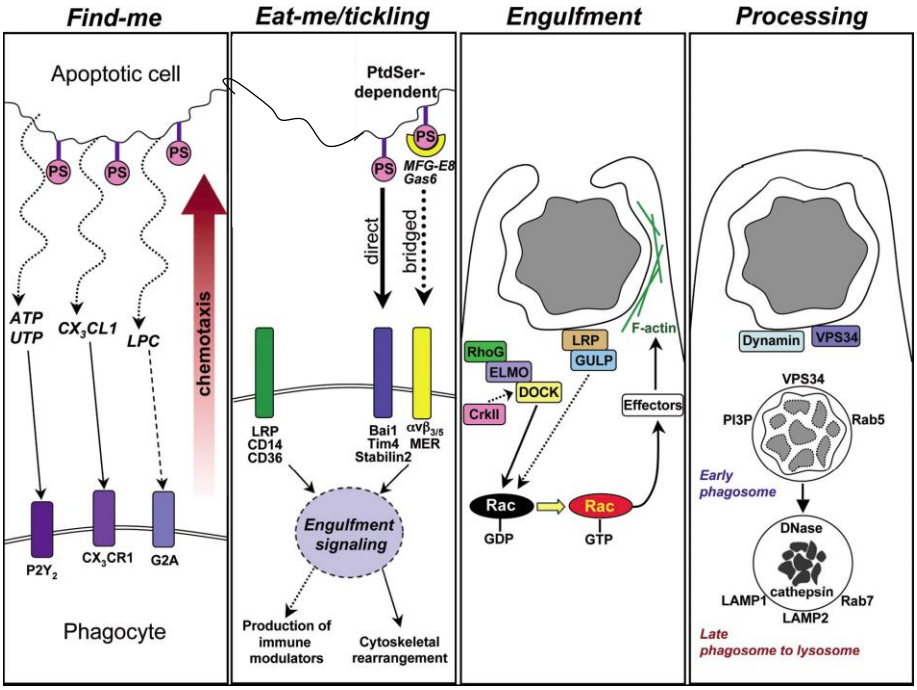


Figure 2. Steps of the apoptotic cell engulfment with some specific modulator. *Apoptotic cells release "find-me" signals (e.g. ATP, UTP, LPC, CX3CL1) to induce chemotaxis of phagocytes to the proximity of dying cells. The broken line from LPC to G2A indicates uncertainty of direct ligand–receptor interaction. Specific recognition of the dying cell depends on the" eat-me" signal exposed on the surface of apoptotic cells. The most characterized is exposure of PS. Phagocytes bind directly through their receptors or via bridging molecules to the apoptotic cells which induce signaling events leading to Rac-dependent cytoskeletal rearrangement and engulfment of the target. After internalization, processing happens through the lysosomal pathway.*

Image by (Elliott et al. 2010).

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. Several molecules such as lysophosphatidylcholine (LPC), sphingosine-1-phosphate (S1P), the chemokine CX3CL1 (Lauber *et al.* 2003; Gude *et al.* 2008; Truman *et al.* 2008) have been proposed to act as "find-me signal", as well as triphosphate nucleotides, ATP/UTP (Elliott *et al.* 2009). 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 (PS) on the surface of apoptotic cells (in healthy cells it is kept exclusively on the inner leaflet of the plasma membrane) – an event which is the most accepted definition of apoptotic cells (Fadok *et al.* 1992; Fadok *et al.* 2000;

Balasubramanian *et al.* 2003). Multiple distinct receptors on the phagocytes can recognize PS, either via direct binding or via soluble bridging molecules. Bai1, Stabilin-2 and TIM-4 are direct-binding receptors but TIM-1 and TIM-3 were also proposed to mediate the uptake of apoptotic cells (Kobayashi *et al.* 2007; Miyanishi *et al.* 2007; Park *et al.* 2007; Ichimura *et al.* 2008; Nakayama *et al.* 2009; DeKruyff *et al.* 2010). Among the bridging molecules MFGE8 (associated with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins), Gas6 and Protein-S (which co-operate with the members of TAM receptor family: Tyro3, Axl and Mer) have been described (Savill *et al.* 1990; Scott *et al.* 2001; Hanayama *et al.* 2004; Lemke *et al.* 2008). Several other molecules have also been shown to contribute to the specific recognition of the apoptotic cells, such as CD31, CD36 and CD14 (Savill *et al.* 1991; Brown *et al.* 2002; Devitt *et al.* 2003). Binding to the PS receptors leads to cytoskeletal reorganization of the phagocyte through the GTPase Rac signaling pathway (Albert *et al.* 2000; Gumienny *et al.* 2001) as a result, engulfment of the apoptotic cells happens. Two separate pathways regulate the activation of Rac. One pathway is initiated by either LRP or Stabilin, as receptors, and GULP, acts as an adaptor protein. The other pathway is mediated by Dock and the migration protein, ELMO. $\alpha_v\beta_5$ integrin-dependent phagocytosis induces signaling through CrkII, Dock and Rac to initiate the early phagosome formation and dominant- negative forms of RhoG or Rac could inhibit the $\alpha_v\beta_3$ integrin-induced apoptotic cell uptake, in the presence of MFGE8, suggesting also the RhoG and Rac involvement in the process.

Many questions still remain to be answered related to PS mediated phagocytosis. For example not only apoptotic cells expose PS, but they are not engulfed (Dillon *et al.* 2000), the binding affinity or avidity of the receptors to PS as well as the expression density varies, the different PS recognition receptors induce different signaling pathways, furthermore the mechanism how an apoptotic cell is induced to expose PS on the cell surface is not well understood (Ravichandran *et al.* 2007). For these reasons participation of additional ligands or helping molecules has to be considered. Several other molecules have been proposed to take part in the phagocytosis of apoptotic cells. Calreticulin was suggested to work together with PS as a signal for removal (Gardai *et al.* 2005), mitochondrial membrane protein Ucp2 of the phagocyte was shown to be critical in the clearance of apoptotic cells (Park *et al.* 2011), PANX1 was identified as a mediator of nucleotide release from apoptotic cells (Chekeni *et al.* 2010). Transglutaminase 2 binds both to integrin β3 (phagocytic receptor) and MFGE8 (bridging molecule) strengthening the interaction between the two. It is required to recruit integrin β3 to the phagocytic cup around the apoptotic cell and is therefore involved in the

formation of engulfing portals (Szondy *et al.* 2003; Falasca *et al.* 2005; Toth *et al.* 2009). Molecules involved in the apopto-phagocytic synapse are shown in Figure 3.

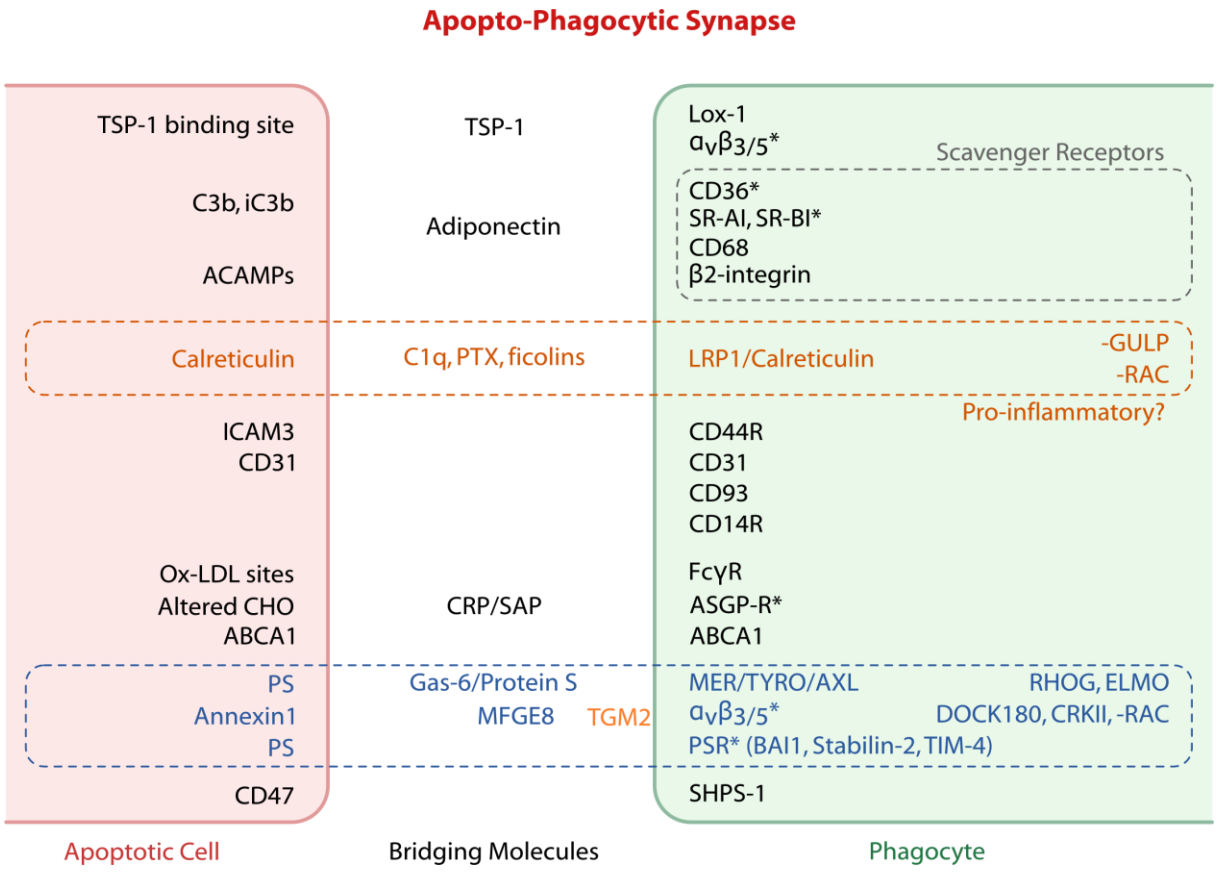


Figure 3. Molecules involved in the apopto-phagocytic synapse. *$\alpha_v\beta_{3/5}$ vitronectin receptor integrins, ABCA-1 (ATP-binding cassette transporter A1), ACAMPs (apoptotic cell-associated molecular patterns), ASGP-R (asyaloglycoprotein receptor); β_2-integrins (CR3, CR4), BAI1 (brain-specific angiogenesis inhibitor 1), C1q (first component of complement), CHO (carbohydrate), CRP (C-reactive protein), CRKII (chicken tumor virus CT10 regulator kinase II), DOCK180 (dedicator of cytokinesis), ELMO (engulfment and migration protein), Gas-6 (growth arrest specific gene-6), GULP (engulfment adaptor protein), iC3 (inactivated complement fragment C3b), ICAM-3 (CD50) (intercellular adhesion moleculae-3), Lox-1 (oxidized low density lipoprotein receptor 1), LRP1(low density lipoprotein receptor-related protein 1), MER (myeloid epithelial reproductive tyrosine kinase), MFGE8 (milk fat globule epidermal growth factor-8), Ox-PL (oxidized phospholipids), PS (phosphatidylserine), PTX (pentraxin), RHOG (Ras homology Growth-related), SHPS-1 (Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1), SR-AI (scavenger receptor AI), SR-BI (scavenger receptor BI), TSP-1 (thrombospondin-1.*Receptor found on non-professional phagocytes.*

Figure adapted from (Majai et al. 2006)

It is important to know that the exact molecular pathway of engulfment can differ depending on the tissue type which is due to the varying expression of surface molecules responsible for apoptotic cell recognition in different cell types. Most of the studies about the phagocytic machinery and the molecules involved in the process have been performed on macrophages.

After the internalization all of the apoptotic 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 loose the membrane integrity while they undergo secondary necrosis and the cell content is released into 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 (Figure 4).

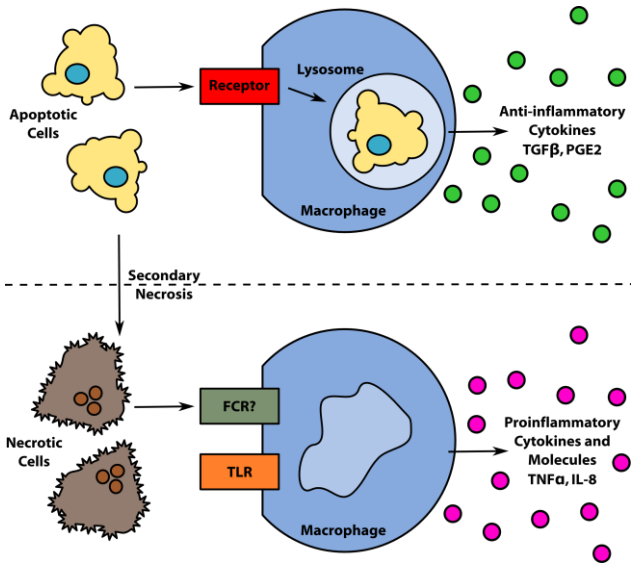


Figure 4. Engulfment of Apoptotic versus Necrotic Cells*. When apoptotic cells are engulfed by macrophages anti-inflammatory cytokines, such as TGFβ and PGE2 are released as a result further macrophage recruitment is inhibited. If apoptotic cells undergo secondary necrosis and they are taken up, macrophages produce proinflammatory cytokines (TNFα, IL-8) to recruit more macrophages.*

Figure adapted from (Nagata et al. 2010)

It has been shown in macrophages that engulfment of apoptotic cells leads to production of anti-inflammatory cytokines and the further recruitment of macrophages to the dying cells is inhibited by TGFβ and PGE2. Recently it was shown that adenosine is a soluble mediator that mediates in paracrin and autocrin way the anti-inflammatory effect of apoptotic cells uptake by mouse macrophages (Koroskenyi *et al.* 2011). The apoptotic cells undergoing secondary necrosis or lysed necrotic cells can initiate proinflammatory response as a result of the cell content that is released (proteases, inflammatory eicosanoids, GM-CSF, macrophage inflammatory preotein-2 (MIP2), IL-8 and TNFα) (Fadok *et al.* 2001).

It also has to be mentioned that the expression of "don't eat-me" signals, even when PS is exposed, is a negation signal for the phagocytes. The best described molecule is CD47 and CD31 (Brown *et al.* 2002; Gardai *et al.* 2005) but the exact mechanism of action is not well understood.

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, atherosclerosis, respiratory diseases, neurological diseases as well as tumorigenesis (all reviewed in (Elliott *et al.* 2010; Nagata *et al.* 2010). Molecules involved in different diseased linked to deficiencies in apoptotic cell clearance are shown in Table 1.

