THE ROLE OF PPARγ IN CLEARANCE OF APOPTOTIC NEUTROPHILS BY HUMAN MACROPHAGES AND DENDRITIC CELLS

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

by

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1. ÖSSZEFOGLALÁS

A makrofágok differenciálódás során igen jelentős fagocitáló képesség növekedést mutatnak. A PPARγ kifejeződése differenciálódás során növekszik. Kísérleteink azt mutatták, hogy PPARγ antagonista hozzáadása differenciálódás során a makrofágokhoz, ezen sejtek fagocitáló képességének a csökkenéséhez vezet. Ez apoptótikus sejt specifikus, mert az antagonista nem befolyásolja opszonizált baktérium bekebelezését. PPARγ$^{fl/-}$(makrofágra sepecifikus PPARγ kiütés) egerekből izolált hasüregi és csontvelői makrofágokat használva észlelhető volt ezen sejtek fagocitáló funkciójának károsodása a PPARγ$^{+/-}$ kontroll egerekhez képest. Gátolva a PPARγ aktivációt olyan gének kifejeződése csökkent, mint a CD36, AXL, TG2, PTX3,

amelyekről ismert, hogy fontos szerepet töltenek be az apoptótikus sejtek bekebelezésében. Gátolva a PPARγ kifejeződését az apoptótikus sejtek és a szintetikus glükokortikoid, dexametazon, gyulladást csökkentő hatását nem tudtuk befolyásolni, de szignifikánsan gátolni tudtuk az IL-10 szekrécióját. Eredményeink arra utalnak, hogy a monocita–makrofág differenciálódás során olyan PPARγ ligandok képződhetnek, amelyek az apoptótikus sejtek eltávolításában résztvevő gének kifejeződését és gyulladásos immunválaszt szabályozzák.

A dendritikus sejtek fagocitáló képességét makrofágokhoz viszonyítva, vizsgálva eredményeink azt mutatták, hogy az apoptótikus neutrofilek fagocitálása főleg a $CD1a^-$ populációnak tulajdonítható és hogy ezt a makrofágokhoz hasonlóan a PPARγ szabályozza. Ellentétben a makrofágokkal, azok a dendritikus sejtek amelyek neutrofileket kebeleznek be aktiválódnak, amelynek következménye IL-8, IL-6, TNF-α és IL-1β gyulladásos citokinek szekréciója. Abban az esetben, ha az éretlen dendritikus sejteket IFNγ és LPS-sel aktiváltuk, ez a citokin termelés többszörös növekedést mutatott. Az apoptótikus neutrofilt fagocitáló dendritikus sejt képes volt az autológ perifériás T sejteket TH1 differenciáció irányába elnyomni és IFNγ szekrécióra késztetni. Abban az esetben, ha a dendritikus sejtek helyett makrofágot használtunk fagocitáló sejtként az IFNγ-t termelő T sejtek nem jelentek meg.

Eredményeink arra utalnak, hogy az apoptótikus neutrofilek bekebelezése eltérő választ vált ki a monocitából differenciáltatott makrofágokban és dendritikus sejtekben. Az apoptótikus sejteket bekebelező makrofágok és dendritikus sejtek egymással való kommunikációja a felszabaduló citokinek és antigén prezentálása révén befolyásolhatja az immunválasz kimenetelét gyulladásos folyamatokban vagy más patológiás körülmények között.

2. INTRODUCTION

2.1. Clearance of apoptotic cells by professional phagocytes

Clearance of apoptotic cells by professional and non professional phagocyting cells has an important role in tissue remodeling and resolution of inflammation protecting tissue from exposure to the inflammatory and immunogenic contents of dying cells. 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 (Fig.1), mainly soluble factors which bind to the distinct molecular pattern of apoptotic cells [Savill et al. 2002; Gregory et al. 2004; Majai et al. 2006]. When human macrophages engulf spontaneously dying neutrophils cell surface CD31 mediates tethering of apoptotic cells; it can discriminate between apoptotic and viable cell by selectively imparting detachment signals to viable cells preventing their ingestion [Brown et al. 2002]. 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 [Savill et al. 1990; Savill et al. 1992; Fadok et al. 1998]. The phagocytic receptor CD14 has been implicated in the ICAM-3 mediated interaction between human macrophages and dying neutrophils [Moffatt et al. 1999; Devitt et al. 2003]. 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 [Szondy et al 2003]. PTX3, an important component of innate immune response [Garlanda et al. 2005], enhances C1q binding and C3 deposition on the surface of apoptotic cells, suggesting a role of PTX3 in complement mediated phagocytosis of apoptotic cell [Nauta et al. 2003].

In dendritic cells the engulfment of apoptotic cells is restricted to their immature state and is mediated by the $\alpha_v\beta_5$ integrin, the scavenger receptor CD36, and the MFG-E8 receptor [Albert et al. 1998; Miyasaka et al. 2004]. Dendritic cells are able to acquire and present antigens from apoptotic cells and stimulate antigen specific class I-restricted $CD8^+$ T cells referred to as ‘cross priming’ [Geijtenbeek et al. 2000]. This T-cell stimulatory capacity is dependent on activation signals mediated by inflammatory stimuli [Albert et al. 1998].

The C-type lectin DC-SIGN (CD209) is expressed by both immature and mature dendritic cells and functions as both an adhesion and pattern recognition receptor. DC-SIGN binds to ICAM-2 [Geijtenbeek et al. 2000] on endothelial cells and to ICAM-3 on T-cells required for the initiation of immune responses [Geijtenbeek et al. 2000; Geijtenbeek et al. 2000]. It was also shown that DC-SIGN does not bind to ICAM-3 present on the surface of neutrophils, but its binding is specific to the polymorphonuclear β_2 integrin Mac1 [van Gisbergen et al. 2005]. As a pattern recognition receptor, DC-SIGN binds to of variety of viral, bacterial, fungal and parasitic pathogens including human immunodeficiency virus 1 [van Kooyk et al. 2003; Hodges et al. 2007], explicable by containing carbohydrate domain with specifity for both high mannose moieties and non-sialylated Lewis antigens [van Gisbergen et al. 2005]. 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 [van Gisbergen et al. 2005]. These suggest that cell specific glycosylation is important in the regulation of cellular interactions between DC and neutrophils.

2.2. Suppression of inflammation by apoptotic cells

2.2.1. Apoptotic cells, unlike necrotic cells, do not induce inflammatory response

In contrast to apoptosis, necrosis is characterized by loss of membrane integrity, swelling and disintegration leading to the release of cellular contents. Macrophages discriminate innately between cells that have undergone a physiological death and those

that have suffered a pathological form of death. Recognition of these two classes of dying cells occurs via distinct and noncompetitive mechanisms [Cvetanovic et al. 2004]. The lysed necrotic cells or apoptotic cells undergoing secondary necrosis can initiate an inflammatory response in macrophages since they can release proteases, inflammatory eicosanoids, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein 2 (MIP-2), IL-8, tumor necrosis factor (TNF)-α and they can engage TLR2. The DNA-binding protein HMGB-1, which elicits a pro-inflammatory response in macrophages through TLR2 and TLR4, can also leak out of necrotic but not of apoptotic cells [Fadok et al. 2001; Scaffidi et al. 2002]. Therefore, leakage of macromolecules should be prevented during the apoptotic process to avoid inflammation; one mechanism for this is the induction and activation of the protein cross-linking enzyme, transglutaminase (TG) 2 in apoptotic cells [Piredda et al. 1997]. The major mechanism to prevent secondary necrosis is the recognition and fast removal of early phase apoptotic cells [Gregory et al. 2004]. However, even cells undergoing secondary necrosis may not necessarily be pro-inflammatory either because of biochemical mechanism sequestering pro-inflammatory molecules inside dying cells or as the result of the anti-inflammatory effects of the co-existing apoptotic cells. Indeed, there is ample evidence to show that apoptotic cells do not only fail to induce but can actively suppress the release of pro-inflammatory cytokines from macrophages which engulf them.

2.2.2. How apoptotic cells suppress inflammation?

Early studies demonstrated that apoptotic cells actively suppress an inflammatory response, and not just fail to provide inflammatory signals. The presence of thymocyte-derived apoptotic cells during monocyte activation increased their

secretion of the anti-inflammatory and immunregulatory cytokine IL-10 and decreased production of the pro-inflammatory cytokines such as TNF-α, IL-1 and IL-12. [Voll et al. 1997] Later, these results were reproduced using apoptotic neutrophils instead of apoptotic lymphocytes [Byrne et al. 2007]. Fadok *et al.* had shown that phagocytosis of apoptotic neutrophils by human macrophages inhibited the production of IL-1β, IL-8, IL-10, GM-CSF, TNF-α, as well as leukotriene C4 and tromboxane B2 trough an autocrine/paracrine mechanism and increased the production of TGF-β1, prostaglandin E2 and platelet activating factor [Fadok et al. 1998]. Moreover, TGF-β neutralizing antibodies largely reversed the inhibitory effect of apoptotic cell uptake and exogenous TGF-β1 down-regulated the synthesis of the chemokines IL-10 and TNF-α [McDonald et al.1999]. There are convincing *in vivo* data that TGF-β1 released by macrophages has an anti-inflammatory effect in inflamed peritoneum and lung [Huynh et al. 2002]. Using the LPS lung inflammation model it was demonstrated that surfactant protein A has a role in induction of alveolar macrophage TGF-ß1 release thereby promoting the resolution of acute inflammation within alveolar space [Reidy et al. 2003]. All these data led to the conclusion that suppression of inflammatory response by apoptotic cells in macrophages is mediated manly by an indirect mechanism through the release of TGF-ß1 [Henson et al. 2001]. In contrast to this it was found by Cvetanovic *et al.* that the initiation of inflammatory modulation occurs as a direct consequence of the interaction of apoptotic target cells with macrophages and without involvement of TGFβ [Cvetanovic et al. 2004]. Nevertheless, the high importance of TGFβ in the clearance of apoptotic cells has been strengthened by the observation that lack of TG2, the cross-linking enzyme which is involved in the biochemical maturation of TGFβ, compromises phagocytosis of apoptotic cells and the anti-inflammatory response to the dying cells [Szondy et al. 2003; Falasca et al. 2005].

The signaling mechanisms that determine the anti-inflammatory mediator release is far from being completely understood. The process appears to be related to the appearance of phosphatidylserine (PS) on the surface of apoptotic cells and recognition of PS by macrophages through a PS receptor was suggested to be the dominant element in the release of TGF-β [Fadok et al. 2000]. Antibody ligation experiments indicate that CD36 and vitronectin receptor, and bridging protein TSP1 can suppress monocyte/macrophage inflammatory responses by stimulating TGF-β release [Voll et al. 1997; Freire-de-Lima et al. 2000]. The endogenous ligand, Annexin-I, inducible by glucocorticoids as well, seems to be capable of inducing IL-10 production and IL-12 synthesis inhibition [Ferlazzo et al. 2003]. A direct suppressive signaling could arise through the tyrosine inhibitory domain of CD31 and kinase domain of MER via Gas-6 [Savill et al. 2002]. Another signaling pathway which does not require soluble factors and can suppress the pro-inflammatory response is the ligation of Src homology 2 domain bearing protein tyrosine phosphatase substrate -1 (SHPS-1) [Tada et al. 2003].

It is important to realize that a maturation process must take place before macrophages can recognize and remove dead cells and display a concomitant anti-inflammatory response [Newman et al. 1982; Giles et al. 2001]. This suggests that gene expression regulation is involved in preparing macrophages to these tasks. Glucocorticoids are the most effective anti-inflammatory and immunosuppressive agents. They have been shown to inhibit in macrophages the expression of cytokines, adhesion molecules, and enzymes involved in the inflammatory process. Although treatment of maturing macrophages with glucoroticoids results in a large increase in the efficiency of macrophages to engulf dying neutrophils [Giles et al. 2001], there is no evidence that the anti-inflammatory effects of apoptotic cells and glucocorticoids are interrelated. Not only the phagocyting cells can release anti-inflammatory cytokines, but

also the cells that are dying by apoptosis can release cytokines with direct immunosuppressive properties. It was shown that apoptotic T cells release TGF-β1 which is not only latent but also bioactive and is localized within the intracellular membrane bound compartments including mitochondria [Chen et al. 2001]. Administration to the eye of antigen-bearing lymphocytes showed that the observed tolerance depends on the ability of dying cells to secrete IL-10 [Gao et al. 1998].

In contrast to previous studies which suggested an anti-inflammatory effect of apoptotic cells, Iyoda *et al.* showed that injection of apoptotic cells into the peritoneal cavity induced the expression of an inflammatory chemokine, MIP-2, and infiltration of neutrophils; anti-MIP-2 antibodies suppressed this infiltration significantly [Iyoda et al. 2004]. They also showed that macrophage mediated uptake and digestion of apoptotic thymocytes was accelerated upon co-culturing them with neutrophils and the latter are recruited for acceleration of apoptotic-cell clearance in tissues with high apoptosis rate [Iyoda et al. 2005].

2.2.3. Loss of the anti-inflammatory response to apoptotic cells leads to diseases

Apoptotic cells also can suppress the inflammatory response elicited by PAMPs through the TLRs. The combination of apoptotic cells and ligands for TLR 2, 4, and 9 mount cytokine responses that differ importantly from those elicited by either class of stimulus alone. TLR ligands induced early secretion of TNF-α, MIP-1α, and MIP-2 with later secretion of IL-10, IL-12, TGF-β1; apoptotic cells alone stimulated TGF-β1 secretion only. The combination of apoptotic cells and TLR ligands enhanced early secretion of TNF-α, MIP-1α, and MIP-2 and increased late TGF-β1 secretion, while suppressing late TNF-α, IL-10, IL-12 by a mechanism which could nevertheless be overridden by IFNγ [Stuart et al. 2003 39]. These results point to the possibility that

inflammatory diseases initiated by microbial pathogens are influenced, very likely attenuated, by apoptosis occurring in the infected tissues and lack of this influence may contribute to a serious outcome in the pathologic response. In some pathologic conditions like chronic granulomatous disease (CGD) phagocytes are severely compromised in their ability to produce anti-inflammatory mediators such as PGD2 and TGF-ß1 during clearance of apoptotic debris and invading pathogens, contributing to persistence of inflammation in CGD [Brown et al. 2003].

