Summary of Thesis for the degree of Doctor of Philosophy (Ph.D.)

by

GYÖNGYIKE EMESE MAJAI, M. D.

The role of PPARγ in clearance of apoptotic neutrophils by human macrophages and dendritic cells

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
MEDICAL AND HEALTH SCIENCE CENTER
UNIVERSITY OF DEBRECEN
DEBRECEN

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Supervisor:

Prof. László Fésüs, M.D., PhD, Member of the H.A.Sc.

Head of the Examination Board:

Members of the Examination Board:

Chair of the Defence Committee:

Reviewers:

Memebers of the Defence Committee:

INTRODUCTION

Clearance of apoptotic cells by professional phagocytes

Clearance of apoptotic cells by professional and non professional phagocyting cells such as macrophages and DCs has an important role in tissue remodeling and resolution of inflammation protecting tissues from exposure to the inflammatory and immunogenic contents of dying cells. Macrophages are specialized to the rapid and efficient clearance of pathogens and dying tissue cells, whereas the major function of DCs is to transport antigens acquired from engulfed apoptotic cells to draining lymph nodes together with transferring co-stimulatory signals to T-lymphocytes. The characteristics that define the macrophage phenotype include not only morphologic features and expression of cell surface receptors but also functional capabilities such as cytokine release, antigen presentation, phagocytic ability and microbicidal activity. A relatively large number of molecules become available during macrophage differentiation to recognize changes occurring on the surface of the apoptotic cells (such as phosphatidylserine exposure), to opsonize the dead cells and to engulf the apoptotic cells. Several components of the innate immune system are utilized in this process, mainly soluble factors which bind to the distinct molecular pattern of apoptotic cells. When human macrophages engulf spontaneously dying neutrophils cell surface CD31 mediates tethering of apoptotic cells; it can discriminate between apoptotic and viable cells by selectively imparting detachment signals to viable cells preventing their ingestion. The important role of CD36 receptor forming an adhesive complex containing thrombospondin and $\alpha_v\beta_3$ integrin receptors was also demonstrated in recognition of apoptotic neutrophils by macrophages. The phagocytic receptor CD14 has been implicated in the ICAM-3 mediated interaction between human macrophages and dying neutrophils. We have recently observed that TG2 is involved in the regulation of the apopto-phagocytic system; its deletion leads to the development of autoimmune disorders. PTX3, an important component of innate immune response, enhances C1q binding and C3 deposition on the surface of apoptotic cells, suggesting a role of PTX3 in complement mediated phagocytosis of apoptotic cells.

In the case of dendritic cells it was shown that engulfment of apoptotic cells is restricted to the immature stage of dendritic cell development and this is associated with the presence of $\alpha_v\beta_5$ which is absent on macrophages . It is known that immature DCs are capable of engulfing apoptotic cells by a mechanism involving CD36, PS receptor, MFGE-8 and

integrins. It was also reported that MERTK is a key receptor for macrophage phagocytosis, while AXL and Tyro seem to be important for phosphorilation of MERtk instead of the phagocytosis process while in case of dendritic cells which also express MERtk the role of AXL and TYRO are more important in the engulfment process. The C-type lectin DC-SIGN (CD209) expressed by both immature and mature dendritic cells functions as both adhesion and pattern recognition receptor. As an adhesion molecule DC-SIGN binds to ICAM-2 on endothelial cells and to ICAM-3 on T cells required for the initiation of immune responses. As a pattern recognition receptor, DC-SIGN binds to of variety of viral, bacterial, fungal and parasite pathogens including human immunodeficiency virus 1, explicable by containing carbohydrate domain with specifity for both high mannose moieties and non-sialylated Lewis antigens. Similarly to T cells neutrophils express ICAM-3 but it was shown that DC-SIGN does not bind to ICAM-3 present on the surface of neutrophils; its binding is specific to the polymorphonuclear β_2 integrin –Mac1 which contains Lewis moieties, absent on other myeloid cells. These suggest that cell specific glycosylation is important in the regulation of cellular interactions between DCs and neutrophils.

How apoptotic cells influence inflammation?