Table 1. Association of diseases with defects of engulfment-related genes

Gene	Disease relationship	References	Human/ mouse
Find me			
G2A	AI	(Lee *et al.* 2001)	M
CX3CR1	Neuropathy	(Cardona *et al.* 2006)	M
CX3CL1	Atherosclerosis	(Combadiere *et al.* 2003)	M
Eat me/tickling			
MER	AI, cancer, neuropathy, atherosclerosis	(Gal *et al.* 2000; Scott *et al.* 2001; Cohen *et al.* 2002; Keating *et al.* 2006; Nandrot *et al.* 2007; Ait-Oufella *et al.* 2008; Thorp *et al.* 2008)	H/M
MFGE8	AI, neuropathy, atherosclerosis	(Hanayama *et al.* 2004; Ait-Oufella *et al.* 2007; Nandrot *et al.* 2007; Toth *et al.* 2009)	M
C1q	AI, neuropathy, atherosclerosis	(Botto *et al.* 1998; Fonseca *et al.* 2004; Bhatia *et al.* 2007)	M
$\alpha_v\beta_{3/5}$	AI, atherosclerosis	(Weng *et al.* 2003; Lacy-Hulbert *et al.* 2007)	M
TIM4	AI	(Rodriguez-Manzanet *et al.* 2010)	M
TGM2	AI, atherosclerosis	(Szondy *et al.* 2003; Boisvert *et al.* 2006)	M
Gas6	Atherosclerosis	(Lutgens *et al.* 2008)	M
Engulfment			
ELMO1	Diabetic nephropathy*	(Shimazaki *et al.* 2005; Leak *et al.* 2009; Pezzolesi *et al.* 2009)	H
GULP1	Arthritis*, schizophrenia*	(Qingchun *et al.* 2008; Chen *et al.* 2009)	H
MEGF10	schizophrenia*	(Chen *et al.* 2009)	H
Post-engulfment			
LXRα/β	AI	(N *et al.* 2009)	M
PPARδ	AI	(Mukundan *et al.* 2009)	M
DNase II	AI	(Kawane *et al.* 2003)	M

*Genes are grouped by their known role in engulfment. AI: autoimmune phenotype, H: human, M: mouse. *there is evidence of genetic linkage but no direct causal relationship was established.*

Table adapted from (Elliott et al. 2010)

2.3. Glucocorticoids

GCs are small lipophilic steroid hormones exerting their effect by binding to the intracellular glucocorticoid receptor (GR), 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 (Pratt *et al.* 2003). After ligand binding GR translocates to the nucleus, and directly or indirectly regulates the transcription of target genes (Ashwell *et al.* 2000). The transcriptional regulation by GR can happen in a DNA-binding independent way as well by tethering of the GR monomer to proinflammatory transcription factors (De Bosscher *et al.* 2009) resulting inhibition of proinflammatory genes and possibly resolution of inflammation by GCs (Karin 1998).

Endogenous GC is synthesized as cortisol in the adrenal cortex in response to excitation of the hypothalamus-pituitary-adrenal (HPA) 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 aminoacids and fatty acids), endogenous GCs also exert a wide range of immunomodulatory activities (Newton 2000).

Glucocorticoids (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 (Hoffman 1993; Wilckens *et al.* 1997; Ito *et al.* 2006) 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 (Almawi *et al.* 1996). In T cells GR affects the immune functions through a variety of mechanisms: GCs can induce T cell apoptosis (Herold *et al.* 2006), can inhibit the transcription of many T cell derived cytokines (Ramirez, Fowell et al. 1996) and enhances T_{reg} subpopulations *in vitro* and in mice (Chen *et al.* 2004; Kang *et al.* 2008).

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 (Giles *et al.* 2001; Ehrchen *et al.* 2007; Barczyk *et al.* 2010). In case of polymorphonuclear leukocytes (PMN, which are the major infiltrating cell type in acute

inflammation, GCs suppress their adhesion by reducing the L-selectin expression and the interactions with integrins and endothelial counterparts (Cronstein *et al.* 1992; Pitzalis *et al.* 2002). An effect of GCs that is utilized in the treatment of neutropenia is that they increase the number of bone marrow-derived neutrophils in combination with G-CSF (Stroncek *et al.* 2001). GCs are also used in B cell dependent pathologies. Reduction of splenic and lymph node B cell number, attenuation of B cell progenitor proliferation, suppressed IgG and enhanced IgE production was observed (Cupps *et al.* 1984; Cupps *et al.* 1985).

In dendritic cells GCs were reported to induce a tolerogenic phenotype and suppress their activation (Rutella *et al.* 2006; Chamorro *et al.* 2009). *In vivo* it was shown that their migration towards the lymph nodes is inhibited (Tuckermann *et al.* 2007). The differentiation and maturation of dendritic cells was also influenced and the mannose receptor-mediated endocytosis was up-regulated by GCs (Matasic *et al.* 1999; Piemonti *et al.* 1999; Piemonti *et al.* 1999). 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 (Majai *et al.* 2010) 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 Dex can suppress the proinflammatory effect of apoptotic neutrophils.

Although most studies 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 (Wilckens *et al.* 1997) on inflammatory and immune response. Results of DNA microarray performed on human peripheral blood mononuclear cells (PBMC) from healthy donors treated with dexamethasone showed up-regulation of proinflammatory mediators, such as complement, or cytokine and chemokine receptors (ex. IL-1RI, IL-8R, INFγI/II, TNFR family members, CCR1/2, C1q, C3, C5 etc) (Galon *et al.* 2002).

2.4. Tissue transglutaminase (TGM2)

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 (Fesus *et al.* 2002).

TGM2 has four distinct domains: the N-terminal β-sandwich domain with integrin and fibronectin binding sites, the catalytic core (it contains the catalytic triad: Cys277, His335 and Asp 358), the transition state stabilizing residue (Trp214) and the two C-terminal β-barrel domains. The enzyme has been shown to exist in at least two profoundly disparate conformations, respectively associated with its two best characterized enzymatic activities (Pinkas *et al.* 2007). Ca^{2+}-bound catalytically active transglutaminase assumes an extended, 'open' conformation in which the four domains are aligned, while in the GTP/GDP-bound conformer the active site is 'closed' and the β1-β2 domains fold over the core domain.

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 (Zemskov *et al.* 2011). Outside the cells either it stays on the cell surface or is deposited in the extracellular matrix, where 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 (Lorand *et al.* 2003).

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 (Nakaoka *et al.* 1994), has protein disulphide isomerase (Hasegawa *et al.* 2003) and protein kinase activity (Mishra *et al.* 2004), binds integrins and fibronectin on the cell surface. On the surface of mouse macrophages it interacts with both integrin β_3 and its phagocytosis ligand MFGE8 (Toth *et al.* 2009). In fibroblasts its externalization depends on syndecan-4 (Scarpellini *et al.* 2009) and its surface localization requires an intact fibronectin binding motif (Akimov *et al.* 2000). 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 (Telci *et al.* 2008). Recently it was

shown that TGM2 is internalized from the cell surface and undergoes lysosomal degradation (Zemskov *et al.* 2007; Belkin 2011).

Many studies report about the involvement of TGM2 in different biological functions such as cytoskeletal rearrangements, cell death, signaling, stabilization of extracellular matrix and phagocytosis (Lorand *et al.* 2003; Toth *et al.* 2009). Disregulations of the enzyme function can lead to different inflammatory diseases including diabetes, neurodegenerative diseases, sclerosis, rheumatoid arthritis and celiac disease (Facchiano *et al.* 2006).

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 CD (Green *et al.* 2003; Dewar *et al.* 2004). 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 (Louka *et al.* 2003). 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 (Molberg *et al.* 1998; van de Wal *et al.* 1998) 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 (Zanoni *et al.* 2006; Hovhannisyan *et al.* 2008; Lindfors *et al.* 2009). 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.

2.5. 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

Materials

Sterile plastics were purchased from TPP (TPP-US, St. Louis, MO, USA), Nunclon (Thermo Fisher Scientific, Roskilde, Denmark) and Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). PBS, BSA, PFA, FBS, LPS, DMSO, May-Grünwald/Giemsa, glutamine, penicillin-streptomycin, Tris–HCl, Triton X-100, $CaCl_2$, EDTA, HEPES, 2-MEA and proteinase inhibitors, RPMI-1640 medium, human AB serum, N-N'dimethyl-casein and LPS were purchased all from Sigma-Aldrich.

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 (Amersham Biosciences, Uppsala, Sweden), $CD14^+$ cells were separated by magnetic sorting with MACS (Miltenyi Biotec, Bergisch Gladbach, Germany, catalog number (Cat. #): 120-000-305), 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 2×10^6 cells/ml and cultured for 2 or 5 days in AIM-V medium (Invitrogen, Carlsbad, CA) containing 800 U/ml GM-CSF (Cat. #: 04-RHUGM-CSF) and 500 U/ml IL-4 (Cat. #: 200-04) (both from Peprotech EC, London, UK). Medium was supplemented with IL-4 and GM-CSF at day 0 and day 3. For glucocorticoid treated samples dexamethasone (Sigma-Aldrich, Cat. #: D1181) 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 (Peprotech, Cat. #: 300-02) 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 (Sigma-Aldrich) and cells were cultured for 16h in IMDM (Invitrogen) supplemented with 10% human AB serum while they underwent spontaneous apoptosis. Autologous lymphocytes (the left-over after magnetic separation of monocytes) 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

Dendritic cells were stained with CellTracker™ Orange CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine) (Cat. #: 2005113) and the freshly isolated neutrophils were labeled with the yellow-green fluorescent dye CFDA-SE (Cat. #: C1157). Both dies were purchased from Invitrogen 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% CO_2 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 (BD Biosciences, Immunocytometry Systems, San Jose, CA, USA). 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 (phagocyting DCs) 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, R&D Systems, Minneapolis, MN USA, Cat. #: MAB8911) or anti CD14 (Abcam, Cambridge, UK, Cat. #: ab6623) antibodies for 15 min at 37°C and 5% CO_2 atmosphere. The antibodies were present during the phagocytosis assay as well. For testing the effect of ADORA3 antagonist on phagocytosis DCs were treated with the selective adenosine A3 receptor antagonist MRS1220 (Tocris Bioscience, Missouri, USA, Cat. #: 1217) for a period of 1h, 37°C and 5% CO_2 atmosphere and the antagonist was present throughout the DC-apoptotic neutrophils co-incubation period.

Cell surface labeling

Prior to each labeling, dendritic cells were incubated in 50% human AB serum for 30 min at 37ºC, 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 (Cat. #: 551265), anti-CD14 (Cat. #: 555398), anti-CD1a (Cat. #: 555807) and anti-CD40 (Cat. #: 555589) antibodies respectively their isotype control antibodies IgG2b (Cat. #: 555743), IgG2a (Cat. #: 559319) and IgG1 (all from BD Pharmingen, San Diego, CA, USA, Cat. #: 555748) 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 (clone 125518, R&D Systems, Cat. #: MAB8912) and anti-CD14 (Abcam, Cat. #: ab6623), respectively IgG1 (isotype control from Sigma-Aldrich, Cat. #: M5284) antibodies followed by FITC-conjugated anti-mouse antibody (Sigma-Aldrich, Cat. #: F8264) which was used at a ratio of 1:50. For CD14 direct labeling with PE-CD14 and PE-IgG2a (both from BD Pharmingen) antibodies were used at a ratio of 1:50.

For surface TGM2 labeling monoclonal anti-TGM2 antibodies were used TG100 (Cat. #: MS-279-P1) and CUB7402 (Cat. #: MS-224-P) (purchased from NeoMarkers, Fremont, CA, USA), 4G3 supernatant (4G3 hybridoma cells were a kind gift of Alexey M. Belkin), H23, G92 (monoclonal antibodies that were kind gift of László Lóránd), pab0062 (Covalab, Lyon, France) respectively mouse IgG1 isotype control antibody (Sigma-Aldrich) at a concentration of 1μg/ 10^6 cells. The secondary antibody was FITC-conjugated anti-mouse antibody used at a ratio of 1:50 (Sigma-Aldrich, Cat. #: F8264).

In each case the labeling was performed on ice for a period of 30 min and 10^6 cells/sample was 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 50mM Tris–HCl containing 0.1% Triton X-100, 1mM 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 (LB), 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 (Sigma-Aldrich) for 1h at room temperature. For loading control mouse monoclonal antibodies 6C5 to GAPDH (purchased from Abcam) or β-actin (Sigma Aldrich) were used overnight at 4ºC. Immunoblots were developed with Immobilon Western chemiluminescent substrate (Millipore, Billerica, MA USA).

Densitometry analysis of Western blots was performed using ImageJ 4.1 software (National Institutes of Health, USA).

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 2×10^6 cells/well. Except for controls, cells were incubated with 25µg/ml 5-BP (Invitrogen), casein (2 mg/ml) and either $CaCl_2$ (2mM) or EDTA (4 mM). Incubation with 1 µg anti-TGM2 antibody (TG100 and CUB7402) was for 30 min at 37 º C before we added 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 (GE-Healthcare, Amersham, UK), 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 (controls and dexamethasone treated) 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 ºC. The concentrations of released TNFα were measured by the human TNFα duo set ELISA kit (R&D Systems, Minneapolis, MN USA, Cat. #: DY210) 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% CO_2 atmosphere. Cells were collected on day 5 and subjected to anti-human IFNγ Ready-Set-Go ELISPOT assay (eBioscience, San Diego, CA, USA, Cat. #: 88-7386) on MultiScreen-HTS PVDF plates (Millipore S.A., Molsheim, France). 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 (BD PharMingen, San Diego, CA, USA). The reaction was stopped by washing with tap water and the air-dried plates were analyzed by a computer-assisted ELISPOT image analyzer (Series 1 ImmunoSpot Analyzer,

Immuno-Spot Version 4.0 Software Academic, Cellular Technology). Secreted IFNγ was measured by the OptiEIA system (BD PharMingen).

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 (Invitrogen). To determine the expression level of genes listed in Table 2, a 384- well TaqMan Low Density Array (TLDA), custom made for the Apopto-phagocytosis research group in our Department (Applied Biosystems, Foster City, CA, USA) 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 $2^{-\Delta\Delta Ct}$. Fold changes (FC) 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".