The clearance of apoptotic cells has a role not only in tissue homeostasis, but provides also a source of antigens for immune tolerance and activation. Macrophages' anti-inflammatory and immunosuppressive tendencies in responding to apoptotic cells make them a potentially powerful regulator of adaptive immune responses, including autoimmune and anti-tumor responses. The engulfment of apoptotic cells by dendritic 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 [Albert et al. 2004]. This process can be beneficial in the development of tumor immunity, but

severely pathogenic when it targets self. Defective clearance of apoptotic cells is often associated with autoimmune diseases: mice with deleted C1q, MER tyrosine kinase, TG2 or MFG-E8 have high titres of autoantibodies and develop autoimmune syndromes [Botto et al. 1998; Scott et al. 2001; Cohen et al. 2002; Hanayama et al. 2004]. In TG2 knock out mice even neutrophils show up in tissues where a high rate of apoptosis has been initiated [Szondy et al. 2003]. On the other hand, lack of CD14 does not lead to autoimmune disease despite of extensive persistence of apoptotic cells in tissues. It has been suggested that defective apoptotic-cell clearance plays a primary role in autoimmune disease pathogenesis only under circumstances when the clearance deficiency is accompanied by a defect in the regulation of anti-inflammatory response by apoptotic cells or it seems to be the case in C1q, MER tyrosine kinase, TG2 or MFG-E8 knock out mice [Gregory et al. 2004].

2.3. The role of PPARγ in monocyte/macrophage and monocyte/dendritic cell differentiation

PPARs (peroxisome proliferator activated receptors) are members of the nuclear receptor superfamily which heterodimerize with the retinoid X receptor [Kliewer et al. 2002]. PPARγ was characterized originally as a key regulator of adipocyte differentiation and lipid metabolism and it also plays an important role in glucose metabolism. PPARγ is activated by diverse synthetic and naturally occurring ligands including anti-diabetic thiazolidindione polyunsaturated fatty acids (these days in clinical use), 15-Deoxy-$\Delta^{12,14}$ Prostaglandin J_2 (15d-PGJ_2) and components of oxidized low density lipoproteins [Lehmann et al. 1995; Willson et al. 1996; Kliewer et al. 1995 and 1997; Krey et al. 1997, , Forman et al. 1995; Nagy et al. 1998]. Recent studies have

suggested that PPARγ may promote monocyte-macrophage differentiation [Tontonoz et al. 1998]. 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 [Ricote et al. 1998; Szanto et al. 2005]. 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 [Ricote et al. 1998; Chung et al. 2000; Li et al. 2000]. It has not been clarified whether PPARγ is required during differentiation of macrophages to prepare them for efficient phagocytosis of apoptotic cells. everal observations suggest that not only monocytes and macrophages, but also dendritic cells express PPARγ receptors at high levels. Monocyte-derived dendritic cells represent a heterogeneous population of cells that can be divided to $CD14^{low}CD1a^{-}$ and $CD14^{-}CD1a^{+}$ subsets that correspond to consecutive differentiation stages [Gogolak et al. 2007]. The $CD1a^{-}$ subtype exhibits higher internalizing capacity of dextrane, latex beads or bacteria as compared to their $CD1a^{+}$ counterparts. The ratio of the $CD1a^{-}$ subset can be increased by the transcriptional activation of the PPARγ receptor gene through its synthetic ligand rosiglitasone [Gogolak et al. 2007, Szatmari et al. 2004].

3. 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

4. MATERIALS AND METHODS

Materials

Sterile plastics were purchased from BD Biosciences and Corning Costar, IMDM was from Gibco, PBS was from Oxoid, RPMI, dexamethasone, Histopaque1119, Histopaque1077, AB serum, propidium iodide, 3,3' dimethoxybenzidine, carboxylate modified fluorescent polystyrene latex beads, brain heart infusion, Ca-Ionophor A23187 and LPS were purchased from Sigma Aldrich Listeria Monocytogenes was from LGC Promochem. Ficoll–Paque Plus was obtained from Amersham Bioscences. CD14 microbeads, human specific were purchased from Miltenyi Biotec, Germany. Human macrophage colony stimulating factor (M-CSF) human granulocyte macrophage colony

stimulating factor (GM-CSF) murine macrophage colony stimulating factor and IL-4 were from Peprotech. The PPARγ ligands GW9662 and Rosiglitasone were purchased from Alexis Biochemicals.

FITC conjugated mouse IgM, PE conjugated mouse IgG1k and mouse, anti human CD36, CD16, CD206 CD209, CD1a monoclonal antibodies were from BD Biosciences Pharmingen, PE conjugated mouse IgG1k and mouse, anti human CD209, CD40, CD1a monoclonal antibodies were from BD Biosciences Pharmingen. Inhibitory anti CD209 antibody was from Abcam (ab13487). PE conjugated mouse IgG2a and monoclonal mouse anti human CD14 antibodies were obtained from Dako Cytomation. 5(6)-CFDA-SE was purchased from Molecular Probes.

Cell Tracker^{TM} Orange CMTMR and 5(6)-CFDA-SE were purchased from Molecular Probes.

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 (Leucomax) and 500 U/ml IL-4 (Peprotech) 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 [Giles et al. 2001]. The apoptosis of neutrophils was verified by light microscopy using May–Giemsa staining and by flow cytometric analysis of propidium iodide stained samples before feeding them to macrophages. 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). Mice carrying null or floxed alleles of PPARγ were created as described previously [Barak et al. 1999; Miles et al. 2000; Hevener et al. 2003]. These mice were backcrossed to the C57BL/6J strain for eight generations. The lysosyme promoter was utilized to express the *cre* recombinase specifically in the macrophages. Mice were bred with Lysozyme-Cre (Lys-Cre) transgene animals by L. Nagy *et al*. in order to create the following genotypes: $PPAR\gamma^{+/-}$ Lys-Cre and $PPAR\gamma^{fl/-}$ Lys-Cre. Genotypes were determined by PCR of tail DNA as described previously [Barak et al. 1999; He et al. 2003]. Recombination was analyzed by RT PCR as previously [Clausen et al. 1999].

Lysozyme-Cre animals were obtained from I. Förster (University of Munich). Testing large number of animals the efficacy of the Lys-Cre mediated recombination was found to be the same in bone marrow and peritoneal macrophages [Szanto et al.]. All animal experiments were carried out under ethical guidelines established by the 28th Act in 1998 of the Parlament of the Republic of Hungary.

Bone marrow cells were isolated from the femur of PPAR$\gamma^{+/-}$ Lys-Cre and PPAR$\gamma^{fl/-}$ Lys-Cre mice, then were washed in saline 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 and assayed 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 at 37°C and 5% CO_2.

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 before their 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. Macrophages were gated on the basis of forward- and side-scatter properties and the percentage of macrophages positive for both CMTMR and CFDA-SE was determined subtracting 4 ^{o}C values from 37 ^{o}C ones.

Phagocytosis of Listeria Monocytogenes

The bacterial strain used in this study is derived from ATCC 35152 (LGC Promochem). Cultures of Listeria Monocytogenes were grown in brain heart infusion at 37 ^{0}C for 15h. Listeria Monocytogenes was labelled with FITC isomer (0,1mg/ml, overnight at room temperature). Next morning the bacteria was washed with PBS and opsonized with 10% serum for 10 min. After opsonization the serum was removed by centrifugation 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 PBS before their 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. Dendritic cells were gated on the basis of forward- and side-scatter properties and the percentage of dendritic cells positive for both PE conjugated CD40 and CFDA-SE was determined subtracting 4 ^{o}C values from 37 ^{o}C ones. For inhibition of membrane CD209 binding to neutrophils the inhibitory anti-CD209 mAb was added to the DC at 5 μg/ml

concentration 30 min before the phagocytosis assay was started and it was present throughout the whole period of the DC – apoptotic neutrophil co- incubation.

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. The sets were factory-loaded into the 384 wells of TaqMan Low Density Array (TLDA). Array format was customized with two replicates per target gene and for each biological sample two parallel analysis were carried out. TaqMan low-density arrays were used in a two-step RT-PCR process. First strand DNA was synthesized from 1 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems). Q-PCR reactions and analysis were then carried out in TLDAs using TaqMan Universal Master Mix and ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). 100 μl sample volume per port (4 ports /sample) was used in 1ng/μl concentrations. Expression levels of target genes were normalized to 18S rRNA as endogenous control. Gene expression values were calculated based on the ΔΔCt method, where one sample was designated as calibrator, through which all other samples were analyzed. ΔCt represents the threshold cycle (Ct) of the target minus that of 18S rRNA and ΔΔCt represents the ΔCt of each target minus that of the calibrator. Relative quantities (RQ or fold changes) were determined using the equation where relative quantity equals $2^{-\Delta\Delta Ct}$.

Confocal microscopy

For confocal laser scanning microscopy, a Zeiss (Göttingen, Germany) LSM 510 system was used. Fluorescein (CFDA) and phycoerythrin (PE) were excited with 488 nm Ar ion and 543 nm HeNe laser and detected through 505-550 nm and 560-615 nm emission filters respectively. For high resolution imaging with a Plan-Apochromat 63×/1.4 NA oil immersion objective pinholes were set to obtain 1 μm optical sections and 512×512 pixel images were taken at 12 bit resolution with pixel times of 3.2 μs and 4× line-averaging. For statistical analysis 75 images of 512×512 pixels (5×10^4 μm^2) were taken from each sample, using a C-Apochromat 40×/1.2 NA water immersion objective at extended depth of focus (8 bit resolution, pixel time 2.5 μs, 2× line-averaging). Image channels were recorded separately and software compensation was used to correct for crosstalk of fluorescein into the PE channel.

Digital Image Processing

Quantitative digital image processing tasks were performed using the C programming environment of SCIL-Image (TNO, Delft, The Netherlands). Binary masks to identify individual cells based on PE fluorescence were created by thresholding the fluorescence image after background subtraction using the rolling ball algorithm. Lower threshold values were determined as average fluorescence from cell-free areas of the image; small objects originating from background noise were removed using another binary mask after Gauss filtering; finally selection was confirmed manually in each case. Each cell area was then treated as an individual ROI and a Kuwahara edge-preserving filter using a circular filter window was applied to identify the membrane region. Occasional holes in the membrane masks were filled with a dilation-erosion cycle using a 3x3 mask and a connectivity of 8 pixels. Crosstalk-corrected, background-subtracted median fluorescence from each cell's membrane region was tabulated, noting also whether the

cell has phagocytosed (presented CFDA signal inside the membrane region). For each treatment category, a distribution histogram was constructed which collectively clearly indicated two populations, with low and high membrane intensity respectively and served to determine the cutoff level (60 arbitrary units of fluorescence intensity) for numerating cells expressing high levels of CD209.

Human IFNγ ELISPOT assay

Monocyte-derived macrophages and dendritic cells were co-cultured with non-labelled apoptotic neutrophils for 8 hours. After thorough washing procedures (CTL wash medium, Cellular Technology, Cleveland, OH) the harvested cells were seeded to 24 wells plate and were co-cultured with autologous lymphocytes at a ratio of 1:25 for 5 days at 37 ^{o}C in 5% CO_2 atmosphere. On day 5 the cell were collected and subjected to a 24-hour IFN-γ Ready Set Go ELISPOT assay (eBioscience, San Diego, CA). The MultiScreen-HTS PVDF plates were purchased from Millipore S.A., Molsheim, France. After 24h at 37 C in 5% CO_2, the cells were removed and the plates were washed with PBS. Detection of cytokine spots was performed by biotinylated IFN-γ-specific antibody and avidin-HRPO conjugate. (BD Biosciences Pharmingen, San Diego, CA). The plates were washed with tap water to stop the reaction and air-dried. The resulting spots were counted using a computer assisted ELISPOT image analyzer (Series 1 ImmunoSpot Analyzer, ImmunoSpot Version 4.0 Software Academic, Cellular Technology, Cleveland, OH) customized for analyzing ELISPOT to meet objective criteria for size, chromatic density, shape, and color.

Determination of cytokine release by human macrophages

Macrophages aged 5 days in the presence of various agents were stimulated with 0.5 μg/ml LPS for 30 min. Part of samples was incubated for 25 min with apoptotic neutrophils following LPS stimulation. After this period the non-ingested neutrophils were removed and the macrophages were incubated in fresh media without serum for 20h. The supernatants were collected and analyzed for the presence of IL-8, IL-6, IL-1β, IL-10, TNF-α, IL-12p70, IL-3, IL-4, IL-5, IL-7, and GM-CSF using the Human Inflammation and Human Allergy Mediators BD Cytometric Bead Assay. TGF-β1 measurements were performed by Elisa using BD OptEIA Set Human TGF-β1 kit (BD Biosciences).

Determination of cytokine release by immature and activated dendritic cells

Dendritic cells aged 5 days in the presence or absence of Rosiglitasone were incubated for 30 min respectively 8 hours with fresh, apoptotic neutrophils or supernatant from apoptotic cells. After this incubation time part of the samples were treated, with 0.1 μg/ml LPS and 10 ng/ml IFNγ for 16 hours. Following this incubation the supernatants 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 Assay.