Macrophages discriminate innately between cells that have undergone physiological death and those that have suffered necrosis. Recognition of these two classes of dying cells occurs via distinct and non-competitive mechanisms. The major mechanism to prevent secondary necrosis of apoptotic cells is their recognition and fast removal not allowing the development of inflammation and autoimmune diseases. There are ample evidences that apoptotic cells do not only fail to induce but can actively suppress the release of pro-inflammatory cytokines such of IL-1β, IL-8, GM-CSF, TNF-α, as well as leukotriene C4 and tromboxane B2 from macrophages which engulf them and potentiate the release of anti-inflammatory cytokines such as TGFβ1, PGE2 and PAF. In dendritic cell studies suggested tolerance induction by apoptotic cells whereas other experiments have shown immunostimulatory effects mediated by apoptotic cells. It was reported that engulfment of apoptotic cells results in down-regulation of IL-12 as well as some markers of DC activation, such as CD86, and release of TGF-β1. However, if antibodies that can bind and opsonize apoptotic cells are present, ligation of Fc receptors will result in DC maturation and production of immunostimulatory cytokines. Also, passive release of HMGB-1, box1, uric

acid and heat shock proteins from necrotic cells have a potential role in stimulating inflammation through the NF-kB mediated pathway. Dendritic cells are unique among phagocytes in being capable of presenting antigenic peptides derived from dying cells on MHC I and MHC II molecules for recognition by $CD8^+$ T cells. In the absence of $CD4^+$ T cell help, dendritic cells that cross-present antigens to $CD8^+$ T cells result in tolerance by a deletion mechanism. By contrast, the ability to activate $CD8^+$ T cells depends on the presence of antigen specific $CD4^+$ T cell help. Once activated, these $CD8^+$ T cells return to the site of inflammation and can destroy target cells. This process can be beneficial in the development of tumor immunity, but severely pathogenic when it targets self.

The role of PPARγ in monocyte macrophagedendritic cell differentiation and their function

PPARs (peroxisome proliferator activated receptors) are members of the nuclear receptor superfamily which heterodimerize with the retinoid X receptor. PPARγ was characterized originally as a key regulator of adipocyte differentiation and lipid metabolism and it also plays an important role in glucose metabolism. Recent studies have suggested that PPARγ may promote monocyte-macrophage differentiation. PPARγ is expressed at low levels in murine bone marrow macrophages but at higher levels in activated peritoneal macrophages and it is highly induced in monocyte derived macrophages. Although the function of PPARγ in macrophages is not fully determined, several reports have proposed that it has an anti-inflammatory role in these cells negatively regulating the expression of pro-inflammatory genes through antagonizing the activities of various transcription factors. It has not been clarified whether PPARγ is required duringdifferentiation of macrophages to prepare them for efficient phagocytosis of apoptotic cells. Several observations suggest that not only monocyte/macrophage but also dendritic cells express PPARγ receptors at high levels. The synthetic ligand rosiglitasone was shown to activate the receptor and also to influence the percentage of $CD1a^-$ population during monocyte dendritic cells differentiation process resulting in a higher internalizing capacity for pathogens.

AIM OF THE STUDIES

1. To set up a human apopto-phagocytic system
2. To clarify whether the capacity of macrophages to engulf apoptotic cells could be influenced by PPARγ agonist or antagonist
3. To decide whether the phagocytosis enhancing effect of dexamethasone is linked or not to PPARγ dependent processes
4. To clarify the role of PPARγ in the secretion of cytokines
5. To study the phagocytic capacity of monocyte differentiated dendritic cells
6. The investigate the role of PPARγ in dendritic cell differentiation and its impact on phagocytosis
7. To identify some of the receptors involved in the clearance of apoptotic neutrophils by dendritic cells
8. To investigate the effect of apoptotic neutrophils on dendritic cell cytokine secretion
9. The influence of apoptotic cell engulfment on T cell activation in macrophages and dendritic cells

MATERIAL AND METHODS

Cell culture procedures

Peripheral human blood mononuclear cells were isolated by density gradient centrifugation with Ficoll–Paque Plus from "buffy coats" obtained from blood donors. $CD14^+$ cells were separated by magnetic sorting with MACS, followed by washing with PBS containing 0.5% BSA, 2mM EDTA. Neutrophils were isolated from "buffy coat" by a density gradient centrifugation with Histopaque1119 and Histopaque1077. Freshly isolated monocytes were cultured for a period of 5 days in IMDM supplemented with 10% AB serum, 5ng/ml M-CSF in the presence or absence of either 1μM dexamethasone or 10μM PPARγ antagonist GW9662, or 2.5 μM PPARγ agonist rosiglitasone. For dendritic cell differentiation freshly isolated monocytes were resuspended into 6-well culture dishes at a density of 2.5×10^6 cells/ml and cultured in AIMV media containing 800 U/ml GM-CSF and 500 U/ml IL-4 in the presence or absence of 2.5 μM PPARγ agonist rosiglitasone. Cells were cultured for 5 or 6 days and the IL-4 and GM-CSF addition was repeated at day 3.