Table 2: Gene list of the apopto-phagocyte panel

Gene symbol (HUGO)	Alias	Gene description	HGNC code	Location
ABCA1	*ABCA1*	ATP-binding cassette, sub-family A (ABC1), member 1	HGNC:29	9q31
ADORA1	*ADORA1*	adenosine A1 receptor	HGNC:262	1q32.1
ADORA2A	*ADORA2A*	adenosine A2a receptor	HGNC:263	22q11.23
ADORA3	*ADORA3*	adenosine A3 receptor	HGNC:268	1p21-p13
ALOX12	*ALOX12*	arachidonate 12-lipoxygenase	HGNC:429	17p13.1
ALOX5	*ALOX5*	arachidonate 5-lipoxygenase	HGNC:435	10q11.2
ANXA1	*ANXA1*	annexin A1	HGNC:533	9q21.13
ANXA5	*ANXA5*	annexin A5	HGNC:543	4q27
ATG12	*APG12L*	ATG12 autophagy related 12 homolog (S. cerevisiae)	HGNC:588	5q21-q22
ATG16L1	*APG16L*	ATG16 autophagy related 16-like 1 (S. cerevisiae)	HGNC:21498	2q37.1
ATG5	*APG5L*	ATG5 autophagy related 5 homolog (S. cerevisiae)	HGNC:589	6q21
APOH	*APOH*	apolipoprotein H (beta-2-glycoprotein I)	HGNC:616	17q23-qter
PYCARD	*ASC*	PYD and CARD domain containing	HGNC:16608	16p11.2
ASGR1	*ASGR1*	asialoglycoprotein receptor 1	HGNC:742	17p13-p11
AXL	*AXL*	AXL receptor tyrosine kinase	HGNC:905	19q13.1
BCAR1	*BCAR1*	breast cancer anti-estrogen resistance 1	HGNC:971	16q22-q23
BECN1	*BECN1*	beclin 1, autophagy related	HGNC:1034	17q21

NAIP	*BIRC1*	NLR family, apoptosis inhibitory protein	HGNC:7634	5q13.2
C1QA	*C1QA*	complement component 1, q subcomponent, A chain	HGNC:1241	1p36.3-p34.1
CD93	*C1QR1*	CD93 molecule	HGNC:15855	20p11.21
C2	*C2*	complement component 2	HGNC:1248	6p21.3
C3	*C3*	complement component 3	HGNC:1318	19p13.3-p13.2
C4A	*C4A*	complement component 4A (Rodgers blood group)	HGNC:1323	6p21.3
CALR	*CALR*	calreticulin	HGNC:1455	19p13.3-p13.2
CAPN1	*CAPN1*	calpain 1, (mu/I) large subunit	HGNC:1476	11q13
CAPN2	*CAPN2*	calpain 2, (m/II) large subunit	HGNC:1479	1q41-q42
NOD2	*CARD15*	nucleotide-binding oligomerization domain containing 2	HGNC:5331	16q12
NOD1	*CARD4*	nucleotide-binding oligomerization domain containing 1	HGNC:16390	7p15-p14
CASP1	*CASP1*	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	HGNC:1499	11q23
CASP5	*CASP5*	caspase 5, apoptosis-related cysteine peptidase	HGNC:1506	11q22.2-q22.3
CD14	*CD14*	CD14 molecule	HGNC:1628	5q22-q32
CD47	*CD47*	CD47 molecule	HGNC:1682	3q13.1-q13.2
CD68	*CD68*	CD68 molecule	HGNC:1693	17p13
NLRP3	*CIAS1*	NLR family, pyrin domain containing 3	HGNC:16400	1q44
CRK	*CRK*	v-crk sarcoma virus CT10 oncogene homolog (avian)	HGNC:2362	17p13
CRP	*CRP*	C-reactive protein, pentraxin-related	HGNC:2367	1q21-q23
CXXC1	*CXXC1*	CXXC finger 1 (PHD domain)	HGNC:24343	18q12
DNASE1	*DNASE1*	deoxyribonuclease I	HGNC:2956	16p13.3
DNASE2	*DNASE2*	deoxyribonuclease II, lysosomal	HGNC:2960	19p13.2-q13.2
DOCK1	*DOCK1*	dedicator of cytokinesis 1	HGNC:2987	10q26.13-q26.3
EDIL3	*EDIL3*	EGF-like repeats and discoidin I-like domains 3	HGNC:3173	5q14
ELMO1	*ELMO1*	engulfment and cell motility 1	HGNC:16286	7p14.1
ELMO2	*ELMO2*	engulfment and cell motility 2	HGNC:17233	20q13
FCGR2B	*FCGR2B*	Fc fragment of IgG, low affinity IIb, receptor (CD32)	HGNC:3618	1q23
FPR2	*FPRL1*	formyl peptide receptor 2	HGNC:3827	19q13.3-q13.4
GAS6	*GAS6*	growth arrest-specific 6	HGNC:4168	13q34
GRLF1	*GRLF1*	glucocorticoid receptor DNA binding factor 1	HGNC:4591	19q13.32
GULP1	*GULP1*	GULP, engulfment adaptor PTB domain containing 1	HGNC:18649	2q32.3-q33
ICAM3	*ICAM3*	intercellular adhesion molecule 3	HGNC:5346	19p13.3-p13.2
IRF8	*ICSBP1*	interferon regulatory factor 8	HGNC:5358	16q24.1
IL10	*IL10*	interleukin 10	HGNC:5962	1q31-q32

IL12B	*IL12B*	interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)	HGNC:5970	5q31.1-q33.1
IL18	*IL18*	interleukin 18 (interferon-gamma-inducing factor)	HGNC:5986	11q22.2-q22.3
IL23A	*IL23A*	interleukin 23, alpha subunit p19	HGNC:15488	12q13.13
IL4R	*IL4R*	interleukin 4 receptor	HGNC:6015	16p12.1-p11.2
IL6	*IL6*	interleukin 6 (interferon, beta 2)	HGNC:6018	7p21-p15
PNPLA8	*IPLA2 (GAMMA)*	patatin-like phospholipase domain containing 8	HGNC:28900	7q31
IRF1	*IRF1*	interferon regulatory factor 1	HGNC:6116	5q23-q31
IRF4	*IRF4*	interferon regulatory factor 4	HGNC:6119	6p25-p23
IRF5	*IRF5*	interferon regulatory factor 5	HGNC:6120	7q32
IRF7	*IRF7*	interferon regulatory factor 7	HGNC:6122	11p15.5
ITGAM	*ITGAM*	integrin, alpha M (complement component 3 receptor 3 subunit)	HGNC:6149	16p11.2
ITGAV	*ITGAV*	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	HGNC:6150	2q31-q32
ITGAX	*ITGAX*	integrin, alpha X (complement component 3 receptor 4 subunit)	HGNC:6152	16p11.2
ITGB2	*ITGB2*	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	HGNC:6155	21q22.3
ITGB3	*ITGB3*	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	HGNC:6156	17q21.32
ITGB5	*ITGB5*	integrin, beta 5	HGNC:6160	3q21.2
LRP1	*LRP1*	low density lipoprotein receptor-related protein 1	HGNC:6692	12q13-q14
MAP1LC3A	*MAP1LC3A*	microtubule-associated protein 1 light chain 3 alpha	HGNC:6838	20q11.22
MERTK	*MERTK*	c-mer proto-oncogene tyrosine kinase	HGNC:7027	2q14.1
MFGE8	*MFGE8*	milk fat globule-EGF factor 8 protein	HGNC:7036	15q25
MSR1	*MSR1*	macrophage scavenger receptor 1	HGNC:7376	8p22
NLRP12	*NALP12*	NLR family, pyrin domain containing 12	HGNC:22938	19q13.42
NFKB1	*NFKB1*	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	HGNC:7794	4q24
OLR1	*OLR1*	oxidized low density lipoprotein (lectin-like) receptor 1	HGNC:8133	12p13.1-p12.3
PECAM1	*PECAM1*	platelet/endothelial cell adhesion molecule	HGNC:8823	17q23.3
PPARG	*PPARG*	peroxisome proliferator-activated receptor gamma	HGNC:9236	3p25
PROS1	*PROS1*	protein S (alpha)	HGNC:9456	3p11-q11.2
PTAFR	*PTAFR*	platelet-activating factor receptor	HGNC:9582	1p35-p34.3
JMJD6	*PTDSR*	jumonji domain containing 6	HGNC:19355	17q25
PTGER2	*PTGER2*	prostaglandin E receptor 2 (subtype EP2), 53kDa	HGNC:9594	14q22
PTK2	*PTK2*	PTK2 protein tyrosine kinase 2	HGNC:9611	8q24.3
SIRPA	*PTPNS1*	signal-regulatory protein alpha	HGNC:9662	20p13
PTX3	*PTX3*	pentraxin 3, long	HGNC:9692	3q25
RAC1	*RAC1*	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	HGNC:9801	7p22
RAP1A	*RAP1A*	RAP1A, member of RAS oncogene family	HGNC:9855	1p13.3
RHOG	*RHOG*	ras homolog gene family, member G (rho G)	HGNC:672	11p15.5-

				p15.4
SCARB1	*SCARB1*	scavenger receptor class B, member 1	HGNC:1664	12q24.32
TGFB1	*TGFB1*	transforming growth factor, beta 1	HGNC:11766	19q13.1
TGFBR1	*TGFBR1*	transforming growth factor, beta receptor 1	HGNC:11772	9q22
TGM2	*TGM2*	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	HGNC:11778	20q12
THBS1	*THBS1*	thrombospondin 1	HGNC:11785	15q15
TNF	*TNF*	tumor necrosis factor	HGNC:11892	6p21.3
TRIO	*TRIO*	triple functional domain (PTPRF interacting)	HGNC:12303	5p14-p15.1
TYRO3	*TYRO3*	TYRO3 protein tyrosine kinase	HGNC:12446	15q15.1-q21.1

**** Gene Symbol defined by HUGO Gene Nomenclature Committee***
(HGNC, http://www.genenames.org)

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 (provided by Merck & Co, NJ, USA) 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 cell were washed in saline, then resuspended and cultured the cells in 25 mM HEPES modified RPMI-1640 medium (Sigma-Aldrich) 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, Cat. #: 315-03) and mouse IL-4 (20 ng/ml, Cat. #: 214-14) for a period of 9 days. Both cytokines were purchased from PeproTech. Every third day half of the old medium were changed to fresh medium containing cytokines. Cells were treated with different concentrations of 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 (Sigma-Aldrich). To generate apoptotic neutrophils the cells were cultured for 24h in IMDM supplemented with 10% mouse serum and stained with CFDA-SE (Invitrogen). The purity of the neutrophils was controlled by May-Grünwald/Giemsa staining. On the 9^{th} 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

For studying phagocytosis of apoptotic cells we used freshly isolated neutrophils that undergo spontaneous apoptosis in 16-24h, an *in vitro* phagocytic system that mimics inflammation sites *in vivo*. 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 (Majai *et al.* 2007). It was reported that *in vitro* differentiated monocyte-derived dendritic cells can engulf apoptotic cells but they are less effective than macrophages (Majai *et al.* 2010). 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, leads to increased phagocytosis (Figure 5).

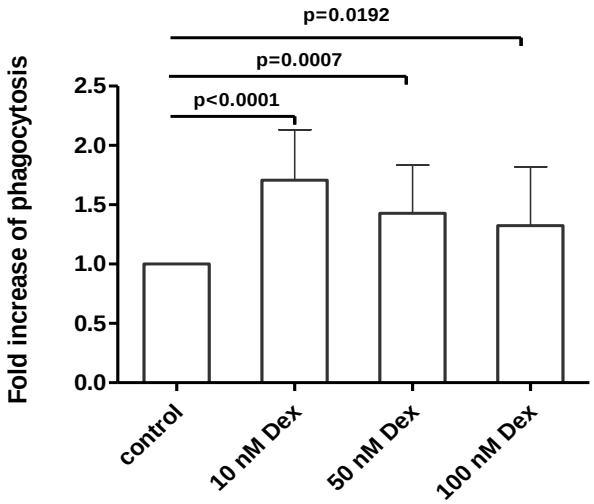


Figure 5. Increased phagocytosis of apoptotic neutrophils by dexamethasone treated human immature dendritic cells. *Human monocytes were differentiated in the absence (control) and presence of different concentrations of dexamethasone (Dex) for 5 days, then phagocytosis assay was performed (n=16) as described in Materials and Methods. The percentage of phagocytic DCs was determined by flow-cytometry. Elevated phagocytic capacity of glucocorticoid treated cells is presented as fold increase compared to the values of the control iDCs which were taken as 1 and statistical analysis was performed. (Error bars indicate SD).*

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. The individual values of phagocytosis are shown in Table 3.

Table 3. Individual phagocytosis values of control and dexamethasone treated human immature dendritic cells.

	% phagocytosis +/- SD			
	control	**10 nM Dex**	**50 nM Dex**	**100 nM Dex**
Donor 1	9.72 +/- 0.59	14.22 +/- 0.17	13.70 +/- 0.6	11.26 +/- 0.35
Donor 2	22.45 +/- 1.20	38.7 +/- 0.57	27.85 +/- 1.06	22.45 +/- 1.63
Donor 3	22.45 +/- 4.20	35.49 +/- 1.38	23.87 +/- 4.26	20.02 +/- 1.29
Donor 4	16.13 +/- 5.1	15.76 +/- 0.25	22.45 +/- 5.03	19.57 +/- 5.83
Donor 5	14.97 +/- 2.87	18.24 +/- 1.57	19.26 +/- 0.95	14.85 +/- 0.96
Donor 6	11.1 +/- 2.18	18.03 +/- 1.63	24.5 +/- 1.16	29.79 +/- 2.72
Donor 7	16.84 +/- 0.11	38.57 +/- 2.52	25.72 +/- 2.58	23.72 +/- 1.59
Donor 8	18.93 +/- 0.26	32.07 +/- 1.36	24.02 +/- 0.03	23.74 +/- 4.16
Donor 9	21.62 +/- 2.15	31.21 +/- 1.67	22.51 +/- 2.45	23.99 +/- 2.27
Donor 10	11.27 +/- 0.33	17.69 +/- 0.06	13.60 +/- 0.95	12.39 +/- 0.99
Donor 11	14.18 +/- 0.13	22.58 +/- 1.05	15.14 +/- 0.02	14.93 +/- 0.43
Donor 12	11.27 +/- 0.33	17.69 +/- 0.06	13.60 +/- 0.95	12.39 +/- 0.99
Donor 13	14.18 +/- 0.13	22.58 +/- 1.05	15.14 +/- 0.02	14.93 +/- 0.43
Donor 14	13.44 +/- 1.53	28.54 +/- 1.03	23.32 +/- 0.07	16.96 +/- 0.45
Donor 15	12.72 +/- 0.44	34.28 +/- 2.67	22.07 +/- 0.55	20.23 +/- 1.44
Donor 16	13.45 +/- 0.09	29.16 +/- 0.72	32.28+/- 0.01	31.27 +/- 1.00

Human monocytes of 16 healthy donors were differentiated in the absence (control) and presence of different concentrations of dexamethasone (Dex) for 5 days then they were co-cultured with apoptotic neutrophils for 8h. The percentage of phagocytic DCs was determined by flow-cytometry. Each experiment was performed in duplicates.