Statistical analysis

Statistical analysis was performed by using unpaired Student t test, Kruskal Wallis non-parametric ANOVA and Wilcoxon-Mann-Whitney post test.

5. RESULTS

5.1. 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 (Fig.2). 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 (Fig.3A, B, C).

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 [Szanto et al. 2005]. 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 (Fig.3A). 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 [Leesnitzer et al. 2002]. 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 (Fig.3B,C). The decrease in phagocytosis capacity has correlated with increasing GW9662 concentrations (Fig.4A). Adding T0070907, another selective antagonist of PPARγ acting by blocking its interaction with coactivators [Lee et al. 2002], also lead to decreased phagocytosis when it was added in 10 μM concentration to differentiating cells; in case of two independent donors the inhibition was 28.5% and 18.8%, respectively. Reduced phagocytic capacity of GW9662 treated macrophages was also confirmed by using carboxylate modified polystyrene latex beads (Fig.3D) which can mimic apoptotic cells [Gumienny et al. 2001; Tosello-Trampont et al. 2001; Tosello-

Trampont et al. 2003]. GW9662 did not inhibit the engulfment of opsonized Listeria Monocytogenes (Fig.3G), suggesting that inhibition of PPARγ mediated processes specifically suppresses uptake of apoptotic cells.

It has been shown that dexamethasone can reprogram monocyte differentiation toward a pro-resolution phenotype, exhibiting increased phagocytosis of apoptotic cells [Giles et al. 2001]. 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 (Fig.3E, F).

Next, we wanted to determine when during the differentiation period GW9662 can interfere with development of the full capacity of engulfing apoptotic cells. During the 5 days maturation period monocyte derived macrophages were incubated with 10μM GW9662 for various time periods prior to the assessment of phagocytosis. GW9662 reduced phagocytosis of apoptotic neutrophils in a time dependent manner and our data indicate that significant inhibition of phagocytosis by PPARγ antagonist requires its presence from the early phase of macrophage maturation (Fig.4B). When GW9662 was added to the macrophages for the duration of the phagocytosis assay itself, it did not influence the uptake of apoptotic neutrophils (data not shown).

5.2. 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 [Huang et al. 1999]. 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 (data not shown). Differentiated bone marrow derived and peritoneal macrophages from PPARγ$^{fl/-}$ Lys Cre mice showed reduced uptake of apoptotic thymocytes as compared to controls (Fig. 5A,B).

5.3. 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 expression of the known PPARγ target gene FABP4 [Graves et al. 1992]. A 3.5 fold induction of PPARγ. and 14 fold induction of FABP4 were observed by Q-PCR analysis during the differentiation period (Fig. 6A). Cells cultured for 5 days in the presence of 10μM 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 [Henson et al. 2006; Savill et al. 1990; Savill et al 1992; Fadok et al. 1998; Szondy et al. 2003; Garlanda et al. 2005] 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. The list of genes which include phagocytosis receptors, cell surface regulatory molecules, bridging molecules, signal transducers, engulfment proteins, effector molecules, transcription factors, inflammatory regulators and cytokines is provided as supplementary data to this manuscript. 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 [Luciani et al. 1996] 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 (Fig. 6A).

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 (Fig. 6A,B). 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. As it is seen in Table 1., 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 (Fig.6B). 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 (Table 1.). Dexamethasone treatment, added to macrophages either alone or in combination with GW9662, did not affect the expression of these differentiation markers.

5.4. Cytokine profile of LPS stimulated macrophages treated by PPARγ antagonist

Macrophages aged 5 days in the presence of various agents were stimulated with LPS for 30 min, and then incubated in fresh media without serum for 20 hours. Following stimulation with LPS a portion of macrophages was incubated for 25 min with apoptotic neutrophils, then the non-ingested neutrophils were removed and the macrophages were incubated in fresh media without serum for 18-20h. The collected supernatants were used for measurement of cytokine concentrations.

Cells differentiated in the presence of GW9662 did not show significant difference in secretion of TNF-α, IL-6, IL-8 and TGF-β upon LPS stimulation but had much reduced IL-10 secretion (Fig.7). 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 (Fig.7).

Several studies have demonstrated that contact with apoptotic cells leads to a potent anti-inflammatory and immunosuppressive response in macrophages through the production of anti-inflammatory cytokines such as TGF-β and suppression of inflammatory mediators like IL-8 and TNF-α [Fadok et al. 1998; Voll et al. 1997]. In accordance with these data apoptotic cells up regulated TGF-β secretion and down regulated IL-6, TNF-α and IL-8 in LPS stimulated macrophages in our study (Fig.7). 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 LPS stimulation. 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 (Fig. 7).

We did not observe changes in the induction of the inflammatory cytokines IL-1β (Fig.7) and IL-12p70 or GM-CSF (not shown) following either GW9662 treatment or incubation of stimulated macrophages with apoptotic cells. Normally, macrophages do not secret cytokines IL-3, IL-4, IL-7, and IL-5. Using the PPARγ antagonist GW9662 during the differentiation period we could not induce the secretion of these

cytokines either by LPS or by apoptotic cells (data not shown) suggesting that PPARγ does not play a role in blocking the production of these cytokines.

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

Our previous results [Majai et al. 2007] showed that the engulfment of apoptotic neutrophils by *in vitro* differentiated human macrophages reached a plateau at 25 min involving about 30% of the phagocytes. Using similar culture conditions we found that immature monocyte-derived DC (DC) are also able to internalize apoptotic neutrophils albeit this process is slow and less efficient than that of macrophages. As shown in Fig.8 the internalization of neutrophils undergoing apoptosis by immature DC requires 8 – 24 hours and results not more than 20 – 35 % of phagocytosing cells. Taken the rapid progress of neutrophil apoptosis and to avoid secondary necrosis, in our further experiments we used an *in vitro* phagocytosis system where immature DC were mixed with an excess of neutrophils (1:5 ratio) previously undergoing spontaneous apoptosis for 16 hours (~35% apoptotic cells, Fig.8), and co-cultured them for an additional 8 hours with DC.

Our previous studies also revealed that PPARγ is up-regulated during the differentiation of monocytes towards both macrophages [Szantó et al. 2005] and DC [Szatmári et al. 2004; Gogolák et al. 2007]. 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 of dextran sulphate, latex beads and pathogens. To assess whether the capacity of DC 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 DC for their capability to internalize apoptotic neutrophils. As it is shown in Fig 9A, the ratio of $CD1a^-$ cells varied individually, but DC 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 (Fig.9B). 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.

5.6. Pro-inflammatory 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 regulate signaling events initiated by pro-inflammatory stimuli [Ferguson et al. 2001; Steinman et al. 1998]. 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 [Majai et al. 2007; Voll et al. 2007]. In order to study the effect of apoptotic neutrophil uptake on cytokine secretion of immature DC apoptotic neutrophils were added to DC for 8 hours and the cell culture supernatants were harvested. As shown in Fig.10 the engulfment of apoptotic neutrophils by DC resulted in the up regulation of IL-8, IL-6, TNF-α and IL-1β secretion in all the three donors tested, however IL-6, TNF-α, and IL-1β cytokine concentrations showed high individual variations. To provide evidence that the measured cytokines were secreted by

dendritic cells we measured the concentration of these cytokines also in the supernatant of apoptotic cells. The culture medium of apoptotic neutrophils, harvested after 16 hours of incubation contained between 969-2430 pg/ml IL-8, whereas IL-6, TNF-α, IL-1β, IL-10 or IL-12 were undetectable (data not shown). Incubation of DC with fresh neutrophils for 30 minutes resulted in similar or even lower amounts of secreted cytokines than that measured in the DC cultures suggesting that the pro-inflamatory cytokine response is the consequence of co-incubation of DC with apoptotic neutrophils (data not shown).

Dendritic cells generated in the presence of rosiglitasone contain predominantly $CD1a^-$ cells (Fig.9A) and engulf more apoptotic neutrophils than those generated in the absence of rosiglitasone (Fig.9B). Remarkably, -treated and neutrophil loaded DC secreted less IL-8, IL-6 and IL-1β but not TNF-α than DC generated in the absence of PPARγ agonist however differences in the concentration of secreted cytokines by DC differentiated in the absence or presence of RSG were not statistically significant (Fig.10A). In accordance with our previous results on the more tolerogenic nature of $CD1a^-$ cells as compared to the $CD1a^+$ subtype [Gogolák et al. 2007] these data suggest that increased expression and activation of PPARγ through RSG favours the generations of CD1a- cells and the uptake of apoptotic neutrophils but does not block the inflammatory response of DC.

5.7. Activation-induced cytokine secretion of dendritic cells incubated with apoptotic neutrophils

To assess how the presence of neutrophils may modulate the cytokine response of DC triggered by additional inflammatory stimuli we washed the neutrophil-loaded DC to remove the autocrine cytokines and activated the cells with LPS+IFNγ for a

further 16 hours (Fig.10B). This treatment dramatically and significantly up regulated the secretion of the pro-inflammatory cytokines IL-8, IL-6 and TNF-α as compared to DC not exposed to apoptotic neutrophils. Most importantly, the activation of DC previously co-cultured with apoptotic neutrophils resulted in the concomitant secretion of IL-12 and IL-10, an atypical cytokine pattern as compared to TLR4-mediated signaling. Nevertheless, both cytokines are referred to as key polarizing factors of inflammatory and regulatory immune responses, respectively.

Similar to non-activated DC the modulatory effect of rosiglitasone resulted in the attenuation of the inflammatory response induced by apoptotic cells and LPS+IFNγ (Fig.10A,B). These results altogether show that the long term contact and the internalization of apoptotic neutrophils by $CD1a^-$ phagocytic DC sensitize the DC for inflammatory IL-12 and also regulatory IL-10 production combined with the trigering of enhanced pro-inflammatory cytokine secretion.

5.8. Dendritic cells incubated with apoptotic neutrophils polarize T-lymphocytes for IFNγ secretion

Based on the inflammatory response induced by internalized apoptotic neutrophils in DC 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 DC 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 APC i.e. monocyte-derived macrophages and dendritic cells derived

from the same donor with autologus T-lymphocytes the ratio of IFNγ-secreting cells was measured by the ELISPOT method. As it is shown in Fig.11A lymphocytes incubated with DCwhich previously engulfed apoptotic neutrophils resulted in significantly higher number of IFNγ-secreting T-lymphocytes than DC not sensitized by apoptotic cells. When macrophages fed by apoptotic neutrophils were used as antigen presenting cells instead of DC no IFNγ-secreting T-lymphocytes could be detected (Fig.11B). These results suggest that both the cytokine response and the antigen presenting potential of macrophages and DC loaded by apoptotic neutrophils is different and have opposing effects on tolerance induction and immunity.

6. DISCUSSION

6.1. The role of PPARγ in macrophage phagocytosis and inflammation process

For the greater benefit and less risk of PPARγ ligands in clinical use the tissue specific biology of this nuclear receptor should be fully understood. It has been shown that macrophages derived by *in vitro* differentiation of PPARγ-deficient mouse embryonic stem cells were comparable to wild type similarly derived macrophages in their expression of macrophage-specific cell surface markers, phagocytic activity toward microbial agents and their inflammatory responses [Moore et al. 2001]. In our study typical cell surface markers of macrophages could be detected when PPARγ

antagonist was present during their differentiation (Table 1.). This means that similarly to the mouse system [Moore et al. 2001], differentiation of human macrophages, at least in terms of commonly used parameters, also can take place without the presence of functioning PPARγ. Maturation of macrophages from monocytes is required before they can recognize and remove dead cells and display a concomitant anti-inflammatory response [Giles et al. 2001; Newman et al. 1982]. 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. Furthermore, macrophage specific deletion of PPARγ in mice led to decreased phagocytosis of apoptotic cells by either differentiated bone marrow cells or peritoneal macrophages. 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.

Our results clearly suggest that a naturally formed ligand of PPARγ acts during macrophage differentiation driving expression of apopto–phagocytic genes. We could demonstrate that macrophages which differentiate in the presence of PPARγ antagonist express significantly less CD36, TG2, AXL and PTX3, suggesting their regulation by PPARγ. Expression of other so far described pro-phagocytic genes was not in influenced by PPARγ antagonist. Cell surface expression of CD36 was decreased and the TG2 content of antagonist treated cells were reduced. Interestingly, concentration of detectably CD14 was also decreased on the cell surface in antagonist treated cells, though level of CD14 messenger RNA did not change. PPARγ was earlier found to modulate CD36 gene expression through direct interaction with the proximal promoter of CD36 via its specific response element [Tontonoz et al. 1998; Chawla et al. 2001].

The TG2 gene is under retinoid regulation in macrophages and it has been recently shown that PPARγ can induce retinoid producing enzymes leading to indirect activation of TG2 [Szatmari et al. 2006]. There is no data available which may link link regulation of AXL or PTX expression to PPARγ ligation. PTX3 production is mainly induced by inflammatory signals [Rovere et al. 2000]; the human PTX3 proximal promoter contains Pu1, AP-1, NF-κB, SP-1 and NF-IL-6 sites [Garlanda et al. 2005].

Are the observed changes in gene expression upon blocking PPARγ activity sufficient to explain the decreased phagocytosis 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 [Henson et al. 2006]. In case of phosphatidylserine (PS) recognition - which is a dominant „eat me" signal when neutrophils undergo apoptosis [Fadok et al. 1998], 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 [Szondy et al. 2003]. With TLDA we could determine that all the necessary molecular elements of PS-dependent recognition and engulfment are expressed in differentiated human macrophages [Petrovski et al. 2007]. 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 [Fadok et al. 1998]. 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 LRP1 [Henson et al. 2006]. 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 [van Rossum et al. 2004]. 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 [Garlanda et al. 2005; Nauta et al. 2003]; by blocking PTX3 expression PPARγ antagonist may significantly reduce efficiency of phagocytosis.