Neutrophils were kept in tissue culture for 24 h in IMDM with 1% AB serum while they underwent apoptosis (50-80% during 24 h). Isolated thymocytes from 4 weeks old NMRI mice were cultured in RPMI medium 1640 supplemented with 10% FCS, 2 mM glutamine, 1 mM Na-pyruvate at 37°C and 5% CO_2. Apoptosis was induced by addition of Ca-Ionophor A23187 (4 μM) for 5 h and the dying cells were added to mouse macrophages.

Generation of mice with macrophage specific deletion of PPARγ. Isolation and culturing of bone marrow and peritoneal cells from these mice

We obtained the macrophage-specific PPARγ knockout mice from L. Nagy (University of Debrecen). Bone marrow cells were isolated from the femur of $PPAR\gamma^{+/-}$ Lys-Cre and $PPAR\gamma^{fl/-}$ Lys-Cre mice, cultured in RPMI 1640 supplemented with 10% FBS, 2mM glutamine, 1 mM Na-pyruvate, penicillin and streptomycin. These cells were differentiated to macrophages by M-CSF (20 ng/ml) for 10 days. Fresh medium containing cytokine was added every third day to complement the old medium. Cells were harvested after 10 days. Thioglycolate-elicited macrophages were harvested by peritoneal lavage 4 days after injection of 3ml 3% thyoglicolate solution. Cells were washed with saline and cultured 2 days in RPMI

1640 supplemented with 10% FCS, 2 mM glutamine, 1 mM Na-pyruvate, streptomycin and penicillin.

Macrophage phagocytosis assays

Dying neutrophils or thymocytes before death stimuli were labelled with 5(6)-CFDA-SE (15μM, overnight), washed free of conditioned media and resuspended in PBS prior addition to a prewashed Cell Tracker^{TM} Orange CMTMR (3.75μM, overnight) labelled macrophage monolayer. Macrophages and apoptotic neutrophils or thymocytes were mixed at a ratio of 1:15 and incubated for 25 min (human cells) or 40 min (mouse cells) either at 37 ^{o}C or 4 ^{o}C in 5% CO_2 atmosphere. Following incubation non-ingested neutrophils or thymocytes were removed by washing three times with PBS. Adherent macrophages were detached from the plate and analyzed by flow cytometry.

Phagocytosis of Listeria Monocytogenes

Cultures of Listeria Monocytogenes were grown in brain heart infusion at 37 ^{0}C for 15h (strain. ATCC 35152, LGC Promochem). Listeria Monocytogenes was labeled with FITC isomer (0.1mg/ml, overnight at room temperature) and opsonized with 10% serum for 10 min. and added to human differentiated macrophages for 25 min. The ratio of macrophage to bacteria was 1:15. The percentage of macrophages taking up labeled bacteria was determined by flow cytometry.

Dendritic cell phagocytosis assays

Freshly isolated neutrophils were labelled with 5(6)-CFDA-SE (15μM, overnight), washed free of conditioned media and resuspended in A-IMV prior addition dendritic cells. Dendritic cells and apoptotic neutrophils were mixed at a ratio of 1:5 and incubated for 8 hours either at 37 ^{o}C or 4 ^{o}C in 5% CO_2 atmosphere. Following incubation the cells were washed three times with PBS, dendritic cells were labelled for CD40, CD209, CD1a cell surface receptors and analyzed by flow cytometry.

Myeloperoxidase staining

Monolayers of macrophages following their exposure to apoptotic neutrophils for 25 min, were washed, then fixed in 2.5% glutaraldehyde and stained for myeloperoxidase using hydrogen peroxide and dimethoxybenzidine. The percentage of phagocytosis was determined microscopically by counting stained cells among 500 macrophages in randomly selected fields.

Flow cytometry

Macrophages were detached from culture plastic with cell scraper using PBS supplemented with 0.5% BSA. After washing with PBS/BSA the cells were stained with PE conjugated anti-CD14, anti-CD16, anti-CD206 (mannose receptor) or FITC conjugated anti-CD36 antibodies for 1h at 4 ^{o}C in the dark and washed twice before analysis on a Becton-Dickinson FACScan.

Western Blotting

Differentiated macrophages were washed with PBS and lysed by incubation with lysis buffer containing 25mM Tris, 1% NP40, 150mM NaCl, and protease inhibitor cocktail (Sigma). Membrane and nuclear fractions were removed by centrifugation at 14,000 x *g*, 4°C, 30 min Lysates containing 2 mg/ml protein were mixed with an equal volume of Laemmli buffer. Electrophoresis was performed in an 8% SDS–polyacrylamide gel. Separated proteins were electroblotted and probed with monoclonal (CUB7402) antibody to TG2.