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

Immature dendritic cells, characterized by the presence of CD209 (DC-SIGN) on the cell surface, were differentiated from CD14$^+$ cells (monocytes) for a period of 5 days. 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 (at day 2) as well. While the surface expression of CD14 should decrease with differentiation, treatment with glucocorticoid prevented this decline (Figure 6b). We also observed that the CD1a$^+$ population of DCs varied among the donors, but dexamethasone treatment decreased the DC1a positivity of the cells, even when applied in the lowest concentration and from the early time point of differentiation (Figure 6a). Control experiment revealed that surface expression of DC-SIGN, expressed by both CD1a$^-$ and CD1a$^+$ cells did not change by the glucocorticoid treatment (Fig. 6c).

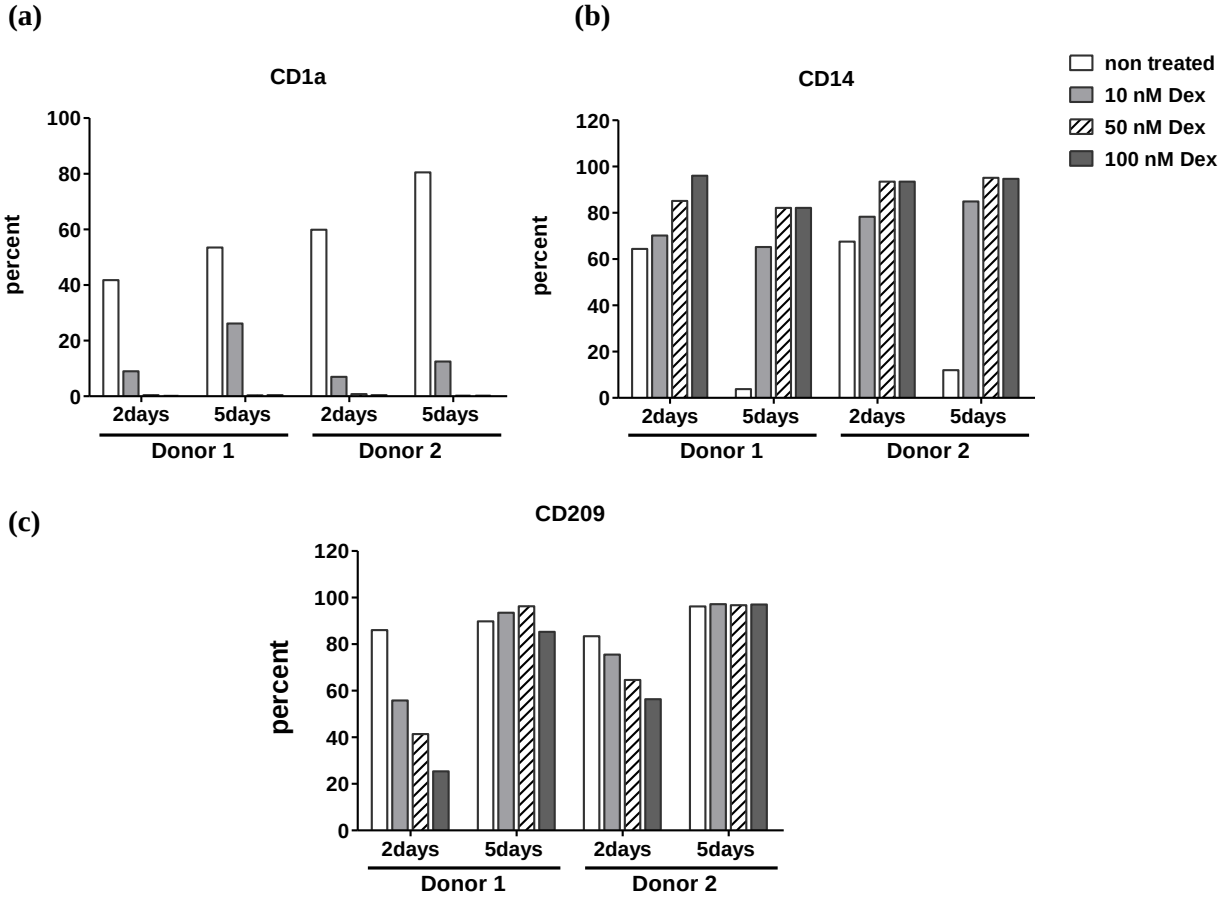


Figure 6. Surface labeling of dendritic cells for CD1a, CD14 and CD209. *Control and Dex treated dendritic cells were stained with anti- anti-CD1a (a), CD14 (b) and anti-CD209 (c) antibodies after 2 days and after 5 days of differentiation. The staining was detected by flow-cytometry and the evaluation was performed compared to the isotype controls.*

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 listed in Table 2.

After evaluating the gene expression pattern of control and Dex treated DCs we focused on up-regulated genes as it follows. The apopto-phagocytic genes down-regulated during the differentiation and by the glucocorticoid treatment will be discussed later in the paragraph 4.8.

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. Table 4 lists the up-regulated genes either during differentiation or by Dex treatment, and their fold change (FC) values. Comparing iDCs to monocytes 17 genes were up-regulated during the 5 days of differentiation (FC1 > 2, Diff.). Among these genes there are 6 which show more than 10 times increase in their relative gene expression (FC1 > 10) compared to the monocytes (Mo): DOCK1, FCGR2B, GAS6, IRF4, PROS1 and PPARG. However, except FCGR2B, these are not the ones which are further and most up-regulated by Dex (showed in bold and italics in Table 4), which will be discussed later on.

Comparing the average relative expressions of iDCs differentiated in the presence of Dex versus non treated cells (Diff. and Dex), 10 apopto-phagocytic genes were up-regulated more than two times (FC2 > 2) listed in Table 4. Among these genes there were 4 (bold and italics) which were already up-regulated during the 5 days of differentiation so they were further increased by the glucocorticoid. Namely these four genes were: the cell surface molecule ADORA3 and FCGR2B, the bridging molecules C1QA and C2.

Table 4. List of apopto-phagocytic genes up-regulated in dendritic cells either during differentiation or by dexamethasone treatment (n= 3)

HUGO gene symbol*	Gene description	FC1 (Diff.)	FC2 (Diff. and Dex)
ADORA1	adenosine A1 receptor	5.889	0.103
ADORA3	***adenosine A3 receptor***	***5.340***	***6.985***
C1QA	***complement component 1, q subcomponent, A chain***	***4.642***	***4.625***
C2	***complement component 2***	***2.250***	***3.053***
CD14	CD14 molecule	0.005	7.474
DNASE2	deoxyribonuclease II, lysosomal	0.106	4.540
DOCK1	dedicator of cytokinesis 1	13.403	0.439
FCGR2B	***Fc fragment of IgG, low affinity IIb, receptor (CD32)***	***17.886***	***2.705***
GAS6	growth arrest-specific 6	16.290	1.263
IL10	interleukin 10	0.323	3.143
IRF4	interferon regulatory factor 4	32.347	0.170
ITGAM	integrin, alpha M (complement component 3 receptor 3 subunit)	7.181	0.192
ITGB5	integrin, beta 5	7.890	0.603
MERTK	c-mer proto-oncogene tyrosine kinase	0.023	15.197
MFGE8	milk fat globule-EGF factor 8 protein	3.971	0.315
MSR1	macrophage scavenger receptor 1	3.890	0.280
NLRP12	NLR family, pyrin domain containing 12	0.003	2.446
OLR1	oxidized low density lipoprotein (lectin-like) receptor 1	4.133	0.053
PPARG	peroxisome proliferator-activated receptor gamma	41.123	0.293
PROS1	protein S (alpha)	142.906	0.815
SCARB1	scavenger receptor class B, member 1	0.997	2.288
TGFBR1	transforming growth factor, beta receptor 1	3.973	0.618
TGM2	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	5.771	0.112

*** Gene Symbol defined by HUGO Gene Nomenclature Committee (HGNC**, http://www.genenames.org**)**

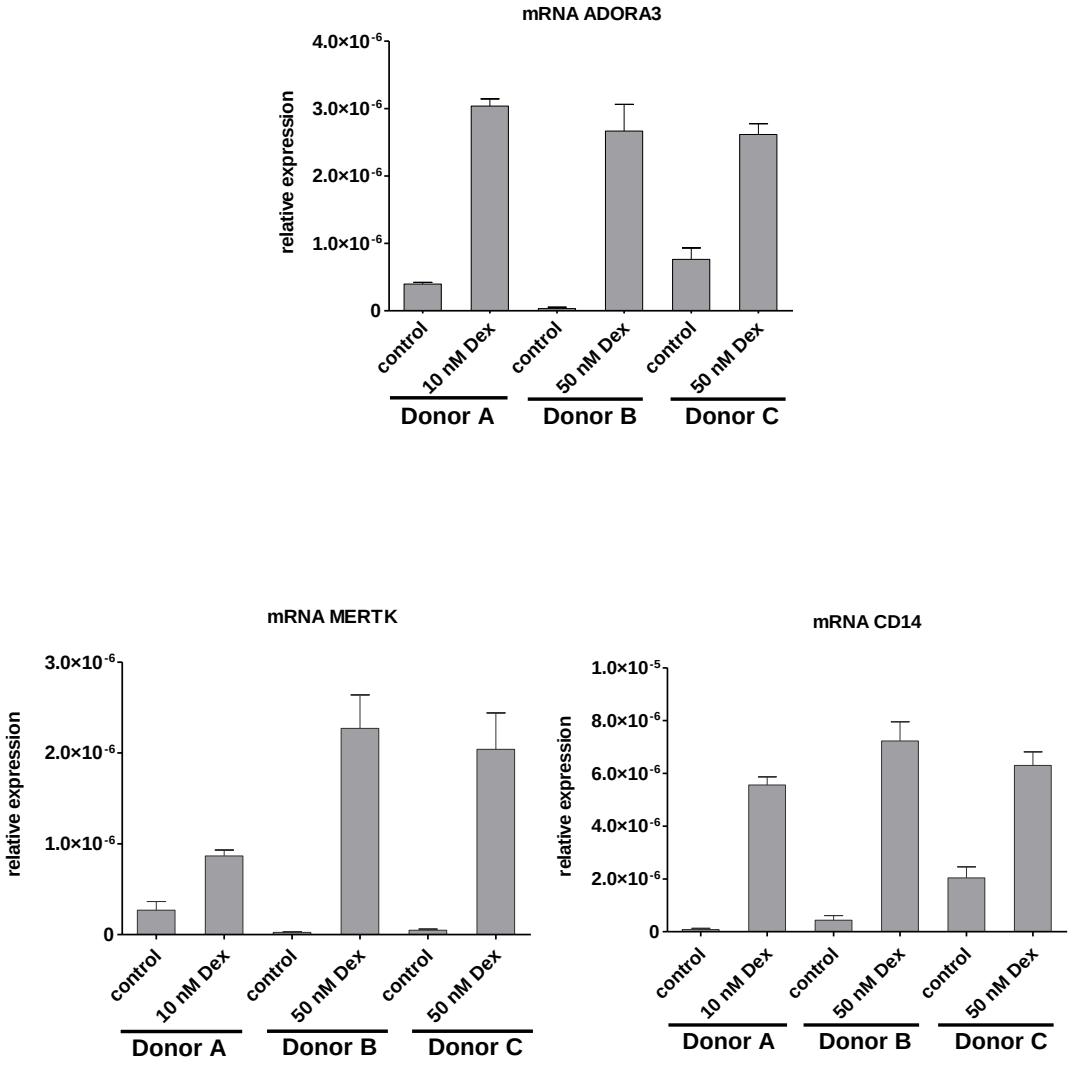
Fold change 1 (FC1): average relative expression iDCs / average relative expression Mo

Fold change 2 (FC2): average relative expression Dex treated iDCs / average relative expression non treated iDCs

Monocytes of 3 donors were differentiated and treated with Dex for 5 days. Controls and treated samples were used to analyze changes of gene expression of 95 preselected apopto-phagocytic genes by TLDA technique. Genes were normalized to the level of 18S rRNA and fold changes were calculated. Genes which were further induced by Dex are shown in bold and italics.

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 shown in Figure 7: 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.



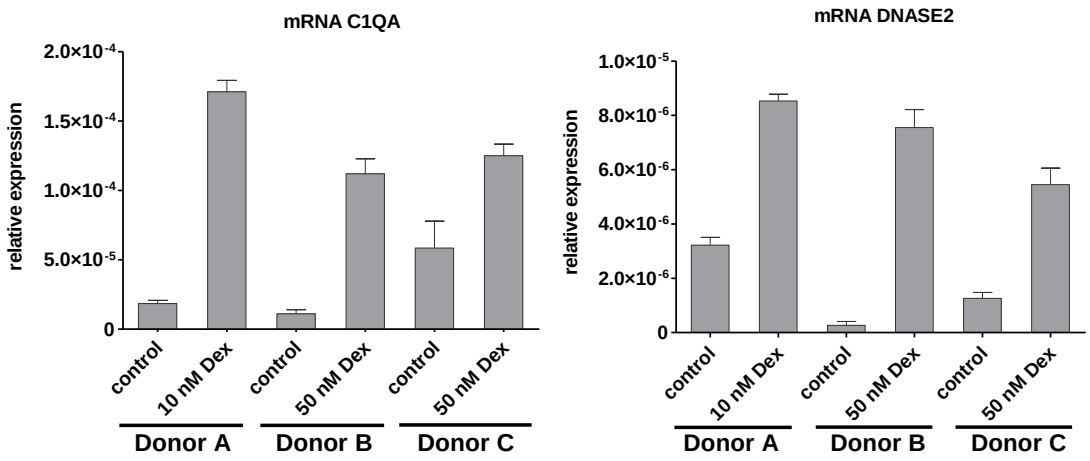


Figure 7. Relative expression of genes up-regulated in all donors by dexamethasone in human immature dendritic cells. *Control and Dex treated dendritic cells of 3 donors differentiated for 5 days were used. Relative expression of genes highly up-regulated in all donors, with a FC>2 in each sample are shown. Error bars indicate SD.*

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. Phagocytosis data with the most effective Dex concentrations, which are not the same as in iDCs, are shown in Figure 8. The up-regulation of the same apopto-phagocytic genes by Dex, observed in iDCs (after 5 day of differentiation) could be detected at the early stage of differentiation as well (Figure 8).