In our system addition of synthetic PPARγ ligand Rosiglitasone to maturing macrophages could not increase either their phagocytic capacity toward apoptotic cells (Fig.3) or CD36 expression (not shown). It was reported that PPARγ ligand treated alveolar macrophages present both enhanced phagocytic capacity and increased CD36 expression [Asada et al. 2004]. It should be noted that alveolar macrophages are highly specialized phagocytic cells which, unlike the M-CSF differentiated macrophages in our study, may become sensitive to PPARγ ligands while they mature in the lung.

There are several studies which have suggested that PPARγ-specific ligands can regulate inflammation inhibiting the expression of various pro-inflammatory proteins. On the other hand, several investigators have proposed that PPARγ activation has no

anti-inflammatory effects. PPARγ agonists have been found to inhibit the secretion of the pro-inflammatory mediators such as gelatinase B, IL-6, TNF-α and IL-1 β in monocytes and also reduced the expression of inducible NOS, scavenger receptor A, metalloproteinase 9, IL-10 and IL-12 in macrophages [Ricote et al. 1998; Jiang et al. 1998; Azuma et al. 2001; Shu et al. 2000]. In contrast, it was reported by others that the PPARγ ligand 15d-PGJ2 induced IL-8 gene expression, suppressed monocyte chemoattractant protein-1 and did not affect the secretion of the chemokine RANTES in human monocyte/macrophages. The PPARγ agonist Rosiglitasone failed to modulate LPS-induced IL-8 secretion [Zhang et al. 2001]. It has been also shown in macrophages with deleted PPARγ that PPARγ expression is not essential for PPARγ ligands to exert their anti-inflammatory effects [Chawla et al. 2001]. From these studies one may conclude that the effects of PPARγ ligands on macrophages inflammatory responses may depend on the type of PPARγ ligand used, the mode by which macrophages are activated and the investigated inflammatory response.

In our experiments inhibition of PPARγ during macrophage differentiation did not influence the basal cytokine production of these cells (data not shown). The most significant consequence of blocking PPARγ-dependent gene expression during macrophage differentiation was the prevention of IL-10 release upon LPS stimulation. IL-10 is an essential anti-inflammatory cytokine that inhibits synthesis of several pro-inflammatory proteins including other cytokines. Mice defective in IL-10 expression develop an inflammatory Crohn's like disease and produce enhanced amounts of TNF-α in response to LPS [Kuhn et al. 1993]. Recently it was found in a mouse model of asthma that administration of the PPARγ agonist may improve the asthmatic features via regulation of IL-10 expression/IL-10 receptor activation, suggesting that PPARγ agonist may have therapeutic potential for the treatment of airway inflammation and

hyper-responsiveness [Kim et al. 2005]. Based on our results one may presume that upon inflammatory stimuli the initial IL-10 secretion from macrophages is driven by a natural PPARγ ligand.

The secretion of the inflammatory IL-1β and IL-8 and the anti-inflammatory TGF-β upon LPS addition were not influenced by treatment of maturing macrophages with PPARγ antagonist. Regarding TNF-α and IL-6 we have not observed significant inhibition of their secretion either – though some studies suggest that they may be controlled by PPARγ ligands. It was found that 15-deoxy- $\Delta^{12,14}$ –PGJ2 inhibited TNF-α and IL-6 production in peripheral blood monocytes, but in the same study other high affinity ligands failed to affect cytokine production [Thieringer et al. 2000]. We also showed that none of the responses of macrophages to apoptotic cells has been altered by blocking PPARγ during macrophage differentiation. 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.

Glucocorticoids represent powerful anti-inflammatory compounds due to their capacity to inhibit inflammatory cell recruitment and to down regulate production of and responsiveness to pro-inflammatory cytokines [Adcock et al. 2001]. Long term exposure of monocytes to the synthetic glucocorticoid dexamethasone programs monocyte differentiation toward a pro-resolution phenotype and this includes increased capacity for phagocytosis of apoptotic cells. The pro-resolution macrophage phenotype was associated with loss of actin containing podosome structures, reduced p130Cas expression, loss of paxillin/pyk2 phosphorylation and high level of active Rac [Giles et al. 2001]. 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). The PPARγ regulated pro-phagocytic genes CD36 and TG2 are down regulated by dexamethasone itself (while they acquire higher phagocytic capacity) and this can not be reversed by PPARγ antagonist suggesting that the effect of the presumed natural PPARγ ligands and glucocorticoids exert their pro-phagocytic effect through different pathways. Further work is needed to clarify the details of these two molecular mechanisms, both leading to enhanced clearance of apoptotic cells.

It was reported that glucocorticoid treatment of maturing macrophages can prevent the induction of TNF-α by LPS, and it also inhibits the release of IL-6 and IL-10 during the differentiation period [Roger et al. 2001; Heasman et al. 2004]. Accordingly, dexamethasone alone down regulated LPS induced secretion of pro-inflammatory cytokines and up regulated the release of anti-inflammatory TGF-β in differentiated macrophages. Furthermore, dexamethasone treatment of differentiating macrophages does not interfere with the anti-inflammatory action of apoptotic neutrophils, rather potentiates it. 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.

6.2. The role of PPARγ in clearance of apoptotic neutrophils by dendritic cells and its impact on cytokine secretion

Macrophages and dendritic cells are tissue resident professional phagocytic cells that derive from common precursors, and exhibit overlapping functions such as

engulfing apoptotic cells, sensing self and pathogenic danger signals and acting as professional APC. Here we show that immature monocyte-derived DC that resemble tissue resident migratory DC [Shortman et al. 2007] are able to engulf apoptotic neutrophils but less efficiently than monocyte-derived macrophages. Furthermore, the slow uptake of apoptotic neutrophils by DC – in contrast to macrophages – results in the activation of DC for pro-inflammatory cytokine secretion and the potential to induce inflammatory T-lymphocyte responses.

In the present study the kinetics of neutrophil uptake, identification of DC subtypes involved in the engulfement of apoptotic neutrophils and the functional consequences of their interaction has been determined. We identified immature $CD1a^-$ DC as a cell type preferentially internalizing apoptotic neutrophils. Although our results showed very different kinetics for the uptake of apoptotic neutrophils by macrophages or DC, the continuous engulfment of neutrophils undergoing apoptosis resulted in ~20 – 30 % loaded DC. The ratio of phagocytosing cells could be increased up to ~50% by the ligand-induced activation of PPARγ. This treatment was shown to shift monocyte-derived DC differentiation to the generation of the $CD1a^-$ subset characterized by more efficient phagocytosis and receptor-mediated endocytosis than monocyte-derived $CD1a^+$ cells [Szatmari et al. 2004; Gogolak et al. 2007].

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 upregulated through PPARγ and LXR activation [Kiss et al. 2006; Gerbod-Giannone et al. 2006]. Macrophage activation is controlled by the cytokines IL-10 and TGFβ as well as by arachidonate metabolites such as PGE2. Macrophages that contact with apoptotic cells acquire sensitivity to autocrine

TGFβ and suppression of the secretion of inflammatory cytokines [Lucas et al. 2006]. Our previous results obtained with an *in vitro* phagocytosis system confirmed this type of regulation and showed that the engulfment of apoptotic neutrophils is able to dampen inflammatory cytokine production induced by LPS [Majai et al. 2007].

Under physiological conditions saturation of immature tissue DC by metabolites or engulfed apoptotic cells induces the steady-state migration to draining lymph nodes (LNs) where DC interact with T-lymphocytes. This cell-to-cell communication is translated to tolerance induction due to the lack of co-stimulatory signals and to the induction of regulatory mechanisms that inhibit signaling pathways and cell communication. Phagocytosis of particulate antigens such as microbes or apoptotic cells by DC results in the sorting of engulfed material into distinct intracellular compartments based on their co-expression of TLR ligands [Blander et al. 2006]. Particles carrying both antigenic structures and TLR ligands induce phagosome maturation and the presentation of antigenic peptides in the context of MHC class II proteins at the DC surface. However, in the absence of coupled TLR signals non-infected apoptotic cells, even internalized together with microbes, will not become immunogenic due to inappropriate phagosome maturation and antigen processing [Blander et al. 2004]. Thus the outcome of apoptotic cell engulfment by DC can lead either to tolerance induction or to priming immune responses, and this decision strongly depends on particle-associated activation signals.

Exogenous lipids transferred by engulfed apoptotic cells to DC may modulate the composition of endogenous lipids and generate 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 and tolerogenic function, we presumed that the loaded DC maintained their anti-inflammatory nature [Gogolak et al. 2007].

However, the secretion of pro-inflammatory cytokines induced by co-culture of apoptotic cells in DC pointed to the co-delivery of both antigenic and co-stimulatory signals by apoptotic neutrophils. This possibility was confirmed by the robust inflammatory cytokine response induced by further activation of the pre-loaded DC by additional stimuli such as LPS+IFNγ. Importantly the LPS+IFNγ-induced IL-12 secretion of DC was accompanied by IL-10 production, a possible effect of DC-SIGN-mediated signaling event [Robinson et al. 2006]. When we compared the T-cell polarizing capacity of activated monocyte-derived DC with or without pre-loaded pre-loaded with apoptotic neutrophils we could demonstrate the strong inflammatory potential of DC preincubated with apoptotic neutrophils. Furthermore, monocyte-derived macrophages of the same donor saturated by apoptotic neutrophils remained immunologically silent.

Remarkably, DC generated in the presence of RSG, loaded with apoptotic neutrophils and activated by IFNγ+LPS secreted in all cases less inflammatory cytokines than those differentiated in the absence of PPARγ agonist. Consistent with previous data these results suggest the anti-inflammatory effect of forced PPARγ expression and activation mediated by the relative tolerogenic nature of PPARγ expressing $CD1a^-$ cells involved preferentially in the engulfment of apoptotic neutrophils.

The neutrophils used in our experiments derived from a different donor than the DC. Their antigenic content could be acquired in the host through the uptake by immune complexes readily engulfed by neutrophils. As microbial products were absent in our experimental system the source of danger signals that might provoke DC for inducing an inflammatory response remains to be identified. However, neutrophils activated by TNF-α were shown to induce T-lymphocyte proliferation through allogenic

DC and resulted in a mixed T-cell response that involved both IL-4 and IFNγ producing effector T-lymphocytes [van Gisbergen et al. 2005]. Furthermore activated autologous peripheral blood mononuclear cells (PBMC) undergoing apoptosis induced the differentiation of IFNγ-producing T-lymphocytes whereas non-actived cells did not [Johansson et al. 2007]. Although the allogeneic response induced by neutrophils can not be ruled out, apoptotic neutrophils by themselves may present endogenous danger signals similar to other mimics of tissue destruction such as adenosine triphosphate (ATP), uric acid or hypotonic stress [Mariathasan et al. 2006; Martinon et al. 2006]. As terminally differentiated short lived effectors, the activated neutrophils rapidly release neutrophil extracellular traps (NET) that involve cytoplasmic proteins bound to intact chromatin [Brinkmann et al. 2004; Urban et al. 2006]. This process has been suggested as a unique cell death program that can be induced by PMA, LPS, IL-8 or type I and II IFN [Fuchs et al. 2007; Wartha et al. 2007]. As immature DC release high level of IL-8 [Gogolak et al. 2007], the induction of NET and the release of cell content emerge as candidate mechanisms to induce DC activation.

Under *in vivo* conditions tissue resident DC are concentrated at mucosal surfaces and similar to macrophages express a wide variety of receptors that are involved in the recognition and uptake of apoptotic cells [Pradhan et al. 1997; Fadok et al.1998; Savill et al. 2002; Gogolák et al. 2003; Lauber et al. 2004]. Microbial invasion recruits neutrophils from the circulation to the site of infection through microbial products, cytokines, chemokines, and factors released by apoptotic cells [Ravichandran et al. 2003]. A recent study also suggested that neutrophils carrying IgG-antigen complexes enter afferent lymphatics, and upon apoptosis they transfer their antigenic content to phagocytic DC residing in the T-cell areas of LN [Maletto et al. 2006]. Inflammatory stimuli also mobilize circulating monocytes and blood DC precursors to the site of

infection [Lauber et al. 2003] where cell death through apoptosis, their clearance by macrophages and the presentation of antigens by DC plays a central role in inducing immunological tolerance to self molecules and immunity against foreign or dangerous structures [Savill et al. 2002]. Thus the uptake of neutrophils by macrophages and DC in both peripheral and lymphoid tissues may have multiple effects on the outcome of immune responses including a strong Th1 response.

Together with our previous results obtained with macrophages in a similar *in vitro* system our new results suggest that the recruitment of neutrophils to inflamed tissues is able to induce opposing responses in monocyte-derived macrophages and dendritic cells. Hence, the cross talk of macrophages and DC loaded by apoptotic neutrophils through their cytokines and antigen presenting functions may modulate the outcome of immune responses under inflammatory or other pathologic conditions.

7. 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 DC 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 DC to engulf apoptotic neutrophils. In contrast with macrophages DC 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 DC for robust inflammatory responses. DC 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 DC 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 DC through their cytokines and antigen-presenting functions may modulate the outcome of immune responses under inflammatory or other pathologic conditions.