RNA isolation and quantification

Total RNA was isolated from cells using Trizol. Transcript quantification was performed via quantitative real time RT PCR using TaqMan probes. Transcript levels in the case of PPARγ, FABP4, ABCA1, CD36, CD14, TG2 were normalized to the expression level of cyclophilin D. For TaqMan low density array (TLDA, Applied Biosystems, Assay ID: 4342379-18S) pre-designed TaqMan probe and primer sets for target genes were chosen from an on-line catalogue. Relative quantities (RQ or fold changes) were determined using the equation where relative quantity equals $2^{-\Delta\Delta Ct}$ (ΔCt represents the threshold cycle (Ct) of the target minus that of 18S rRNA).

Human IFNγ ELISPOT assay

Differentiated macrophages and dendritic cells were incubated with non-stained apoptotic neutrophils. Following co-incubation the autologous PBLs were added to dendritic cells and macrophages and incubated 5 days at 37 ^{o}C in 5% CO_2 atmosphere. Finally, the cells were collected at day 5 for an IFN-γ ELISPOT assay. The resulting spots were counted using a computer assisted ELISPOT image analyzer (Series 1 ImmunoSpot Analyzer, ImmunoSpot Vesion 4.0 Software Academic, Cellular Technology, Cleveland, OH) customized for analyzing ELISPOTs to meet objective criteria for size, chromatic density, shape, and color.

Cytokine determination assay

Supernatants from macrophages and dendritic cells engulfing apoptotic neutrophils were collected and analyzed for the presence of IL-8, IL-6, IL-1β, IL-10, TNF-α, IL-12p70 using the Human Inflammation BD Cytometric Bead Array (CBA) (BD Biosciences).

RESULTS AND DISCUSSION

PPARγ antagonists inhibit phagocytosis of apoptotic neutrophils and its glucocorticoid dependent enhancement

Less than 6% of freshly isolated human monocytes can engulf apoptotic neutrophils. After their differentiation program about 30% of macrophages could take up dying neutrophils during a 25 min co-incubation period. Similar data were obtained by either myeloperoxidase staining of engulfed neutrophils or detecting pre-stained dead cells inside macrophages by flow cytometry. Since the two techniques showed good correlation, in subsequent experiments mainly flow cytometry was used to estimate phagocytic activity of macrophages.

Recent findings have indicated the presence of a link between PPARγ expression and macrophage differentiation and it has also been shown that synthetic PPARγ ligands can regulate various macrophage functions. To clarify whether the capacity of macrophages to engulf apoptotic cells could be influenced by PPARγ agonists, monocytes of different blood donors were cultured for 5 days in the presence of 2.5μM rosiglitasone, a PPARγ agonist. There was no significant difference in the phagocytic capacity of rosiglitasone treated macrophages as compared to controls. To determine whether the observed basic phagocytic capacity of macrophages was dependent on PPARγ activation, we added the PPARγ antagonist GW9662 to the culture fluid for the entire differentiation period of monocytes. GW9662 is a potent and selective antagonist of PPARγ leading to irreversible loss of ligand binding and it has no effect on transcription mediated by either PPARα or PPARδ. When differentiating monocytes were cultured *in vitro* for 5 days in the presence of 10μM GW9662 the matured macrophages showed significantly decreased phagocytic capacity as determined by both myeloperoxidase staining and flow cytometer analysis. The decrease in phagocytosis capacity was correlated with increasing GW9662 concentrations (Fig.3A). Adding T0070907, another selective antagonist of PPARγ acting by blocking its interaction with co-activators, also lead to decreased phagocytosis. Reduced phagocytic capacity of GW9662 treated macrophages was also confirmed by using carboxylate modified polystyrene latex beads, which can mimic apoptotic cells, GW9662 did not inhibit the engulfment of opsonized Listeria Monocytogenes, suggesting that inhibition of PPARγ mediated processes specifically suppresses uptake of apoptotic cells. Our results clearly show that blocking PPARγ by an

effective antagonist during the maturation process of human monocytes, that is mimicking deletion of this nuclear receptor, leads to differentiated macrophages which are compromised in their ability to engulf apoptotic cells. On the other hand, the cellular phagocytosis machinery is not affected under such circumstances since antagonist treated human macrophages can take up opsonized bacteria as efficiently as untreated ones.

It has been shown that dexamethasone can reprogram monocyte differentiation toward a pro-resolution phenotype, exhibiting increased phagocytosis of apoptotic cells. In order to decide whether this phenomenon is linked to PPARγ dependent processes monocytes were cultured *in vitro* for 5 days in the presence of 1μM dexamethasone alone or in combination with either rosiglitasone or GW9662. Macrophages treated with rosiglitasone in combination with dexamethasone displayed a phagocytic capacity similar to that treated by dexamethasone. However, the stimulatory effect of dexamethasone could be reversed by co-incubation of macrophages with GW9662. Our experiments clearly demonstrate that PPARγ antagonist could reverse the highly increased uptake of apoptotic neutrophils by dexamethasone treated macrophages. This reversal effect is not connected to cytoskeletal changes since the loss of podosomes could not be reversed by treatment with PPARγ antagonist (our unpublished observation).