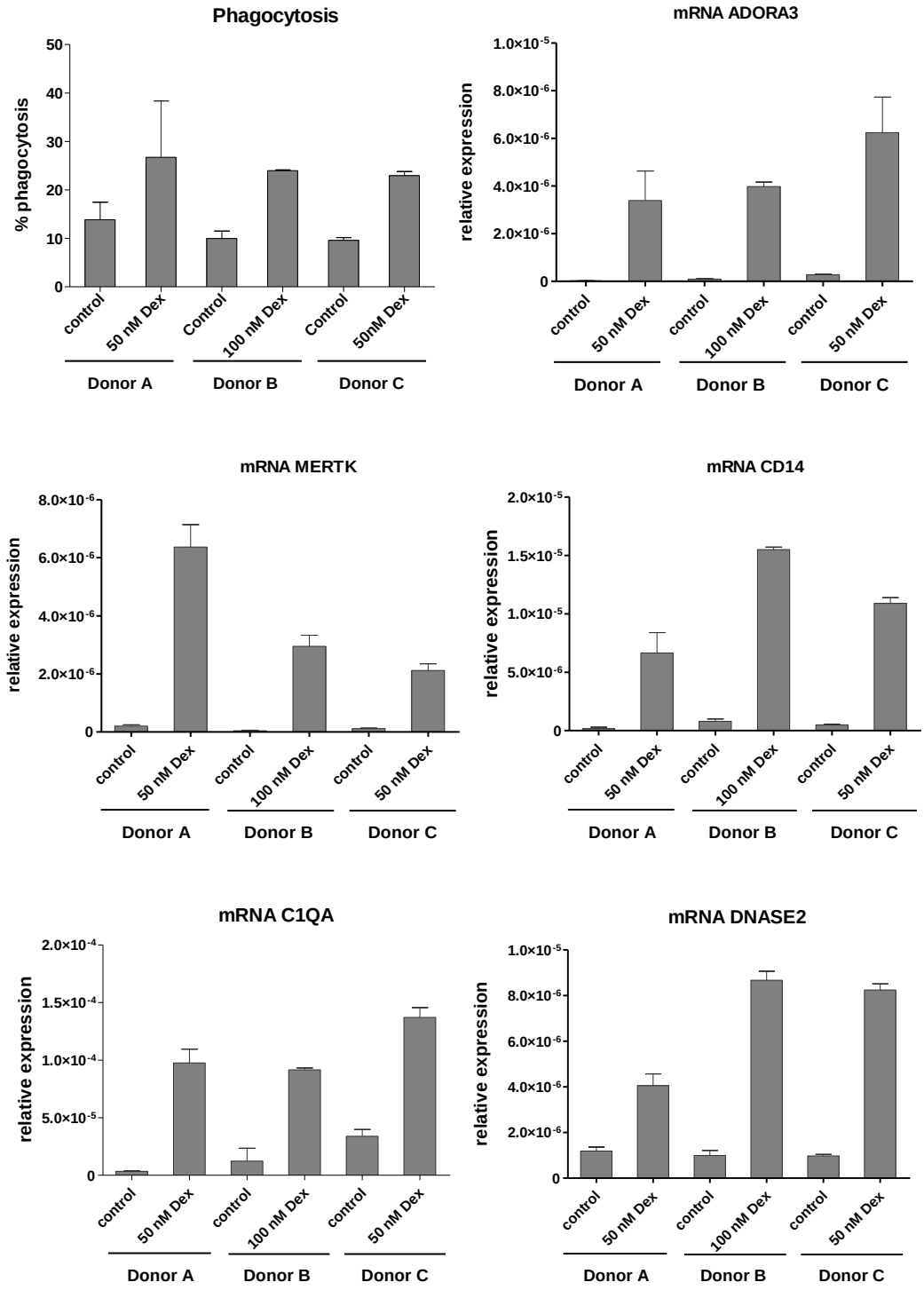


Figure 8. Relative expression of genes up-regulated in all donors by dexamethasone in human dendritic cells at early stage of differentiation. *Control and Dex treated dendritic cells of 3 donors differentiated for 2 days were used for TLDA analysis. Phagocytosis capacity of apoptotic neutrophils was also tested and data of the most effective Dex concentration are shown. The relative expression level of genes highly up-regulated in all donors, with a FC>2 in each sample are presented. Error bars indicate SD.*

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. One representative example of 3 donors is shown in Figure 9.

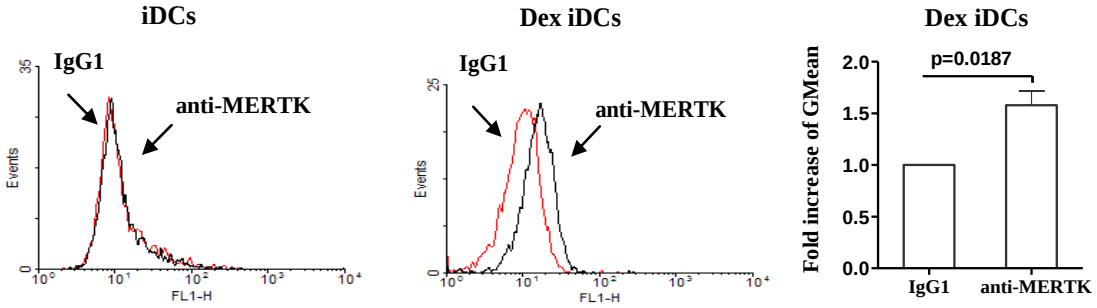


Figure 9. Cell surface labeling of dendritic cells with antibodies specific for MERTK. *Control and Dex treated (100nM) dendritic cells were labeled with anti-MERTK and control (IgG1) antibodies on the 5th day of differentiation. Samples were measured by flow-cytometry. One representative example of cell surface labeling and fold increase of GMean values of Dex treated iDCs compared to the isotype control (IgG1) are presented for 3 donors.*

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 (McColl *et al.* 2009; Zahuczky *et al.* 2011), 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 (Figure 10).

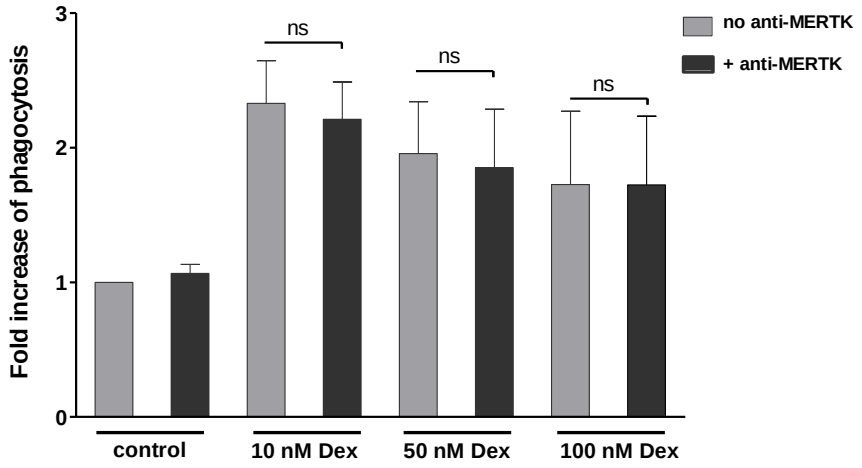


Figure 10. Effect of blocking antibody specific for MERTK on the phagocytosis of apoptotic neutrophils by human dendritic cells differentiated in the presence of Dex. *Dendritic cells on day 5 of differentiation were pre-incubated with 10 μg/ml anti-MERTK antibody for 15 min at 37°C and 5% CO_2 atmosphere, then loaded with labeled apoptotic neutrophils for 8h. After fixation samples were measured by flow-cytometry and fold increase of phagocytosis was calculated to the Dex and antibody non treated control, which was taken as 1. Bars represent the mean ± SD (n=3).*

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 up-regulated in iDCs upon glucocorticoid treatment. The increased cell surface expression the protein could be detected as well with both direct (Figure 11a), which was a control staining and indirect (Figure 11b) labeling.

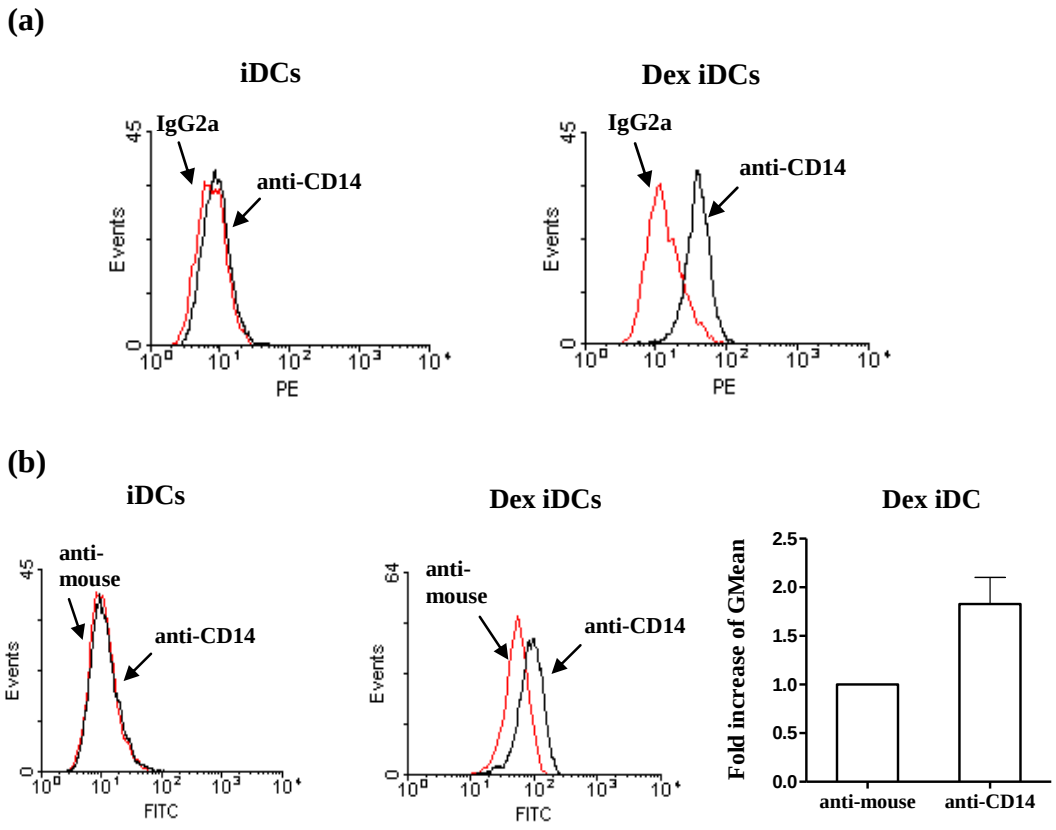


Figure 11. Cell surface labeling of dendritic cells with antibodies specific for CD14. *Control and Dex treated (50nM) dendritic cells were stained either directly (a) or indirectly (b) with anti-CD14 and control antibodies (IgG2a respectively anti-mouse antibody) on day 5. Samples were measured by flow-cytometry. One representative example of cell surface labeling and fold increase of GMean values of Dex treated iDCs compared to the anti-mouse control are shown (n=2).*

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 (Figure 12).

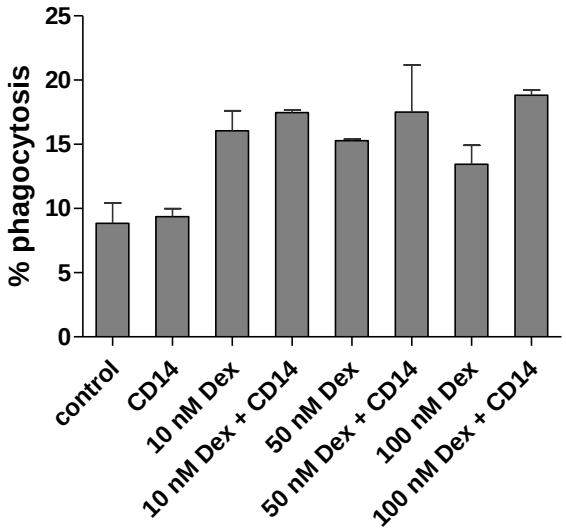


Figure 12. Effect of blocking anti-CD14 antibody specific on the phagocytosis of apoptotic neutrophils by human dendritic cells differentiated in the presence of Dex. *Dendritic cells on day 5 of differentiation were pre-incubated with 10 μg/ml anti-CD14 antibody for 15 min at 37°C and 5% CO_2 atmosphere, then loaded with labeled apoptotic neutrophils for 8h. After fixation samples were measured by flow-cytometry. Bars represent the mean ± SD.*

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, referred to as MRS (Kim *et al.* 1996). iDCs were pre-incubated with the antagonist and then loaded with apoptotic neutrophils. As seen in Figure 13. 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.

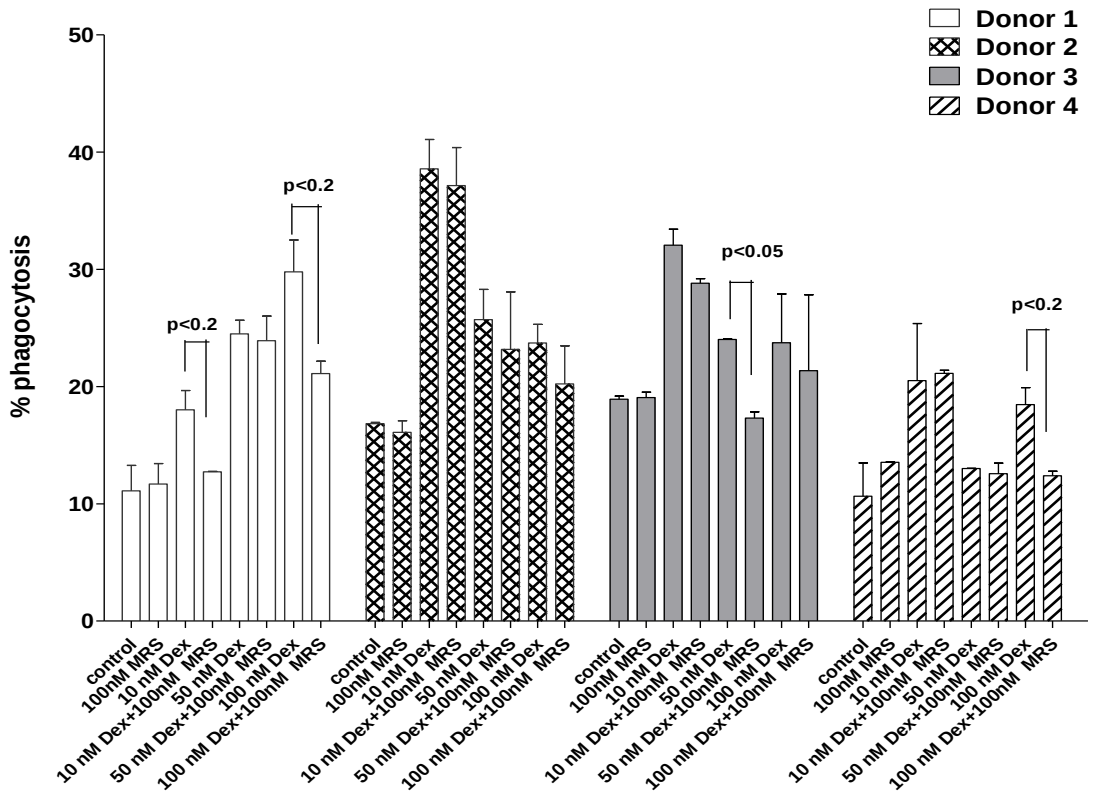


Figure 13. Effect of the highly selective ADORA3 antagonist (MRS) on the Dex induced increase of apoptotic neutrophils uptake in iDCs*. Monocytes of 4 donors were differentiated in the absence or presence of Dex and on day 5 the cells were harvested, pre-incubated with 100 nM human adenosine A3 receptor antagonist for 1h, 37ºC and phagocytosis assay was performed as described previously. Samples were fixed and analyzed by flow-cytometry. Bars represent the mean ± SD and the statistical analysis was performed.*

The involvement of ADORA3 in the Dex effect was tested in a murine system as well. Dendritic cells 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 (Figure 14). It was also noted that WT dendritic cells were somewhat less efficient in engulfing apoptotic neutrophils than the KO ones (data not shown).