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9. FIGURE LEGENDS

Figure 1. Molecules involved in the apopto-phagocytic synapse. αvβ3/5 vitronectin receptor integrins; ABCA-1, ATP-binding cassette transporter A1; ACAMPs, apoptotic cell-associated molecular patterns; ASGP-R, asyaloglycoprotein receptor; β2GPI, β2 glycoprotein I; β2GPI-R, β2GPI-receptor; β2-integrins (CR3, CR4); C1q, first component of complement; CHO, carbohydrate; CRP, C-reactive protein; Del-1, developmental endothelial locus-1; Gas-6, growth arrest specific gene-6; iC3b, inactivated complement fragment C3b; ICAM-3 (CD50), intercellular adhesion moleculae-3; Lox-1, oxidized low density lipoprotein receptor 1; MER, myeloid epithelial reproductive tyrosine kinase; MFG-E8, milk fat globule epidermal growth

factor-8; Ox-PL, oxidized phospholipids; PS, phosphatidylserine; PSR, putative PS receptor; 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.

Figure 2. Phagocytic capacity of freshly isolated monocytes compared to 5 days aged macrophages as determined by flow cytometry. The phagocytosis assay was performed using either apoptotic neutrophils or polystyrene latex beads as targets. Solid line shows the 25 min phagocytosis capacity (per cent of macrophages with engulfed apoptotic neutrophils) at 37 ^{0}C while the bold line represents controls run at 4 ^{0}C; the differences between the two were 5.2% (monocytes) and 29.5% (macrophages). M1 represents gating for engulfing cells.

Figure 3. Effect of GW9662 on the uptake of apoptotic neutrophils, polystyrene latex beads and opsonized bacteria by macrophages. ***A*** and ***E,*** The phagocytic ability of macrophages in the presence or absence of 10μM GW9662 or 2.5μM rosiglitasone (Ros), 1μM dexamethasone (Dex.) as determined by flow cytometry. Each triangle (▲) represents mean of duplicate measurements from one donor; * indicates $p<0.01$ considering means of results from different goups of donors. ***B*** and ***F,*** Representative flow cytometry data of one donor. ***C,*** Myeloperoxidase staining showing phagocytosis of untreated (left) and GW9662 treated (right) macrophages. ***D***, Phagocytic capacity of macrophages toward latex beads. ***G,*** Phagocytosis of fluorescent labelled opsonized Listeria Monocytogenes. The results were obtained using macrophages from 3 different donors (mean±SD); *, $p<0.05$.

Figure 4. Time and concentration dependence of the effect of GW9662. ***A,*** Phagocytic capacity of monocyte derived macrophages treated for 5 days with different concentration of GW9662 before assessment of phagocytosis. ***B,*** Phagocytic capacity of monocyte derived macrophages that were incubated with 10μM GW9662 for the indicated time periods during the 5 days differentiation. Data represents results of 3 experiments using different donors (mean±SD); *, $p<0.05$.

Figure 5. Clearance of apoptotic tymocytes by mouse macrophages with deleted PPARγ. Bone marrow derived ***(A)*** and peritoneal macrophages ***(B)*** from PPARγ$^{fl/-}$ Lys Cre mice present decreased phagocytic capacity as compared to control PPARγ$^{+/-}$ Lys Cre mice. The experiment was performed from 4 PPARγ$^{fl/-}$ Lys Cre and 4 PPARγ$^{+/-}$ Lys Cre mice in the case of bone marrow differentiated macrophages (results show mean± SD; $p<0.1$). In the experiments with peritoneal macrophages reults (mean± SD of three separate assays in each) were obtained from 2 PPARγ$^{fl/-}$ Lys Cre and 2 PPARγ$^{+/-}$ Lys Cre mice.

Figure 6. The effect of GW9662 on the expression genes involved in phagocytosis of apoptotic cells. ***A,*** RT-Q-PCR assays: data shows the mean± SD of triplicate measurements from 3 different donors; *, $p<0.05$. ***B,*** The effect of GW9662 on TG2 expression at mRNA (left, RT-PCR) and protein level (right), as detected by Western-blotting; *, $p<0.05$. ***C,*** TLDA assays: the results represent the mean± SD of triplicate measurements obtained TaqMan Low Density Array from 3 donors.

Figure 7. Cytokine profiles of monocyte derived macrophages treated with GW9662, dexamethasone, or GW9662 in combination with dexamethasone for 5 days. ”Control”

as 100% represents the level of cytokines produced by 5 days aged untreated macrophages stimulated on the 5th day with 0.5μg/ml LPS for 30 min. The level of basal cytokine secretions (unstimulated vs. stimulated) were the followings: IL-6 (34.29 vs. 4329.31 pg/ml), TNF-α (8.53 vs. 799.56 pg/ml), IL-1β (78.96 vs. 74.48 pg/ml), IL-8 (4256.71 vs. 35592.78 pg/ml), IL-10 (3.5 vs. 254 pg/ml), TGF-β1(864.61 vs. 673.2 pg/ml). All data are represented as mean ± SD from four independent experiments using different donors. *, $p<0.05$.

Figure 8. Characterization of the internalization process of neutrophils undergoing apoptosis by immature dendritic cells. Monocyte-derived immature DC were co-cultured with apoptotic neutrophils for the indicated periods of time ***(A)***, and the ratio of apoptotic neutrophils was measured by flow cytometry using Annexin V and PI staining ***(B)***. The preferential uptake of apoptotic neutrophils by the CD1a$^-$ immature DC subsets was demonstrated by flow cytometry ***(C).***

Figure 9. The effect of PPARγ activation on the phagocytosis of apoptotic neutrophils by monocyte-derived immature dendritic cells. Monocyte-derived immature DC were generated by the standard method as described in Materials and Methods (■) or with RSG present throughout the 5-day culture (▲).The effect of RSG on the ratio of CD1a$^-$ and CD1a$^+$ immature DC measured on day 5 (n=6) was determined by flow cytometric analysis of membrane CD1a expression shown as percentage of total DC ***(A)***. The phagocytic activity of immature DC, generated in the absence (■) or presence of 2.5 μmol RSG (▲), was measured by flow cytometric analysis of the cells internalizing green fluorescence-labeled neutrophil granulocytes in the same donors (n=6) shown as percentage of phagocytosing cells ***(B)***.

Figure 10. Cytokine profile of monocyte-derived dendritic cells co-cultured with apoptotic neutrophils. Cytokine secretion of DC differentiated in the absence or presence of 2.5 μmol RSG and incubated with washed neutrophils, undergoing spontaneous apoptosis induced by *in vitro* culture for 16 hours, at a DC:neutrophil ratio of 1:5. After an 8-hour co-culture period the supernatants of the co-cultured DC were collected and their cytokine contents were measured by the cytokine bead array technique. All data are presented as mean values ± standard errors (SD) of pg/ml concentrations calculated from three independent experiments using neutrophil-loaded dendritic cells derived from different healthy blood donors. * indicates $p<0.05$ ***(A)***. The effect of neutrophil phagocytosis on the cytokine secretion of DC activated by LPS and IFNγ. Immature DC were mixed and incubated with neutrophils undergoing apoptosis and than activated by 100ng/ml LPS in combination with 10ng/ml IFNγ for an additional 16 hours. Cytokine concentrations of the cell culture supernatants were measured by the cytokine bead array and are shown as compared to the cytokine concentrations measured in the culture supernatant of IDC not fed with apoptotic neutrophils. * indicates $p<0.05$ ***(B)***.

Figure 11. Phagocytosis of apoptotic neutrophils by immature dendritic cells induces inflammatory T cell activation. Immature DC and differentiated macrophages were incubated with neutrophils undergoing apoptosis for 8 hours and 25 minutes, respectively. Following phagocytosis, dendritic cells ***(A)*** and macrophages ***(B)*** were washed, mixed with autologous lymphocytes at a ratio of 1:10 and co-cultured for 5 days. The non-adherent lymphocytes were harvested and subjected to a 24-hour ELISPOT assay to measure the frequency of IFNγ cytokine-secreting cells as described

in the Materials and Methods. The number of spots was counted and the area covered by the spots was calculated by a computer assisted ELISPOT image analyzer. The data were given as mean ± SD of 3-5 parallel measurement performed with the cells of three different donors.

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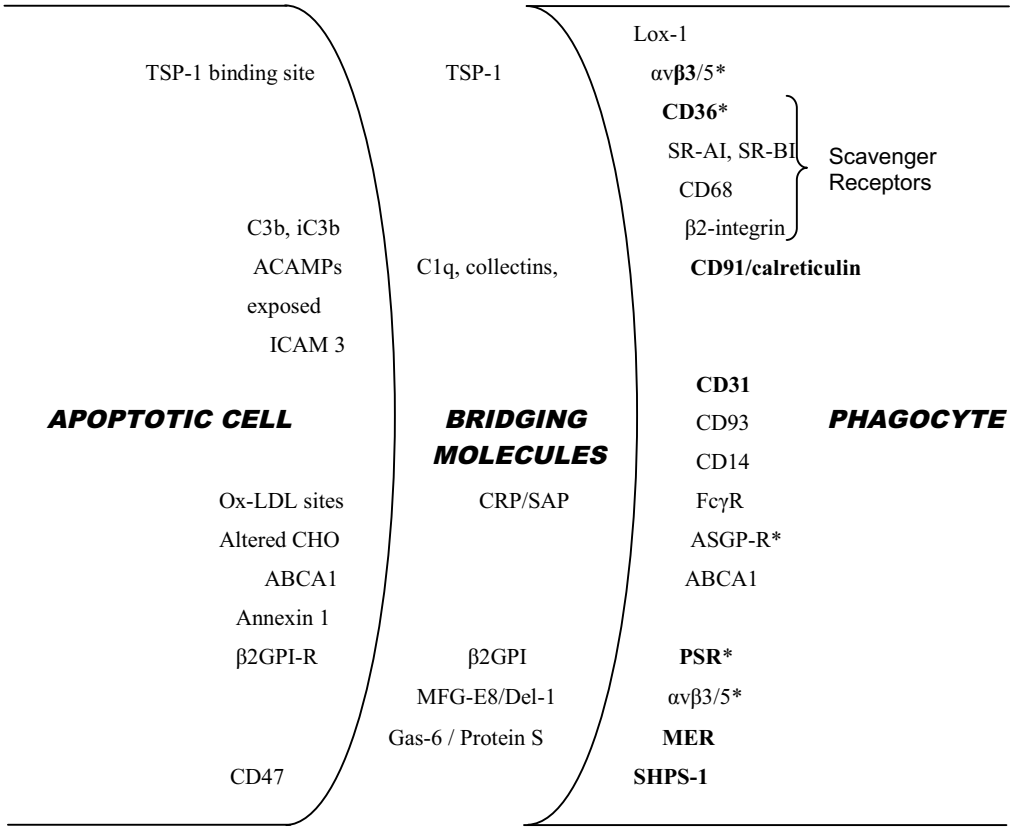
11. FIGURES AND TABLES

Table 1. The effect of GW9662 on expression of different cell surface receptors. Data shows one representative flow cytometry experiment.

	Expression (fluorescence mean)				
	Monocyte	Control Macrophage	GW9662 treated Macrophage	Dex. treated Macrophage	Dex.+GW9662 treated Macrophage
CD206	8.06	47.37	31.19	59.08	52.65
CD16	12.56	78.41	40.06	71.79	39.48
CD36	435.53	200.355	100.235	42.68	39.09
CD14	251.58	283.92	203.02	280.88	228.99

Figure 1.



(*) Receptors found on non-professional phagocytes
Receptors suggested of being involved in the regulation of the inflammatory response are shown in bold

Figure 2.

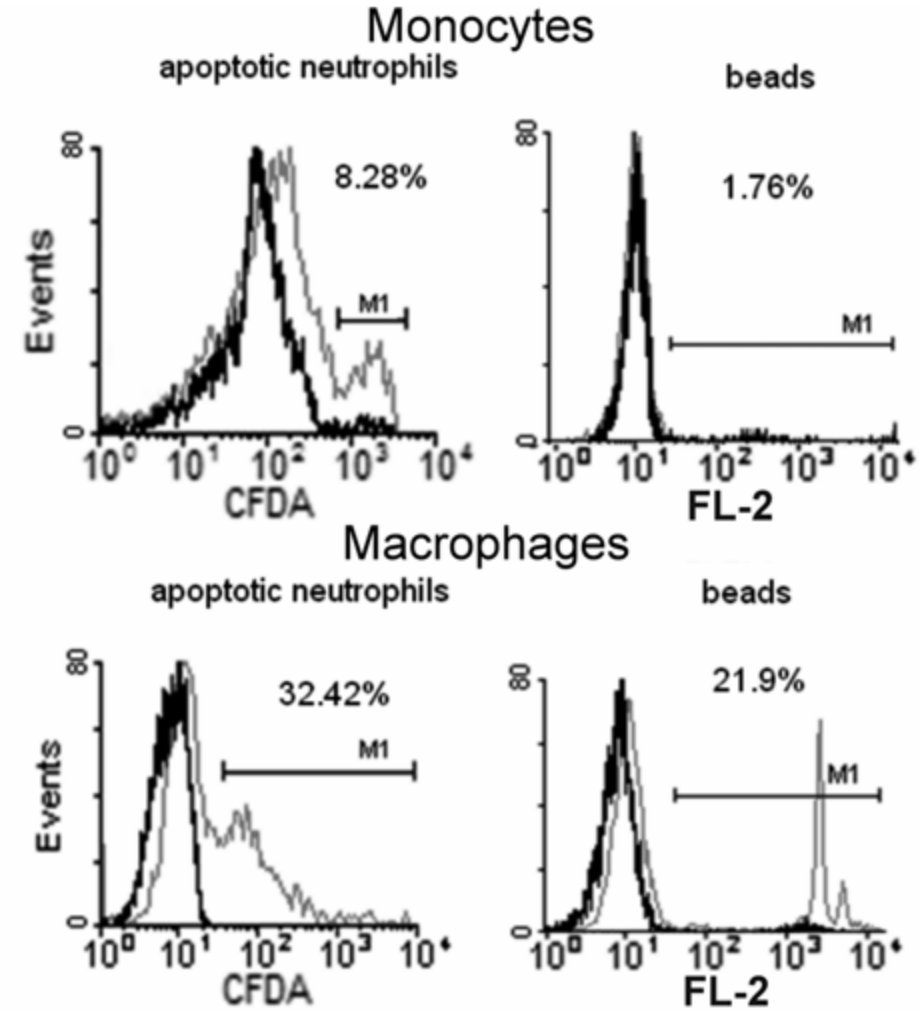

Monocytes
apoptotic neutrophils
beads
8.28%
1.76%
M1
M1
Events
80
0
10^0 10^1 10^2 10^3 10^4
CFDA
FL-2
Macrophages
apoptotic neutrophils
beads
32.42%
21.9%
M1
M1
Events
CFDA
FL-2

Figure 3.