Deletion of PPARγ leads to defective phagocytosis of apoptotic cells

To get more evidence for the involvement of PPARγ in the regulation of phagocytic capacity of differentiated macrophages toward apoptotic cells we used PPARγ$^{fl/-}$ Lys Cre (with macrophage specific deletion of PPARγ) and the parental PPARγ$^{+/-}$ Lys Cre (control) mice. The anti-inflammatory IL-4 was found to induce the expression of PPARγ in peripheral blood monocytes Unlike in the heterozygous controls induction of PPARγ was not observed in macrophages of PPARγ$^{fl/-}$ Lys Cre mice in the presence of IL-4 and rosiglitasone Differentiated bone marrow derived and peritoneal macrophages from PPARγ$^{fl/-}$ Lys Cre mice showed reduced uptake of apoptotic thymocytes as compared to controls

PPARγ dependent gene expression contributes to effective phagocytosis of apoptotic cells

In order to provide evidence that during the differentiation process PPARγ dependent gene expression takes place, we have examined changes in mRNA expressions. A 3.5 fold induction of PPARγ and a 14 fold induction of FABP4 (a well known PPARγ target gene) were observed by Q-PCR analysis during the differentiation period. Cells cultured for 5 days in the presence of GW9662 showed a strong inhibition of FABP4 expression clearly showing that the PPARγ antagonist functions as expected in differentiating macrophages. CD36, TG2, AXL and PTX3, genes which are involved in the phagocytosis of apoptotic cells were down regulated at the transcription level in these cells as compared with control macrophages. While the mRNA level of CD36 and TG2 were estimated by single quantitative real time PCR assays, the AXL and PTX3 results were obtained by using TaqMan low density arrays, parallely determining the expression level of all the so far described genes involved in the phagocytosis of apoptotic cells. Apart from CD36, TG2, AXL and PTX3 the presence of PPARγ antagonist did not alter significantly (more than 2 times) the level of expression of other pro-phagocytic genes; there was a small increase in mRNA of the ABCA1 pro-phagocytic gene while we did not observe changes in the level of others not listed but exampled here by showing data of CD14 transcripts by RT-Q-PCR

In dexamethasone treated cells down regulation of FABP4 and TG2, decreased expression of CD36, up regulation of ABCA1, and no changes in the level of PPARγ and CD14 were observed. Cells differentiated in the presence of dexamethasone and GW9662 showed down regulation of FABP4, PPARγ, TG2, decreased expression of CD36 and a two fold increase of ABCA1 as well as CD14 mRNAs .The expression of PTX3 and AXL varied considerably in dexamethasone treated cells showing differences of one to three orders of magnitude which precluded the systematic assessment of the effect of the antagonist.

Flow cytometric analysis was also used to determine whether the effect GW9662 on phagocytosis was associated with changes of CD36 and CD14 expression at the protein level. Addition of GW9662 led to down regulation of CD36 as well as CD14 on the surface of macrophages. In accordance with mRNA levels an increase of TG2 protein content was found in differentiated macrophages, which was partially prevented by GW9662 treatment. Interestingly, dexamethasone itself down regulated CD36 and TG2 also at the protein level and this was not changed by the presence of GW9662. The antagonist did not prevent expression of macrophage differentiation markers unrelated to phagocytosis, since CD16 and CD206 (mannose receptor) were present on the surface of GW9662 treated cells; their expression is 3 or 4 fold higher, respectively, as compared to monocytes, though not reaching

the ones observed on control macrophages. Dexamethasone treatment, added to macrophages either alone or in combination with GW9662, did not affect the expression of these differentiation markers.