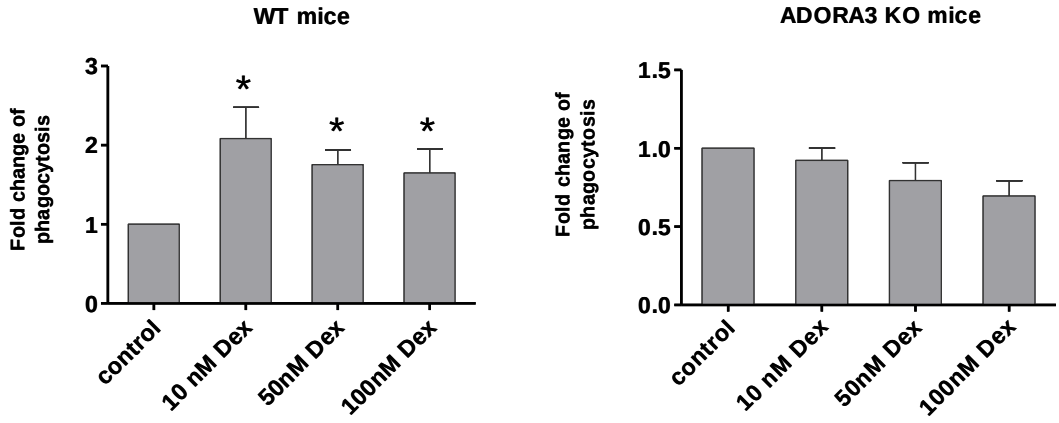


Figure 14. The effect of genetic ablation of ADORA3 on apoptotic neutrophils phagocytosis by DCs. *WT and ADORA3 KO mouse bone marrow progenitors were differentiated to dendritic cells as described previously. Dexamethasone was given at day 0 of differentiation. On day 9 the cells were incubated with murine apoptotic neutrophils then flow-cytometry was performed. Fold change in phagocytic activity was calculated relative to the control. Bar represent the mean ± SD of 4 independent experiments (*p<0.05).*

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

It was shown previously that human monocyte-derived DCs activated by LPS and IFNγ respond to allogeneic apoptotic neutrophils with inflammatory cytokine production and the engulfment of apoptotic neutrophils lead to the activation of T cells (Majai *et al.* 2010). 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 an additional 16h when the concentration of secreted TNFα in the culture supernatants was measured. 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 (Figure 15). The most effective Dex concentration leading to this response varied among individuals.

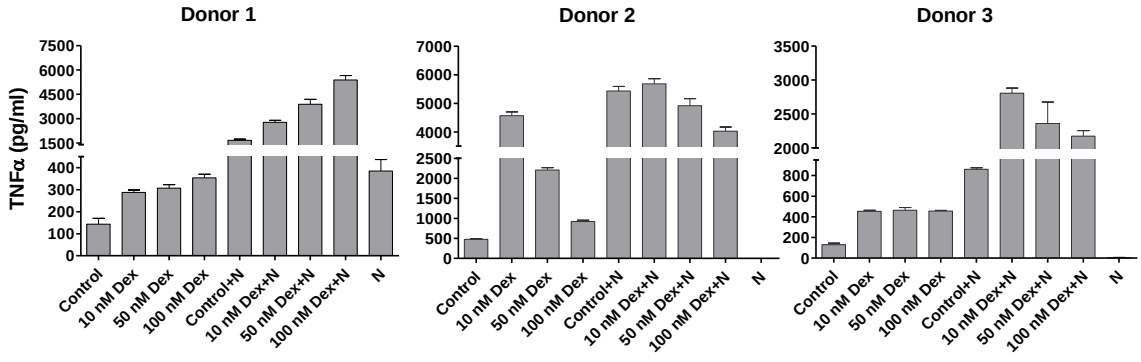


Figure 15. Dex treated iDCs release higher amount of TNFα in response to LPS and IFNγ after apoptotic cell engulfment. *Control and Dex treated iDCs of 3 donors were fed with apoptotic neutrophils (N) for 8h followed by LPS and IFNγ stimulation for additional 16h. The TNFα concentration was determined in the cell-free supernatants by ELISA. Bars represent the mean ± SD of pg/ml cytokine concentration. The concentration of basal TNFα secretion of non-stimulated, immature DCs was 18.88 +/- 15.33 pg/ml (range 3.33- 33.99 pg/ml).*

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 (Fig 16). 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.

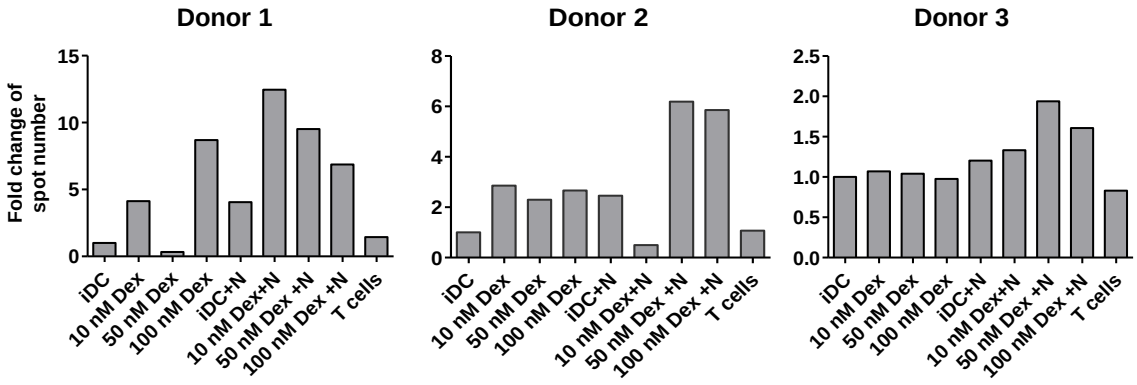


Figure 16. Effect of dexamethasone on apoptotic neutrophil-loaded dendritic cell-induced T cell activation. *Immature DC from the same donors were incubated with apoptotic neutrophils and co-cultured with autologous lymphocytes at a ratio 1:25 and for 5 days. The nonadherent lymphocytes were harvested and subjected to a 48h IFNγ ELISPOT assay. Data were analyzed by a computer-assisted ELISPOT image analyzer and fold changes of spot numbers were calculated compared to the iDC control.*

As differences in the internalizing capacity, cytokine and chemokine profile and T cell polarizing potential were shown between the $CD1a^+$ and $CD1a^-$ DCs (Gogolak *et al.* 2007), cells of the same donors were stained for CD1a. iDCs were CD1a positive in 40-60%, and the glucocorticoid treatment, similar to the previous results, skewed the DC differentiation to a CD1a negative subtype (Figure 17).

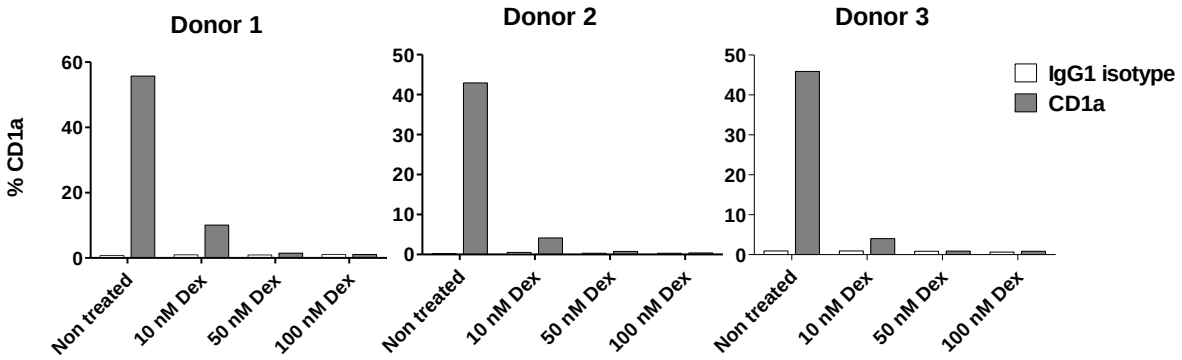


Figure 17. CD1a labeling of immature dendritic cells. *Control and Dex treated iDCs from the same donors were labeled with PE-conjugated CD1a and isotype control (IgG1) antibodies. Samples were analyzed by flow-cytometry.*

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

4.8.1. Genes down-regulated during the 5 days of differentiation

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 (FC < 0.5). Among these genes listed in Table 5, there are 8, seen in bold and italics, which are in common with the genes down-regulated by Dex. Namely these genes were: the surface molecule adenosine A2a receptor, the phagocytosis (tethering/tickling) receptors as AXL, ITGAX, ITGB, LRP1, the bridging molecules like complement component 3 (C3) and ICAM3 and the interferon regulatory factor 1 (IRF1). We also could see that in line with the cell surface labeling data showed in Figure 6, the expression of CD14 at mRNA level is decreased during the 5 days of differentiation to immature dendritic cells (FC = 0.005).

Table 5. Genes down-regulated during iDC differentiation (n= 3)

	HUGO gene symbol	Gene description	FC
Differentiation	***ADORA2A***	***adenosine A2a receptor***	***0.015***
	ALOX5	arachidonate 5-lipoxygenase	0.052
	ANXA1	annexin A1	0.498
	ANXA5	annexin A5	0.225
	APG16L	ATG16 autophagy related 16-like 1 (S. cerevisiae)	0.495
	ASC	PYD and CARD domain containing	0.498
	AXL	***AXL receptor tyrosine kinase***	***0.355***
	BECN1	beclin 1, autophagy related	0.194
	C3	***complement component 3***	***0.097***
	CAPN1	calpain 1, (mu/I) large subunit	0.245
	CAPN2	calpain 2, (m/II) large subunit	0.085
	CARD15	nucleotide-binding oligomerization domain containing 2	0.248
	CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	0.149
	CASP5	caspase 5, apoptosis-related cysteine peptidase	0.015

	CD14	CD14 molecule	0.005
	CD47	CD47 molecule	0.303
	CD93	CD93 molecule	0.039
	DNASE2	deoxyribonuclease II, lysosomal	0.106
	ELMO1	engulfment and cell motility 1	0.456
	FPRL1	formyl peptide receptor 2	0.005
	GRLF1	glucocorticoid receptor DNA binding factor 1	0.204
	ICAM3	***intercellular adhesion molecule 3***	***0.198***
	IL10	interleukin 10	0.323
	IRF1	***interferon regulatory factor 1***	***0.179***
	IRF5	interferon regulatory factor 5	0.475
	IRF7	interferon regulatory factor 7	0.451
	ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	0.303
	ITGAX	***integrin, alpha X (complement component 3 receptor 4 subunit)***	***0.496***
	ITGB2	***integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)***	***0.296***
	LRP1	***low density lipoprotein receptor-related protein 1***	***0.162***
	MERTK	c-mer proto-oncogene tyrosine kinase	0.023
	NALP12	NLR family, pyrin domain containing 12	0.003
	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.465
	NLRP3	NLR family, pyrin domain containing 3	0.060
	PECAM1	platelet/endothelial cell adhesion molecule	0.140
	PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa	0.138
	PTK2	PTK2 protein tyrosine kinase 2	0.042
	PTX3	pentraxin 3, long	0.338
	RHOG	ras homolog gene family, member G (rho G)	0.230
	TGFB1	transforming growth factor, beta 1	0.142
	THBS1	thrombospondin 1	0.002

Fold change (FC): average relative expression iDCs / average relative expression Mo

Monocytes of 3 donors were differentiated for 5 days to immature dendritic cells. Changes of gene expression of 95 preselected apopto-phagocytic genes were analyzed by TLDA technique. Genes were normalized to the level of 18S rRNA and fold changes were calculated using the average relative expression.

4.8.2. Genes down-regulated by dexamethasone treatment

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. The genes with at least a 2 fold decrease in their relative expression (FC < 0.5) are listed in Table 6 and in bold and italics are shown those 8 gene which are further decreased by the glucocorticoid and were already mentioned above.

Table 6. Genes down-regulated by the presence of Dex during iDC differentiation (n= 3)

	HUGO gene symbol	**Gene description**	**FC**
Differentiation and Dex	ADORA1	adenosine A1 receptor	0.103
	ADORA2A	***adenosine A2a receptor***	***0.291***
	AXL	***AXL receptor tyrosine kinase***	***0.334***
	BCAR1	breast cancer anti-estrogen resistance 1	0.254
	C3	***complement component 3***	***0.484***
	DOCK1	dedicator of cytokinesis 1	*0.439*
	ICAM3	***intercellular adhesion molecule 3***	***0.046***
	IL12B	interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)	0.373
	IRF1	***interferon regulatory factor 1***	***0.486***
	IRF4	interferon regulatory factor 4	*0.170*
	ITGAM	integrin, alpha M (complement component 3 receptor 3 subunit)	0.192
	ITGAX	***integrin, alpha X (complement component 3 receptor 4 subunit)***	***0.285***
	ITGB2	***integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)***	***0.221***
	LRP1	***low density lipoprotein receptor-related protein 1***	***0.473***
	MAP1LC3A	microtubule-associated protein 1 light chain 3 alpha	0.297
	MFGE8	milk fat globule-EGF factor 8 protein	0.315
	MSR1	macrophage scavenger receptor 1	0.280
	OLR1	oxidized low density lipoprotein (lectin-like) receptor 1	0.053
	PPARG	peroxisome proliferator-activated receptor gamma	*0.293*

	RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	0.342
	TGM2	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	0.112
	TNF	tumor necrosis factor	0.239

Fold change (FC): average relative expression Dex treated iDCs / average relative expression non treated iDCs

Monocytes of 3 donors were differentiated and treated with Dex for 5 days. Controls and Dex treated samples were used to analyze changes of gene expression of 95 preselected apopto-phagocytic genes by TLDA technique. Genes were normalized to the level of 18S RNA and fold changes were calculated using the average relative expression.