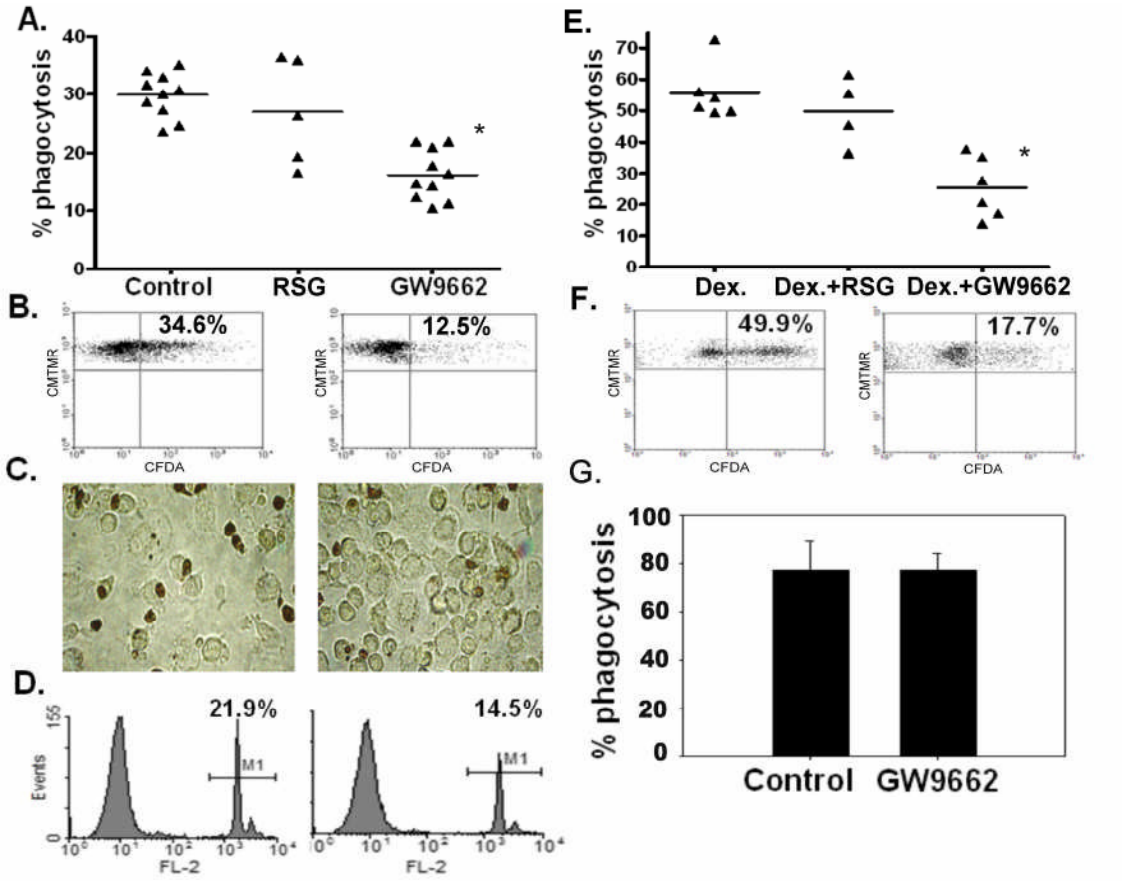

A.
% phagocytosis
Control
RSG
GW9662
*
B.
34.6%
12.5%
CMTMR
CFDA
C.
D.
21.9%
14.5%
M1
Events
FL-2
E.
% phagocytosis
Dex.
Dex.+RSG
Dex.+GW9662
*
F.
49.9%
17.7%
CMTMR
CFDA
G.
% phagocytosis
Control
GW9662

Figure 4.

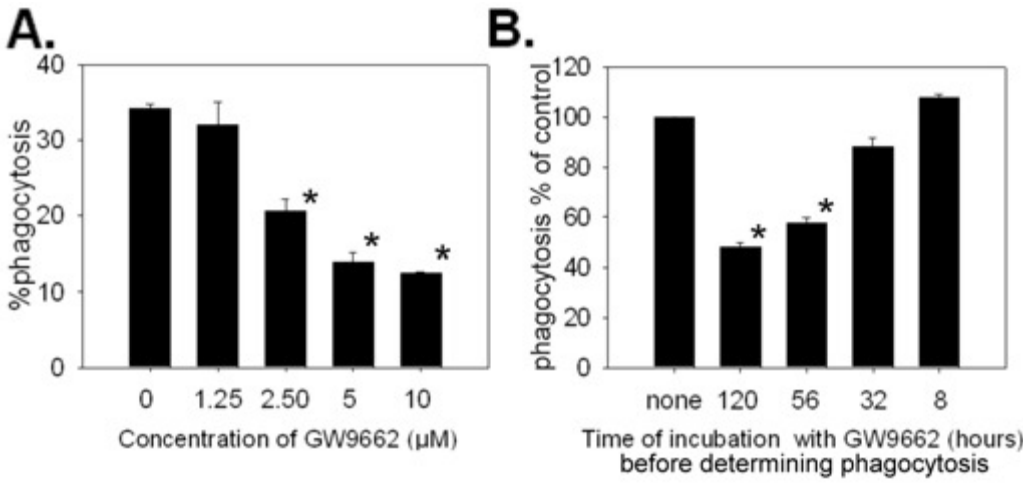

A.
%phagocytosis
40
30
20
10
0
*
*
*
0
1.25
2.50
5
10
Concentration of GW9662 (μM)
B.
phagocytosis % of control
120
100
80
60
40
20
0
*
*
none
120
56
32
8
Time of incubation with GW9662 (hours)
before determining phagocytosis

Figure 5.

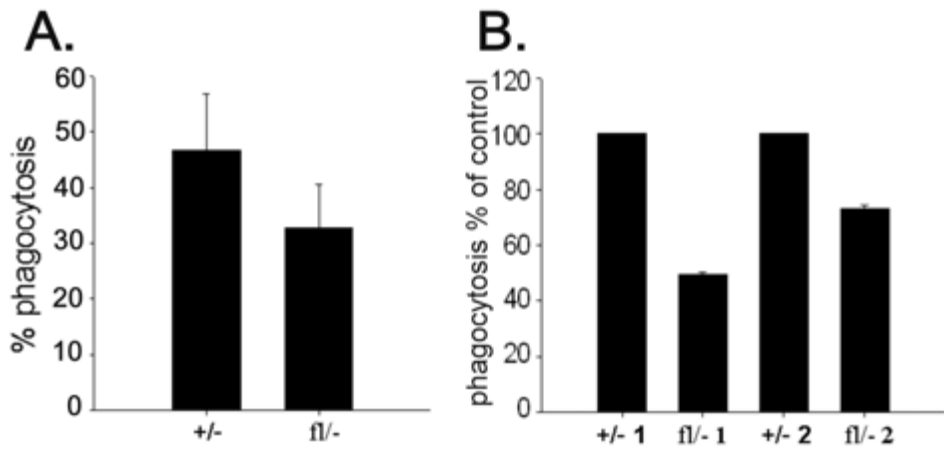

A.
% phagocytosis
60
50
40
30
20
10
0
+/-
fl/-
B.
phagocytosis % of control
120
100
80
60
40
20
0
+/- 1
fl/- 1
+/- 2
fl/- 2

Figure 6.

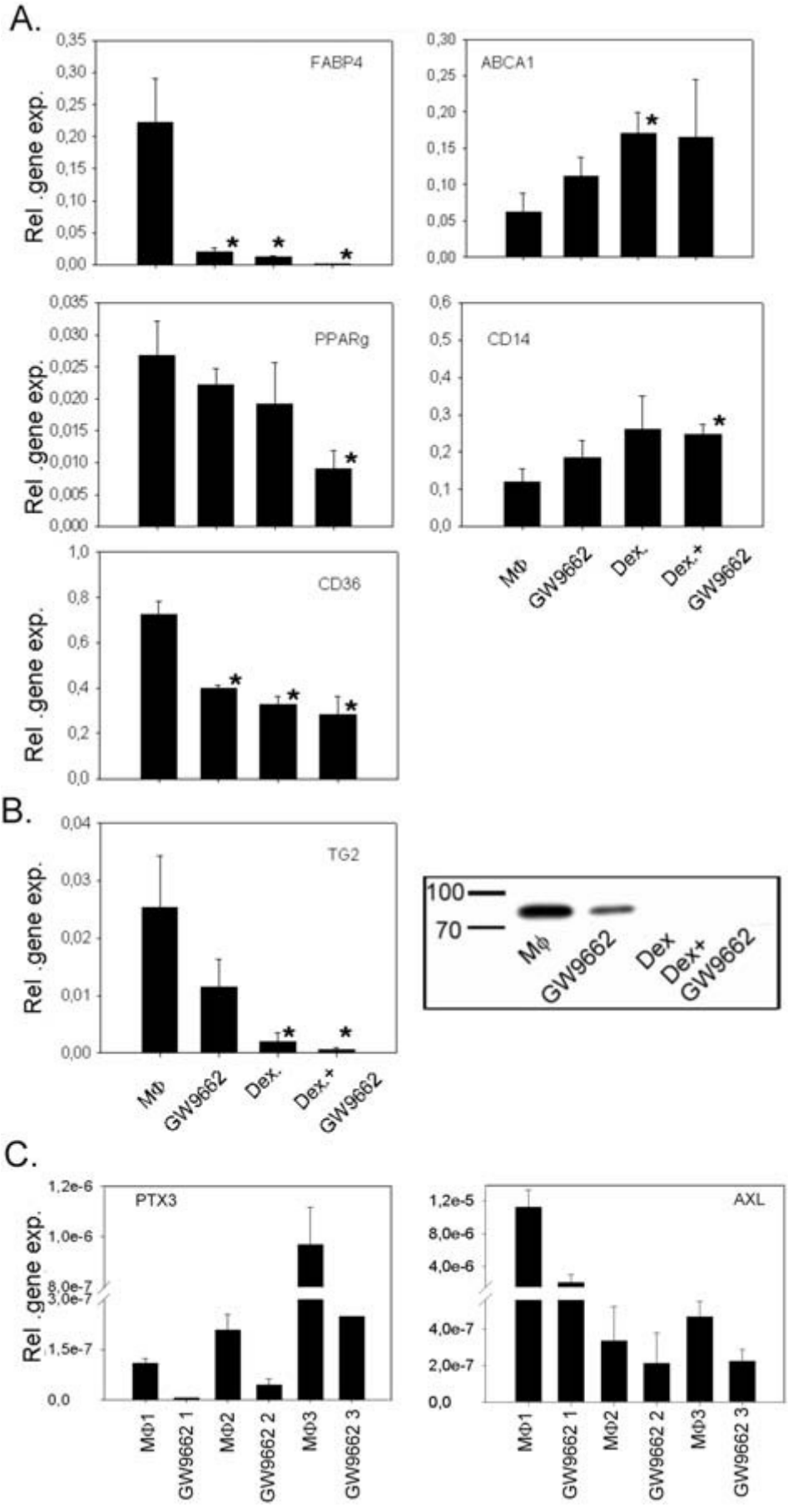

A.
FABP4
ABCA1
PPARg
CD14
CD36
Rel .gene exp.
MΦ
GW9662
Dex.
Dex.+ GW9662
B.
TG2
100
70
Mφ
GW9662
Dex
Dex+ GW9662
C.
PTX3
AXL
MΦ1
GW9662 1
MΦ2
GW9662 2
MΦ3
GW9662 3

Figure 7.