Are the observed changes in gene expression upon blocking PPARγ activity sufficient to explain the decreased phagocytic capacity of macrophages toward apoptotic cells? It has been suggested that different surface receptors on the phagocytes are involved in tethering (recognition and binding of cell corpses) and tickling (internalization and activation of downstream signaling) processes, leading via at least two major pathways to activation of Rac which is obligatory for uptake of dead cells. In case of phosphatidylserine (PS) recognition - which is a dominant „eat me" signal when neutrophils undergo apoptosis, at least one tethering and one internalization receptor is needed for efficient clearance. Tethering is co-opted by integrins and integrin-associated proteins, such as CD36 working in contact with the bridging molecule MFGE8. Tickling is mediated by Gas or protein S through one of the tyrosine kinase receptors (MERTK, AXL or TYRO3) leading to activation of Rac engaging TRIO, RhoG, DOCK180, CrkII and ELMO. Transglutaminase 2 facilitates the process of PS exposure and TGFβ activation needed for efficient phagocytosis. Using TLDA we could determine that all the necessary molecular elements of PS-dependent recognition and engulfment are expressed in differentiated human macrophages. Though in the presence of antagonist there was no significant change in the expression of the majority of genes involved in the PS pathway, down regulation of CD36, AXL and TG2 may sufficiently decrease the efficiency of the PS-dependent uptake of dead cells by macrophages to explain the observed results. The importance of down regulating CD36 by the antagonist is underlined by the observation that addition of anti-CD36 antibody to human macrophages engulfing apoptotic neutrophils led to about 50% decrease of phagocytosis. Exposed calreticulin on the apoptotic cell surface is recognized by collectins and ficolins (such as C1QA, PTXs) serving as bridging molecules, then the opsonized dead cells are bound to phagocytosis receptors on the surface, including calreticulin in conjunction with LRP. From this pathway only PTX3 was down regulated by the PPARγ antagonist in our study. It was reported that PTX3 inhibits phagocytosis of late apoptotic neutrophils by macrophages. However, we have used early apoptotic neutrophils which can bind PTX3 secreted from the macrophages and enhance C1q binding (also released from macrophages) as well as C3 deposition on the apoptotic cell surface facilitating their phagocytosis by blocking PTX3 expression PPARγ antagonist may significantly reduce efficiency of phagocytosis.

Cytokine profile of LPS stimulated macrophages treated by PPARγ antagonist

Cells differentiated in the presence of GW9662 did not show significant difference in secretion of TNF-α, L-6, IL-8 and TGFβ upon LPS stimulation but had much reduced IL-10 secretion. As expected, dexamethasone treated cells secreted significantly less IL-6, TNF-α and IL-10 but not IL-8, and more TGF-β following LPS stimulation. Cells differentiated in the presence of dexamethasone and GW9662 showed similar cytokine secretion patterns as ones treated with dexamethasone only, excluding IL-10 which was secreted at a lower level. Blocking PPARγ activation did not influence the anti-inflammatory effect of apoptotic cells. Dexamethasone treatment, even with addition of GW9662, could down regulate secretion of IL-6 and TNF-α and increased the release of TGF-β upon LPSstimulation. Apoptotic cells neither abolished nor enhanced this macrophage response – except in case of TGF-β where apoptotic cells could enhance the effect of dexamethasone and this was not influenced by GW9662. Apoptotic cells could down regulate LPS-induced IL-8 secretion in macrophages differentiated in the presence of dexamethasone either alone or in combination with PPARγ antagonist.

The release of the anti-inflammatory cytokine IL-10 following LPS treatments, as it is shown above, was significantly less in GW9662 treated macrophages as compared to controls. This inhibitory effect was also observed after incubating these cells with apoptotic neutrophils. Like in other settings, GW9662 did not block the effect of apoptotic cells on IL-10 secretion, rather enhanced it and this was the case with dexamethasone treated cells as well.

Our results clearly showed that in the down regulation of IL-10 secretion apoptotic cells, glucocorticoid-mediated signals and PPARγ antagonist seem to act in a synergistic way. The LPS-induced IL-8 secretion, on the other hand, is not sensitive to glucocorticoid or PPARγ regulation, but still responds to apoptotic cells. These data further emphasize the unique nature of the anti-inflammatory action of apoptotic cells. This suggests that the so far unrevealed anti-inflammatory signalling pathway elicited by the contact of apoptotic cells with recognition receptors of macrophages does not depend on PPARγ -mediated gene expression - even in case of IL-10.

PPARγ agonist up regulates the engulfment of apoptotic neutrophils by human monocyte-derived dendritic cells

Dendritic cells and macrophages are phagocytic cells that derive from common precursors, reside in peripheral tissues and exhibit overlapping functions such as engulfing apoptotic cells, sensing self and pathogenic danger signals and acting as professional APCs. As we showed, the engulfment of apoptotic neutrophils by *in vitro* differentiated human macrophages reached a plateau at one hour involving about 30% of the phagocytes. Using similar culture conditions we found that immature monocyte-derived DCs (IDCs) are also able to internalize apoptotic neutrophils albeit this process is slower and less efficient than that of macrophages. As our results shown the incubation of apoptosing neutrophils with IDCs for 8, 16 or 24 hours resulted in not more than 20% of phagocytosing cells.