Among the most down-regulated apopto-phagocytic genes we further investigated TGM2 which is down-regulated both mRNA and protein level from early time points of differentiation (Figure 18).

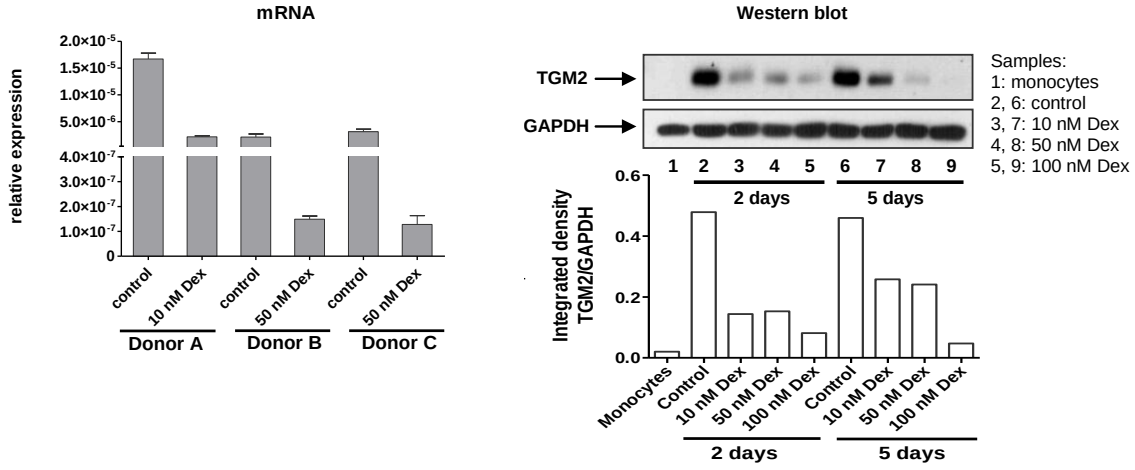


Figure 18. Expression of tissue transglutaminase (TGM2) in human dendritic cells treated with dexamethasone. *Control and Dex treated dendritic cells of 3 donors differentiated for 2 and for 5 days were used. Expression TGM2 at mRNA in iDCs (left) and protein level (Western blot on right) are shown. The Western blot and the quantification by densitometry (ImageJ software) represent one example of 3 donors. Error bars indicate SD.*

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. Intestinal antigen-presenting cells associated with celiac lesions are suspected to originate from peripheral blood derived monocytes (PBMCs) (Raki *et al.* 2007). In accord with previous reports (Le Naour *et al.* 2001; Szatmari *et al.* 2006) we found that dendritic cells differentiated *ex vivo* from human PBMCs express high levels of tissue transglutaminase induced during the differentiation process (Figure 19a). 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 (data not shown except for 4G3), the TG100 monoclonal antibody bound iDCs efficiently (Figure 19b). 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 (Figure 19c).

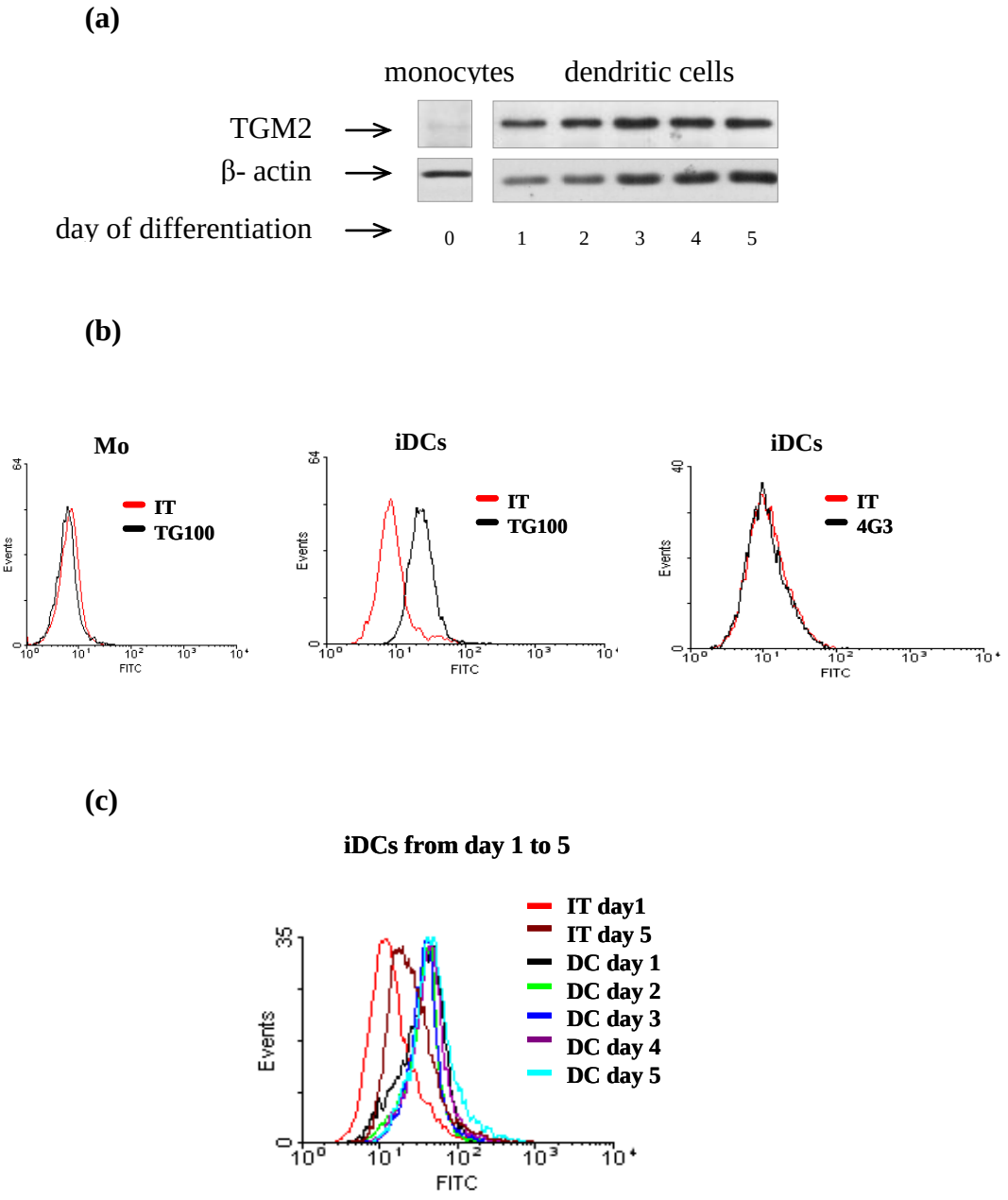


Figure 19. TGM2 detection on the surface of dendritic cells. *Human monocytes (Mo) and dendritic cells were collected on the indicated days and TGM2 protein expression was detected in cell lysates by Western blotting with 4G3 hybridoma cell supernatant (a). Monocytes and iDCs were labeled with antibodies specific for TGM2 (TG100, 4G3 hybridoma supernatant) or with isotype control and analyzed by flow-cytometry (b). Dendritic cells were labeled for csTGM2 with TG100 and isotype control antibody on consecutive days of differentiation and the staining was evaluated by flow-cytometry(c).*

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 csTGM2 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 csTGM2 (Figure 20, donor 2). 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 csTGM2 could be observed (Figure 20), thought it is known that both antibodies inhibit TGM2 activity in solution (Esposito *et al.* 2002).

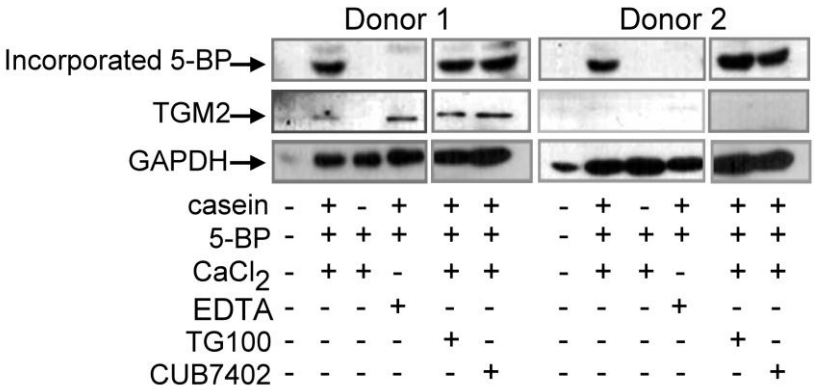


Figure 20. TGM2 is active on the surface of iDCs*. Immature dendritic cells were pre-incubated with the TGM2-specific antibodies, TG100 and CUB7402, and then co-incubated with the indicated compounds. Cell-free supernatants were collected and Western blot was performed. 5-BP incorporation was detected with HRP–streptavidin, for TGM2 detection the membranes were probed by TGM2 specific antibody (CUB7402), and the loading control was GAPDH.*

4.9.3. LPS-treated monocyte-derived iDCs 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 (Hovhannisyan *et al.* 2008). We used bacterial lipopolysaccharide (LPS) to simulate the activation of iDCs upon exposure to a putative pathogenic bacterium and in case of 16h of LPS stimulation and mature dendritic cells (mDC) we found an increased surface expression of TGM2 detected by TG100 (Figure 21).

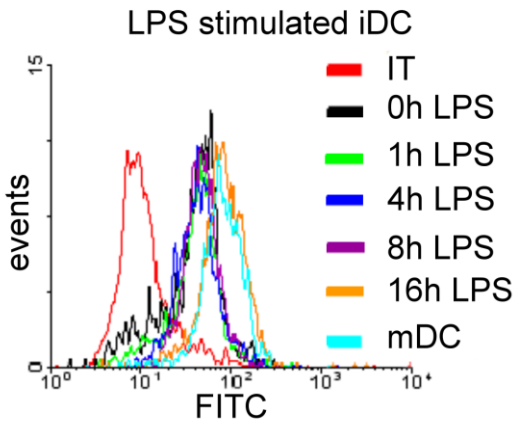


Figure 21. Enhancement of surface TGM2 expression upon LPS stimulus*. iDCs were stimulated with LPS and mDCs were generated with LPS and INFγ. Cells were collected at the indicated time points and labeling was performed for csTGM2 with TG100 and isotype control antibodies. Samples were analyzed by flow-cytometry*

5. Discussion

Glucocorticoids are stress hormones naturally released during the course of an immune response (Besedovsky *et al.* 1975) and they represent an essential link between the central nervous system and the regulation of the innate immune system (Sternberg 2006).

Endogenous glucocorticoids exist in two different forms, being either active (cortisol) or inactive (cortisone). Both are normally present in the serum in a concentration of $10^{-7} – 10^{-9}$ M, but with an excess of inactive cortisone (Sternberg *et al.* 1992). Within target cells or tissues the action of GC depends not only on the plasma hormone level, receptor expression and receptor-effector coupling, but also on local glucocorticoid metabolism in which 11β-hydoxisteroid dehydrogenases enzymes appear to play the major role. Cortisol has prominent immunosuppressive effect but several *in vitro* and *in vivo* studies demonstrated immunostimulating effect of cortisol in both humans and rodents, concluding that higher doses would lead to the suppression and the lower doses to the stimulation of the immune system (for review see ref. (Buttgereit *et al.*)).

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, in spite of the severe side effects (atrophy of the skin, muscle weakness, osteoporosis, insulin resistance and diabetes) associated with the long-term therapy. Understanding the molecular mechanisms of glucocorticoid action can help to develop more specific therapies. 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 (Amsterdam *et al.* 2002), particularly on macrophages and dendritic cells. However, while the effect of GCs on the morphology and function of human monocyte-derived macrophages was widely investigated, only a few studies have been so far devoted to dendritic cells.

Monocytes are essential part of the innate immune system and they can be differentiated into macrophages and dendritic-cells, which are crucial in the initiation of adaptive immunity, clearance of infectious particle and the resolution of inflammation (Mosser 2003). Different subtypes of macrophages and DCs have been described with proinflammatory as well as anti-inflammatory functions depending on the stage of differentiation and the mechanism of activation (Gordon 2003; Gordon *et al.* 2005). Early exposure of differentiating monocytes to glucocorticoids induces a distinct functional, pro-

resolution phenotype with increased phagocytosis of apoptotic neutrophils which was associated with decreased tyrosine phosphorylation of paxillin and pyk2 (important components of adhesion contacts), and decreased expression of p130Cas, a mediator of adhesion signaling (Giles *et al.* 2001). It was observed that supernatants collected from dexamethasone treated macrophages significantly enhanced the engulfment of apoptotic polymorphonuclear leukocytes and this was attributed to Dex induction of annexin1 expression (Maderna *et al.* 2005). Furthermore, it was proposed that glucocorticoid augmentation of apoptotic neutrophil phagocytosis by human macrophages is associated with a switch from serum-independent to a serum dependent apoptotic cell recognition mechanism which can be recapitulated with purified protein S (McColl *et al.* 2009).

As dendritic cells can take part in the induction of tolerance, prevention of autoimmunity, induction of anti-tumor immunity and protection against infectious agents (Torres-Aguilar *et al.*) investigating the effect of the widely used glucocorticoid on their morphology and various functions is an important issue. DCs represent a heterogeneous cell population specialized for capture, processing and presentation of antigens to naïve, memory, effector and/or regulatory T cells. For *in vitro* generation of these cells most of the protocols describe differentiations in medium supplemented with serum, but DCs for clinical application are cultured also in serum free medium (Goxe *et al.* 2000; Guyre *et al.* 2002; Pullarkat *et al.* 2002). We used monocyte-derived dendritic cells generated in serum free AIM-V medium in the presence of GM-CSF and IL-4 obtaining immature dendritic cells characterized by high capacity of antigen uptake and processing.

It was suggested that glucocorticoids can influence dendritic cells (reviewed in (van Kooten *et al.* 2009) by freezing them at an immature stage indicating that GCs may act at the very first step of the immune response by modulating DC differentiation, maturation and function (Piemonti *et al.* 1999). It was also shown that dexamethasone enhanced both mannose receptor-mediated and fluid-phase endocytosis (Piemonti *et al.* 1999). When we tested the effect of glucocorticoids on human immature monocyte-derived dendritic cells, which resemble tissue resident migratory DC (Shortman *et al.* 2007), we also observed the morphological changes (less adherent cells with abundant cytoplasm) reported earlier (Piemonti *et al.* 1999). Phagocytosis of apoptotic cells by these cells, investigated with an *in vitro* phagocytic system established previously (Majai *et al.* 2007) to mimic events at *in vivo* inflammation sites, was increased in GC treated DCs. Upon Dex treatment DCs are differentiated to a prominently CD1a negative population, showed previously to exhibit higher phagocytic capacity then $CD1a^+$ subtypes (Gogolak *et al.* 2007), but they express the

dendritic cell marker CD209. 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 (Oakley *et al.* 2010).