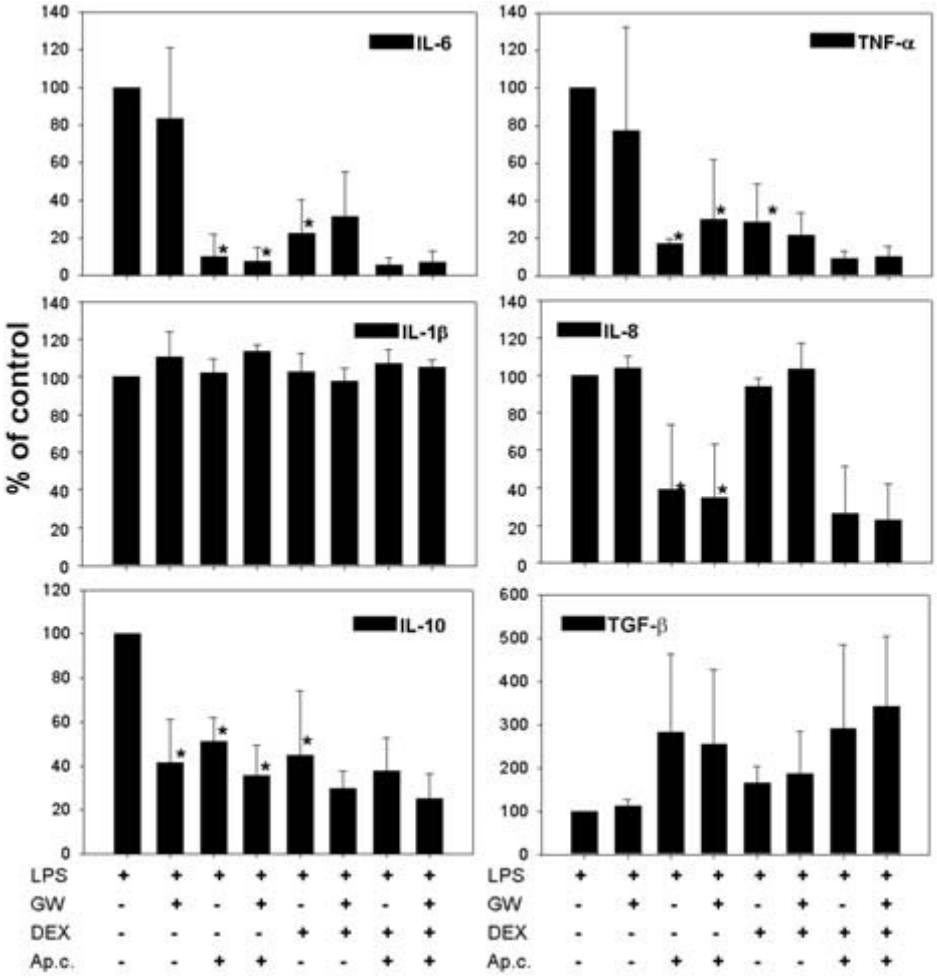


Figure 8.

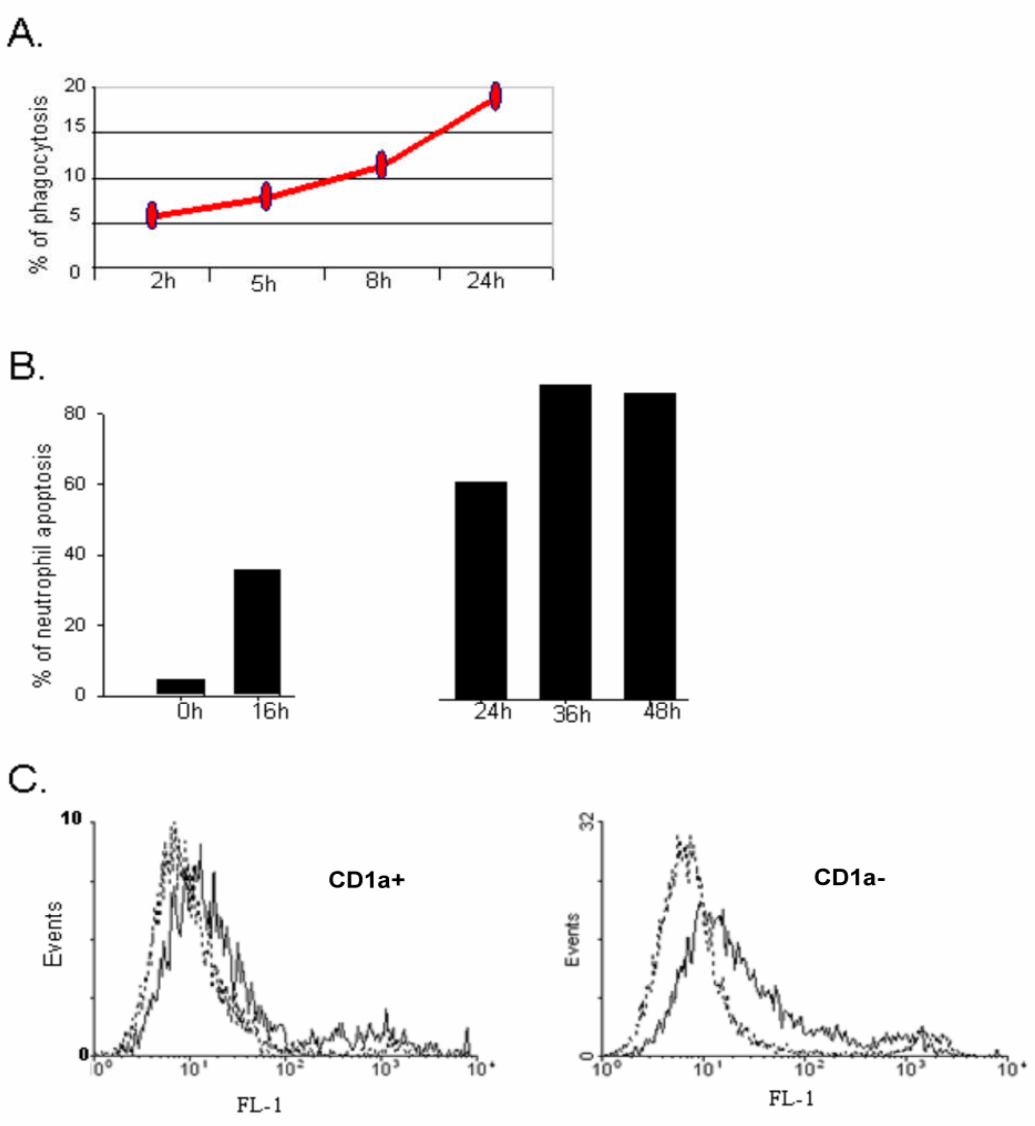

A.
% of phagocytosis
20
15
10
5
0
2h
5h
8h
24h
B.
% of neutrophil apoptosis
80
60
40
20
0
0h
16h
24h
36h
48h
C.
Events
10
0
CD1a+
FL-1
Events
32
0
CD1a-
FL-1

Figure 9.

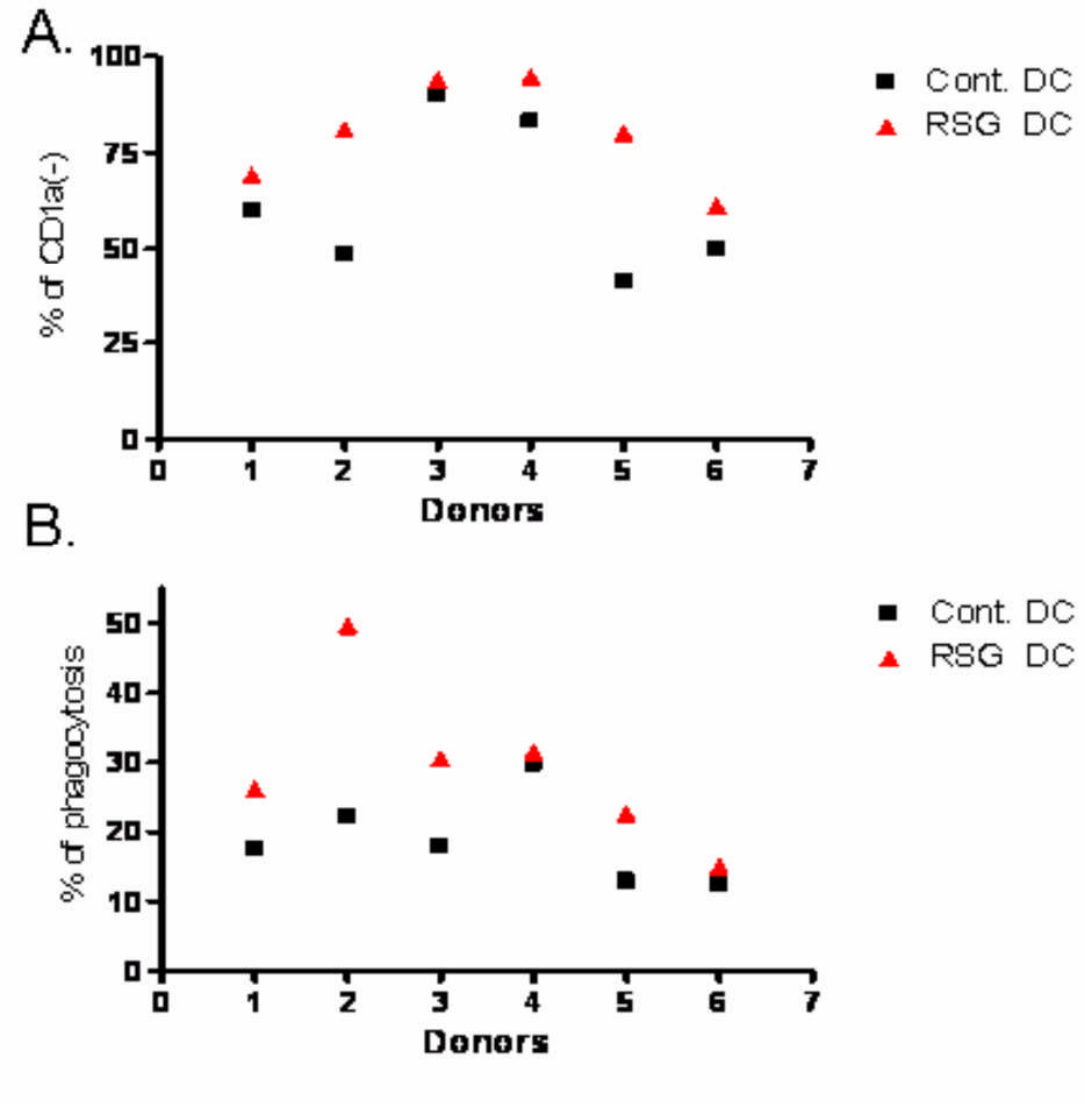

A.
% of CD1a(-)
100
75
50
25
0
0 1 2 3 4 5 6 7
Donors
Cont. DC
RSG DC
B.
% of phagocytosis
50
40
30
20
10
0
0 1 2 3 4 5 6 7
Donors
Cont. DC
RSG DC

Figure 10.

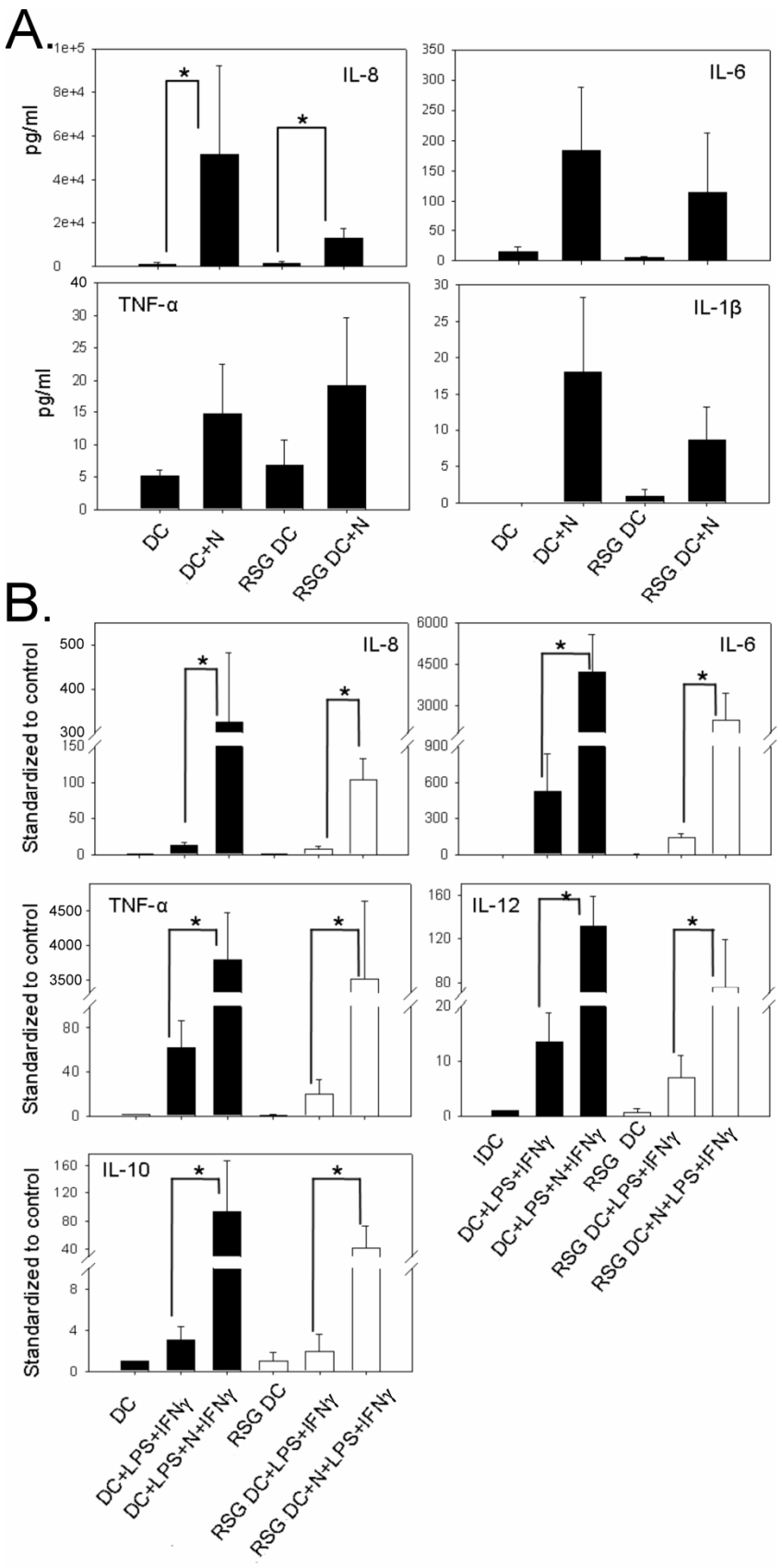
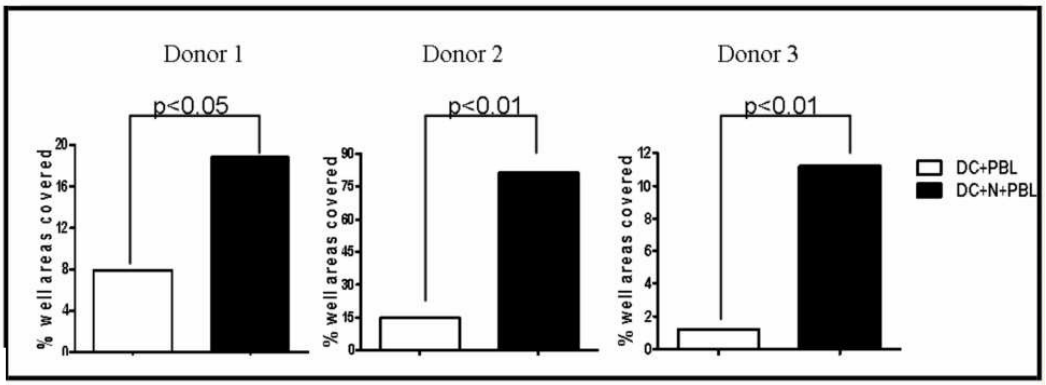
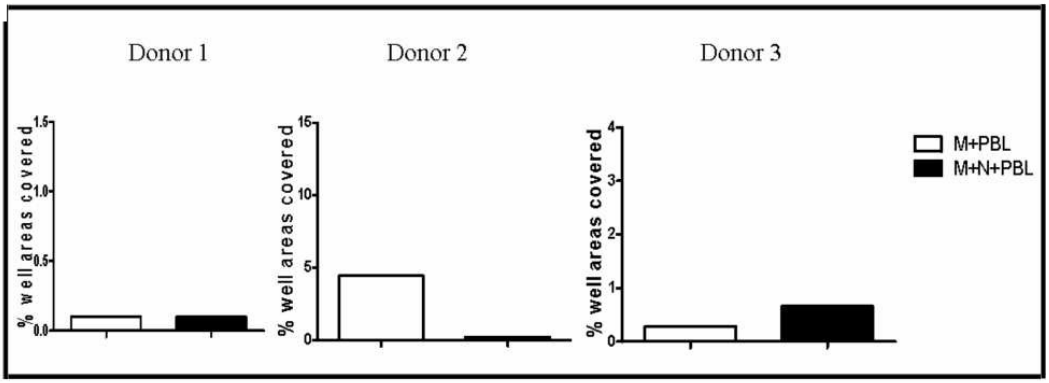