Previous studies have revealed that PPARγ is up-regulated during the differentiation of monocytes towards both macrophages and DCs. The maturation program and the phagocytic activity of macrophages is down regulated by PPARγ antagonists, whereas the agonistic ligand rosiglitasone is able to shift monocyte-derived DC differentiation to the generation of $CD1a^-$ cells previously characterized by high phagocytic and internalizing capacity. To assess whether the capacity of IDCs to engulf apoptotic neutrophils could also be influenced by PPARγ, freshly isolated human monocytes were cultured for 5 days in the presence of 2.5µmol rosiglitasone and the IDCs were tested for their capability to internalize apoptotic neutrophils. The ratio of $CD1a^-$ cells varied individually, but IDCs generated in the presence of rosiglitasone contained a higher proportion of $CD1a^-$ cells than those differentiated without rosiglitasone. In line with our previous results the agonist-induced activation of PPARγ not only resulted in a shift of $CD1a^-$ ratios but also induced increased net phagocytosis obvious in four out of six donors. These results demonstrated for the first time that $CD1a^-$ cells, differentiated from monocytes either in the absence or presence of PPARγ agonists are preferentially involved in the engulfment of apoptotic cells.

Cytokine secretion of immature dendritic cells loaded with apoptotic neutrophils

Uptake of apoptotic cells, unlike necrotic ones, in most cases does not provoke an inflammatory response and can even down regulates signaling events initiated by pro-inflammatory stimuli. Our previous results revealed that monocyte-derived macrophages fed by apoptotic neutrophils down regulated the secretion of LPS-induced inflammatory cytokines and induced the production of anti-inflammatory mediators. In order to study the effect of uptake of apoptotic neutrophils on cytokine secretion of immature DCs apoptotic neutrophils were added to IDCs for 8 hours and the cell culture supernatants were harvested.

A fraction of the cells were treated with LPS+IFNγ for a further 16 hours to see how IDC maturation influences the cytokine response. The engulfment of apoptotic neutrophils by IDCs resulted in the up regulation of IL-8, IL-6, TNF-α and IL-1β secretion in all the three donors tested. Dendritic cells generated in the presence of rosiglitasone contain predominantly $CD1a^-$ cells and engulf more apoptotic neutrophils than those generated in the absence of rosiglitasone. Remarkably, rosiglitasone-treated and neutrophil loaded DCs secreted significantly less IL-8, IL-6 and IL-1β but not TNF-α than DCs generated in the absence of PPARg agonist. These data confirm our previous results on the more tolerogenic nature of $CD1a^-$ cells as compared to the $CD1a^+$ subtype. Uptake of apoptotic cells results in a twofold increase of cellular content due to the lipid, protein, nucleotide and cholesterol components of the acquired cells. Loading of the host cells is compensated by various mechanisms such as the induced efflux of cholesterol by the ABCA1 transporter that is up regulated through PPARγ and LXR activation. Lipids transferred by engulfed apoptotic cells to DCs, however may modulate the composition of endogenous lipids and generate lipid ligands for PPARγ activation. As the uptake of apoptotic neutrophils was confined to a DC subset that previously was associated with high expression of PPARγ associated with tolerogenic function we presumed that the loaded DCs maintained their anti-inflammatory nature.

Further activation of apoptotic neutrophil-loaded DCs by LPS+IFNγ dramatically up regulated the secretion of the pro-inflammatory cytokines IL-8, IL-6 and TNF-α. Most importantly, loading of DCs by apoptotic neutrophils resulted in the secretion of IL-12 and IL-10, the key polarizing cytokines of inflammatory and regulatory immune responses, respectively. Similar to non-activated DCs the modulatory effect of rosiglitasone resulted in the attenuation of the pro-inflammatory response. These results altogether show that the internalization of apoptotic neutrophils by $CD1a^-$ phagocytic IDCs induces the secretion of pro-inflammatory cytokines in DCs and also sensitizes the phagocytosing cells for high IL-12 and IL-10 production, two important cytokines involved in T lymphocyte differentiation.