To study the mechanism behind the GC induced increase in phagocytosis in human dendritic cells we examined the changes in expression of an apopto-phagocytic gene panel in dexamethasone treated dendritic cells. The self-designed TaqMan Low Density Array used for this purpose contained genes involved in the apopto-phagocytic process described in macrophages and selected based on data from the literature They 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 (Table 2). The genes with no detectable expression in iDCs were mainly bridging molecules (APOH, CRP, EDIL3, RAP1A), but ATG12, GULP1, the phagocytosis receptor ASGR1and the signaling molecule ALOX12 could not be detected as well.

The genes found up-regulated by dexamethasone during the differentiation to immature dendritic cells are members of cell surface molecules (ADORA3, FCGR2B), bridging molecules (C1QA, C2), phagocytosis-tethering/tickling receptors (CD14, MERTK, SCARB1), effectors (DNASE2), cytokines (IL10) and inflammatory regulators (NLRP12), totally 10 in number. 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 resulted 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. The extensive change in the gene expression pattern was also manifested in a large number of down-regulated apopto-phagocytic genes. One may presume that the products of these genes have additional functions unrelated to phagocytosis or they may block GC-mediated molecular events, thus should be switched off for optimal GC response. For example, one of the down-regulated genes is transglutaminase 2 (TGM2), which will be discussed later on.

Galon et al. used DNA microarray analysis to investigate the effect of dexamethasone on human PBMC. They found that dexamethasone could regulate genes with critical roles in innate and adaptive immune responses. Furthermore, they pointed to the role of GC not only as immunosuppressant but also as a major immuno-permissive and immuno-enhancing agent (Galon *et al.* 2002). Ehrchen et al. described the GC dependent regulation of 133 genes in

human monocytes by microarray technology. Treatment with the low concentration of glucocorticoid fluticasone propionate even for a shorter period (16h) was enough to see the induction of genes responsible for apoptosis, adherence, cellular motility, chemotaxis phagocytosis and reactive oxygen metabolism. GC treatment did not cause a global suppression of monocytic effector functions but reprogrammed the cells toward an anti-inflammatory phenotype (Ehrchen *et al.* 2007). Among the GC up-regulated genes described in their study there are 4, namely ADORA3, MERTK, C1QA, IL-10, which were also induced in our study and overlap with up-regulated genes in monocyte derived dendritic cells where ADORA3, the phagocytosis receptor MERTK and CD14, the bridging molecule C1QA and the effector DNASE2 were the most up-regulated genes. The up-regulation of these genes was present already after 2 days of differentiation, showing that Dex have effect even from the early stage of differentiation. MERTK is a member of the TAM (TYRO3, AXL, MER) receptor protein tyrosine kinase family with important role in innate immunity. With the participation of GAS6 and protein S they can bind phosphatidylserine on apoptotic cells (Rothlin *et al.* 2007). In human macrophages MERTK is up-regulated by Dex (Zahuczky *et al.* 2011) and plays a key-role in the GC induced phagocytosis enhancement (McColl *et al.* 2009). We show here that 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 the GC induced enhancement of phagocytosis by iDCs could not be diminished. 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 (reviewed in (Gregory 2000)). GC treated DCs are positive for surface CD14 staining (Piemonti *et al.* 1999) 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 is an endogenous purine nucleoside produced under metabolic stress, like hypoxic conditions, acute or chronic inflammatory tissue insult. Released into the extracellular environment it binds to four types of G-protein coupled adenosine receptors (A1, A2a, A2b and A3) expressed on various immune cells and modulates immune response in

different inflammatory conditions (Hasko *et al.* 2008; Kumar *et al.* 2009). The physiological levels of adenosine, which is lower than 1 μM can activate A1, A2a and A3 receptors. The cellular response to adenosine depends on adenosine concentration at the cell surface but the receptor expression defines the magnitude of the response. 10^{-6} M of adenosine can stimulate ADORA3 and mediate adenylate cyclase inhibition.

The signaling of the adenosine receptors goes through the adenylyl cyclase-cAMP system, but various other pathways can be involved as well. The downstream signaling of cAMP is mediated by its interaction with effector molecules protein kinase A or exchange proteins directly activated by cAMP, which have been shown to modulate phagocyte functions (Serezani *et al.* 2008). It was shown that elevation of cAMP in macrophages specifically inhibits phagocytosis of apoptotic neutrophils (Rossi *et al.* 1998). It was also shown that in human monocyte-derived dendritic cells the cAMP signaling through protein kinase A (PKA) could down-regulate uptake of FITC-dextran by DCs (Garay *et al.* 2010).

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 (Panther *et al.* 2001; Schnurr *et al.* 2004). In human monocytes ADORA3 was found to be up-regulated by GC (Ehrchen *et al.* 2007). 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.

Previously it was shown that Dex down-regulates the immuno-stimulatory capacity of DCs but this was measured either with exposure of DC to TNFα/ CD40 or by testing the presentation of tetanus toxin to autologous T cells (Piemonti *et al.* 1999). We loaded the immature dendritic cells differentiated in the presence of Dex with allogeneic apoptotic human neutrophils and this was followed by their exposure to autologous T cells. We showed previously that in contrary to human macrophages DCs respond to these stimuli in an unexpected way by secreting proinflammatory cytokines and T cell activation (Majai *et al.* 2010). 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 (Galon *et al.* 2002). 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 (Ding *et al.* 2010), which is an other example when GCs are not uniformly immunosuppressive and their effect depends on cell type, dose and timing of exposure.

Dexamethasone down-regulated the multifunctional enzyme TGM2 in iDCs. We reported previously that the loss of TGM2 leads to delayed phagocytosis of apoptotic cells by macrophages and to development of autoimmunity in mice (Szondy *et al.* 2003). It was also proposed that TGM2 takes part in the formation of phagocytic portals by interacting with both integrin β_3 and MFGE8. In the absence of the enzyme the formation of the engulfing portals is less efficient and the uptake of apoptotic cells becomes slow and random (Toth *et al.* 2009).

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 (csTGM2) 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. For example CUB7402 antibody (Birckbichler *et al.* 1985) can detect csTGM2 on fibroblasts and endothelial cells (Gaudry *et al.* 1999; Gaudry *et al.* 1999) did not recognize the enzyme on immunocompetent cells, e.g. dendritic cells (Raki *et al.* 2007). It was hypothesized that this elusive behavior of the enzyme is due either to a different

conformational state, which the molecule adopts on the cell surface or to masking of the antigen recognition sites by interaction partners.

Among extracellular (plasma membrane or ECM) proteins the interaction of TGM2 with fibronectin is best characterized. TGM2 was found in clusters with fibronectin by several investigators (Radek *et al.* 1993; Gaudry *et al.* 1999; Akimov *et al.* 2000). It was also shown that TGM2 interacts with β1 and β3 integrins, even though, no interaction motif has been identified, as yet. It was suggested that TGM2 mediates the formation of ternary complexes of integrins and fibronectin (Akimov *et al.* 2000). Recently it was shown in our group that TGM2 also binds milk fat globule-EGF factor 8 (MFGE8) and anchors it to this complex (Toth *et al.* 2009). TGM2 is co-transported to the cell surface with heparin sulphate proteoglycans (HSPG), syndecan-4 in particular, which positively affects its activity (Scarpellini *et al.* 2009). Finally TGM2 binds to the scavenger receptor LRP1, when it is destined for degradation, through its catalytic core domain and also to the structurally related VLDLR (Zemskov *et al.* 2007). Macrophages and dendritic cells express β1 and β3 integrins (Krissansen *et al.* 1990; McCarthy *et al.* 1997), LRP1 and VLDLR (Fogelman *et al.* 1988; Basu *et al.* 2001), and HSPGs (de Witte *et al.* 2007). Altogether, there are multiple intermolecular interactions which can sterically hinder the binding of one or several antibodies.

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 (Hovhannisyan *et al.* 2008). Inhibition of TGM2 with antibodies or selective inhibitors, therefore, can be a valid approach to palliate celiac disease (Esposito *et al.* 2007; Schuppan *et al.* 2009).

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

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. Perspectives

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.

Monocyte-derived dendritic cells are a heterogeneous population, $CD14^+$ cells differentiate toward both $CD1a^+$ and $CD1a^-$ cells. Previous studies showed that $CD1a^+$ DCs secreted more INFγ then $CD1a^-$ cells (Gogolak *et al.* 2007). In our T cell polarizing assays the control cells of the donors were highly $CD1a^+$ therefore it would be an interesting issue whether donors with predominantly $CD1a^-$ control DCs would also give an inflammatory response to apoptotic neutrophils. Sorting the cells after differentiation is not possible since Dex treated cells are $CD1a^-$, therefore for this project a large number of donors would be necessary, and the CD1a characterization could be done from control cells. If any difference could be seen, we might speculate that there is a predisposition for proinflammatory response in the presence of Dex. This could be predicted from the ratio of DC subtypes differentiated *in vitro* from peripheral blood $CD14^+$ cells.

There is no clear evidence of why the different donors respond to the glucocorticoid with different efficiency. The discovery of a large cohort of GR subtypes changed the traditional view that glucocorticoids act through a single GR protein. The receptor isoforms differ in their expression, functional and gene regulatory profiles. The GR diversity is further expanded by the posttranslational modification of these proteins; therefore correlation could be searched between the GR molecular heterogeneity and polymorphism and proinflammatory response of the APCs to allogeneic neutrophils.

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.

Összefoglalás

A szöveti homeosztázis fenntartásában jelentős szerepet játszik a természetes sejthalállal elpusztult sejtek folyamatos eltakarítása. Ebben a folyamatban jelentős szerepet vállalnak a professzionális fagocita sejtek, a dendritikus sejtek, amelyek egyben az immunválasz fő szabályozói is, azon képességük révén, hogy kiválthatnak mind aktivációt mind toleranciát.

A dexametazon a gyógyászatban igen gyakran használt glükokortikoid, amely az immunrendszer minden sejtjét befolyásolja. *In vitro* körülmények között, monocita eredetű dendritikus sejtek fagocitáló képességére kifejtett hatását vizsgálva, azt tapasztaltuk, hogy jelentősen megnövelte az allogén apoptótikus neutrofil felvételt. Az apopto-fagocita génekre való hatása pedig ezek kifejeződésének megváltoztatásában nyilvánult meg. Olyan gének expressziója nőtt meg a dexametazon jelenlétében, mint a fagocitózis receptor MERTK és CD14, hídmolekulák közül a C1QA, DNASE2 és ADORA3. Amennyiben a sejteket ADORA3 antagonistával előkezeltük, a dendritikus sejtek fagocitáló képessége lecsökkent. Valamint a génhiányos egerekből differenciáltatott dendritikus sejtek, nem növelik meg fagocitózis képességüket a glükokortikoid kezelés hatására. A MERTK szerepének tisztázására, megvizsgáltuk fehérje szinten a sejtfelszíni expressziót. Noha detektálható a felszínen a fehérje és a glükokortikoid hatásra megnő a felszíni expresszió, specifikus antitestek nem gátolták a fagocitózis Dex indukálta megnövekedését.

Dexametazon hatására, apotótikus allogén neutrofil bekebelezése valamint LPS és IFNγ stimulus után, a dendritikus sejtek, TNFα gyulladási citokint termelnek. A megkezelt sejtek képesek T sejt aktiválásra, amit az apoptótikus neutrofilek fagocitózisa képes tovább fokozni.

Az apopto-fagocita gének közül, a TGM2 expresszója csökkent. Egér makrofágokon kimutatták, hogy ez az enzim szükséges az apotótikus sejtek fagocitózisához. Feltételezés szerint humán rendszerben, lisztérzékeny betegek estén, a fő antigén prezentáló sejtek (dendritikus sejtek) felszínén lévő transzglutamináznak szerepe lehet a gliadin deamidálásában és a glutén-reaktív T-sejtek fokozott aktiválódásában. Azonban nem ismert olyan adat, amely a humán antigén prezentáló sejtek felszíni TGM2 expresszióját alátámasztaná, valamint a deamidálás pontos helye sem ismert. Eredményeink azt mutatják, hogy a humán monocita eredetű dendritikus sejtek kifejezik a sejtfelszínen ezt a fehérjét és az aktív. Valamint LPS-el való stimulálás esetén a felszíni expresszió megnő. A tény, hogy a glükokortikoid hatásra a TGM2 kifejeződése mind gén, mind fehérje szinten lecsökken, miközben a sejtek fagocitáló képessége megnő, arra utal, hogy Dex hatására alternatív útvonalak aktiválódnak, amelyek helyettesíthetik a transzglutamináz apoptótikus sejtfelvételben játszó szerepét.

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8.2. Publicaton list prepared by the KenézyLife Scieneces Library

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Doctoral School: Doctoral School of Molecular Cell and Immune Biology

List of publications related to the dissertation

1. **Hodrea, J.**, Majai, G., Doró, Z., Zahuczky, G., Pap, A., Rajnavölgyi, É., Fésüs, L.: The glucocorticoid dexamethasone programs human dendritic cells for enhanced phagocytosis of apoptotic neutrophils and inflammatory response.
   J. Leukoc. Biol. "accepted for publication", 2011.
   IF:4.626 (2010)

2. **Hodrea, J.**, Demény, M.Á., Majai, G., Sarang, Z., Korponay-Szabó, I., Fésüs, L.: Transglutaminase 2 is expressed and active on the surface of human monocyte-derived dendritic cells and macrophages.
   Immunol. Lett. 13 (1-2), 74-81, 2010.
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List of other publications

3. Petrovski, G., Ayna, G., Majai, G., **Hodrea, J.**, Benkő, S., Mádi, A., Fésüs, L.: Phagocytosis of cells dying through autophagy induces inflammasome activation and IL-1beta release in human macrophages.
   Autophagy. 7 (3), 321-330, 2011.
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4. Majai, G., Gogolák, P., Ambrus, C., Vereb jr., G., **Hodrea, J.**, Fésüs, L., Rajnavölgyi, É.: PPARgamma modulated inflammatory response of human dendritic cell subsets to engulfed apoptotic neutrophils.

H-4032 Debrecen, Egyetem tér 1. e-mail: publikaciok@lib.unideb.hu

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J. Mol. Recognit. 19 (4), 270-274, 2006.
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9. Keywords

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

Tárgyszavak

Dendritikus sejtek, dexametazon, fagocitózis, apopto-fagocita gének, apoptótikus neutrofilek, T sejt aktiválás, gyulladás, szöveti transzglutamináz

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