Apoptotic neutrophil-loaded phagocytosing dendritic cells polarize T-lymphocytes for IFNγ secretion

Based on the inflammatory response induced by internalized apoptotic neutrophils in DCs we presumed that these antigen-presenting cells are able to polarize autologous T-

lymphocytes to Th1 differentiation associated with IFNγ secretion. Using our *in vitro* phagocytosis system for mimicking an *in vivo* inflammation site, monocyte-derived IDCs were incubated for 8 hours with apoptotic neutrophils and then incubated with autologous lymphocytes for 5 days. In a parallel experiment the same monocytes were differentiated to macrophages, fed by the same apoptotic neutrophil fraction for 1 hour and co-cultured with the same autologous lymphocyte population for 5 days. After incubation of both APCs with T-lymphocytes the ratio of IFNγ-secreting cells was measured by the ELISPOT method. Lymphocytes incubated with DCs loaded by apoptotic neutrophils resulted in significantly higher number of IFNγ-secreting T-lymphocytes than DCs not sensitized by apoptotic cells. When macrophages fed by apoptotic neutrophils were used as antigen-presenting cells instead of DCs no IFNγ-secreting T-lymphocytes could be detected. These results suggest that both the cytokine response and the antigen-presenting potential of macrophages and DCs loaded by apoptotic neutrophils is different and have opposing effects on tolerance induction and immunity. Together with our previous results obtained with macrophages in a similar *in vitro* system these results suggest that the recruitment of neutrophils to inflamed tissues induces different types of responses in monocyte-derived macrophages and dendritic cells. Hence, the crosstalk of apoptotic cell-loaded macrophages and DCs through their cytokines and antigen-presenting functions may modulate the outcome of immune responses under inflammatory or other pathologic conditions.

SUMMARY

Macrophages acquire their capacity for efficient phagocytosis of apoptotic cells during their differentiation from monocytes. The peroxisome proliferator activated receptor gamma (PPARγ) is highly up-regulated during this maturation program. We have shown that addition of PPARγ antagonist during differentiation of human monocytes to macrophages significantly reduces the capacity of macrophages to engulf apoptotic neutrophils, but did not influence phagocytosis of opsonized bacteria. Macrophage-specific deletion of PPARγ. in mice also resulted in decreased uptake of apoptotic cells. The antagonist acted in a dose-dependent manner during the differentiation of human macrophages and could also reverse the previously observed augmentation of phagocytosis by glucocorticoids. Blocking activation of PPARγ led to down-regulation of molecular elements (CD36, AXL, TG2 and PTX3) of the engulfment process. Inhibition of PPARγ dependent gene expression did not block the anti-inflammatory effect of apoptotic neutrophils or synthetic glucocorticoid but significantly decreased production of IL-10 induced by LPS. Our results suggest that during differentiation of macrophages natural ligands of PPARγ are formed regulating the expression of genes responsible for effective clearance of apoptotic cells and macrophage-mediated inflammatory response.

Studying the effects of apoptotic neutrophil engulfment on DCs as compared to macrophages we have shown that apoptotic neutrophils are preferentially taken up by the $CD1a^-$ DC subset and similar to macrophages the activation of PPARγ regulates the capacity of DCs to engulf apoptotic neutrophils. In contrast with macrophages DCs internalizing apoptotic neutrophils get activated during the phagocytic process resulting in secretion of L-8, IL-6, TNF-α and IL-1β. In the presence of additional inflammatory stimuli such IFNγ and LPS, the uptake of apoptotic neutrophils sensitizes DCs for robust inflammatory responses. DCs engulfing apoptotic neutrophils were able to polarize autologous T cells resulting in Th1 differentiation associated with IFNγ secretion. When macrophages fed by apoptotic neutrophils were used instead of DCs no IFNγ secreting T cells were observed.

Our results suggest that the recruitment of neutrophils to inflamed tissues induces different types of responses in monocyte-derived macrophages and dendritic cells. Hence, the crosstalk of apoptotic cell-loaded macrophages and DCs through their cytokines and antigen-

presenting functions may modulate the outcome of immune responses under inflammatory or other pathologic conditions.

***In extenso* publications related to the thesis:**

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IF: 6.708

Posters:

First author of posters on the following meetings:

Gyöngyike Majai, Zsolt Sarang, Krisztián Csomós, László Fésüs. PPARgamma dependent regulation of human macrophages in phagocytosis of apoptotic cells ECDO, Chania, Crete, Greece 2004.

Gyöngyike Majai, Zsolt Sarang, Krisztián Csomós, László Fésüs. PPARgamma dependent regulation of human macrophages in phagocytosis of apoptotic cells ECDO, Budapest, Hungary, 2005.

Gyöngyike Majai, Zsolt Sarang, Krisztián Csomós, Gábor Zahuczky, László Fésüs PPARgamma dependent regulation of human macrophages in phagocytosis of apoptotic cells ECDO, Chia, Sardinia, Italy, September 29-October 4, 2006.

Co-author:

Klára Katona, Gyöngyike Majai, Ralph Rühl, Gábor Zahuczky and László Fésüs. Dynamic molecular regulation of phagocytosis in the apoptotic cells by macrophages**.** ECDO, Chia, Sardinia, Italy, September 29-October 4, 2006.

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