A.
IL-8
IL-6
TNF-α
IL-1β
pg/ml
DC
DC+N
RSG DC
RSG DC+N
B.
Standardized to control
IL-8
IL-6
TNF-α
IL-12
IL-10
IDC
DC+LPS+IFNγ
DC+LPS+N+IFNγ
RSG DC
RSG DC+LPS+IFNγ
RSG DC+N+LPS+IFNγ

Figure 11.

A.


Donor 1
Donor 2
Donor 3
p<0.05
p<0.01
p<0.01
% well areas covered
DC+PBL
DC+N+PBL

B.


Donor 1
Donor 2
Donor 3
% well areas covered
M+PBL
M+N+PBL

SUPPLEMENT 1.

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Short review

Inflammation and the apopto-phagocytic system

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Abstract

Although under normal conditions many cells die daily mainly by apoptosis in human tissues, inflammation does not occur. The redundant function of a relatively large number of molecules are available to recognize changes occurring on the surface of apoptotic cells, to opsonize the dead cells and to engulf the apoptotic cells previously opsonized or not. 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. These cells, unlike necrotic ones, do not induce the expression of inflammatory cytokines in phagocytic cells, they can even inhibit such a response and engage an active signaling process to elicit a direct anti-inflammatory effect. The molecular details of these signaling processes have not been clarified yet. Both professional and "amateur" cells can engulf apoptotic cells and mediate an anti-inflammatory action. Disturbance of these processes have significant roles in development of autoimmune diseases and highly malignant tumors.

Keywords: Apoptosis; Macrophages; Non-professional phagocytes; Innate immunity; Pro-inflammatory and anti-inflammatory cytokines; Signaling; Autoimmunity; Tumors

1. Introduction

In the human body close to 500 billion cells die each day – mainly by apoptosis – and the huge number of dead cells are either lost directly to the environment from body surfaces or continuously removed by a remarkably efficient apopto-phagocytic system while inflammation and scar formation do not occur [1,2]. The surface with complex molecular patterns and dynamic interactions between the dying and engulfing cells (macrophages, immature dendritic cells (DCs) and non-professional phagocytes) is often called the third synapse by analogy to the synapses in the nervous and immune (antigen presenting) system. The "anatomical" description of this synapse is probably almost complete (see the schematic description on Fig. 1) and it includes the large number of receptors and opsonins which bind to cellular ligands exposed during the various stages of apoptotic cell death [3]. However, in spite of the remarkable progression in the field, we are still far from understanding the complex biochemical and regulatory processes which take place when dying cells are cleared from tissues.

One of the most intriguing aspects of the apopto-phagocytic system is the active and dynamic interrelationship between the dying and the engulfing cells. The apoptotic cells can induce migration of phagocytes by releasing lipid-derived attraction signals [4]. The engulfing cells are capable of influencing the fate of their neighbors by promoting either death or survival [5–11]. This cooperative relationship between apoptotic cells and their phagocytes is even more significant in determining whether inflammation occurs or it does not in tissues with high apoptotic rate under normal or pathologic conditions. In this paper the encounter between the apopto-phagocytic and inflammatory systems will be reviewed with particular emphasis on three closely related issues, namely (i) what elements of innate immunity are involved in the clearance of apoptotic cells; (ii) how apoptotic cells can suppress the inflammatory response; and (iii) how inflammation is prevented when the dead cells are cleared by non-professional phagocytes, for example by parenchymal or epithelial cells of various tissues.

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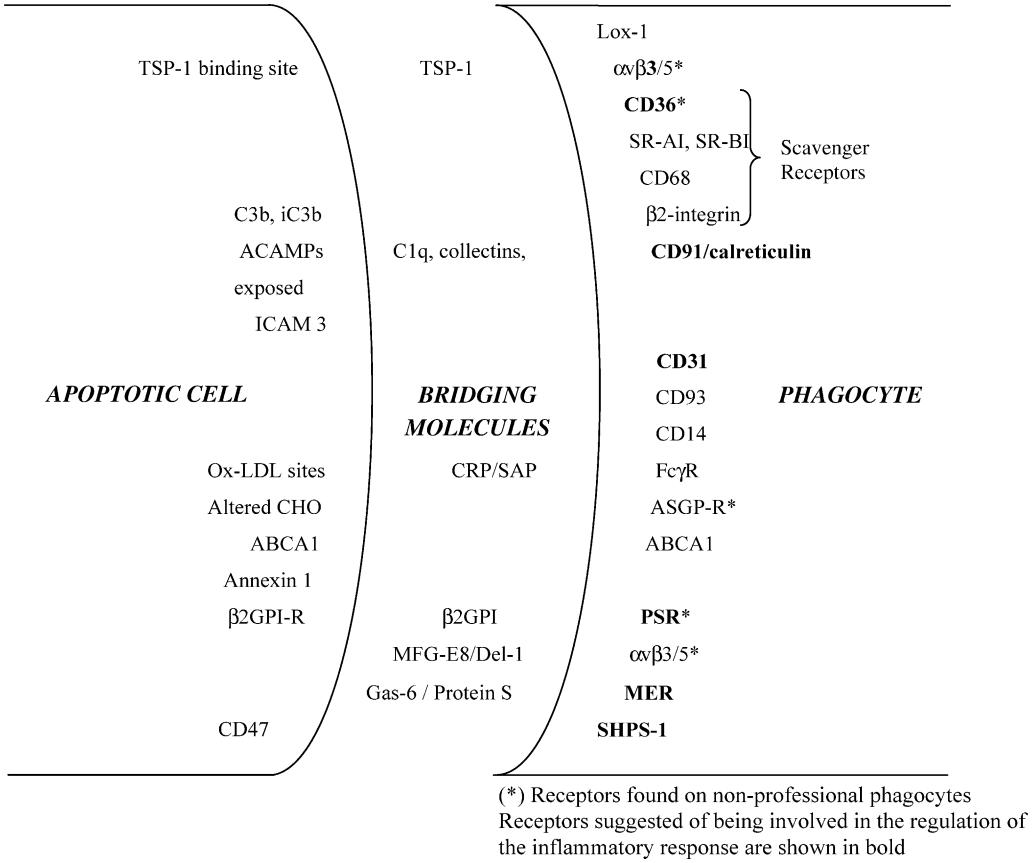

Fig. 1. Molecules involved in the apopto-phagocytic synapse. αvβ3/5 Vitronectin receptor integrins; ABCA-1, ATP-binding cassette transporter A1; ACAMPs, apoptotic cell-associated molecular patterns; ASGP-R, asyaloglycoprotein receptor; β2GPI, β2 glycoprotein I; β2GPI-R, β2GPI-receptor; β2-integrins (CR3, CR4); C1q, first component of complement; CHO, carbohydrate; CRP, C-reactive protein; Del-1, developmental endothelial locus-1; Gas-6, growth arrest specific gene-6; iC3b, inactivated complement fragment C3b; ICAM-3 (CD50), intercellular adhesion moleculae-3; Lox-1, oxidized low density lipoprotein receptor 1; MER, myeloid epithelial reproductive tyrosine kinase; MFG-E8, milk fat globule epidermal growth factor-8; Ox-PL, oxidized phospholipids; PS, phosphatidylserine; PSR, putative PS receptor; 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.

2. Common elements between the apopto-phagocytic system and the innate immunity

None of the so far revealed elements of the apopto-phagocytic system seems to be specifically dedicated to the clearance of apoptotic cells. Changes on the surface of dying cells involve elimination, modification or translocation of molecules which have well established roles in living cells. Cell surface receptors and the intermediate molecules participating in the phagocytic events are designed for other biological processes as well, such as functional organization of the extracellular matrix, wound healing, tissue repair, lipid metabolism or others. Among these shared processes the utilization of the innate immune system is particularly relevant to our understanding of the intimate relationship between the apoptotic-cell clearance and the inflammatory response.

Invading pathogens are primarily recognized by a large number of pattern-recognition molecules including soluble factors (complement proteins, members of the collectin and pentraxin family), calreticulin, C-type lectins, CD14 and Toll-like receptors (TLRs). Many components of this innate immunity are also involved in the clearance of apoptotic cells (see Fig. 1) as they present conserved molecular patterns—apoptotic cells associated molecular pattern (ACAMP), similarly to microbes which are decorated by pathogen-associated molecular patterns (PAMPs) like lipopolysaccharides (LPS), to innate immune molecules [2]. It is likely that molecular groupings forming repetitive structures and topological associations in Annexin-I-, phosphatidylserine (PS)-, and CD43-rich distinct domains on cells undergoing apoptosis are among the critical determinants of ACAMP. The multiplicity of ACAMP structures provides redundancy making the recognition of dead cells a failsafe mechanism but it may also reflect early and late stages of the apoptotic cell surface which participate in distinct chronological events, such as recognition, binding (“tethering”), signaling (“tickling”) and engulfment during the clearance process.

Molecules of the innate immune response are particularly active in opsonization of apoptotic cells; actually, it has been proposed that C1q and other opsonins prevent autoimmunity and maintain self-tolerance by supporting the efficient clearance of apoptotic material, as well as by actively modulating phagocyte function [12]. However, it should be noted that while some PAMP receptors do participate in clearance of apoptotic cells (e.g. CD14), most of them do not and the response of

macrophages to ACAMPs is usually independent of PAMP receptors. Furthermore, the response of macrophages to PAMPs is pro-inflammatory but to ACAMP it is anti-inflammatory. The reason of this crucial difference is related to signaling through TLRs: while engagement of PAMPs by TLRs leads to a full-blown inflammatory response, there is no TLR response to ACAMP, that is the various TLRs do not recognize ACAMPs. On the other hand, necrotic cells have been shown to engage TLR2 and induce pro-inflammatory and tissue repair genes [13].

3. Suppression of inflammation by apoptotic cells

3.1. Apoptotic cells, unlike necrotic cells, do not induce inflammatory response

Clearance of apoptotic cells has an important role in tissue remodeling and resolution of inflammation protecting tissue from exposure to the inflammatory and immunogenic contents of dying cells. In contrast to apoptosis, necrosis is characterized by loss of membrane integrity, swelling and disintegration leading to the release of cellular contents. Macrophages discriminate innately between cells that have undergone a physiological death and those that have suffered a pathological form of death. Recognition of these two classes of dying cells occurs via distinct and non-competitive mechanisms [14]. The lysed necrotic cells or apoptotic cells undergoing secondary necrosis can initiate an inflammatory response in macrophages since they can release proteases, inflammatory eicosanoids, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein 2 (MIP-2), IL-8, tumor necrosis factor (TNF)-α and they can engage TLR2 (see above). The DNA-binding protein HMGB-1, which elicits a pro-inflammatory response in macrophages through TLR2 and TLR4, can also leak out of necrotic but not of apoptotic cells [15,16]. Therefore, leakage of macromolecules should be prevented during the apoptotic process to avoid inflammation; one mechanism for this is the induction and activation of the protein cross-linking enzyme, transglutaminase (TG) 2 in apoptotic cells [17]. The major mechanism to prevent secondary necrosis is the recognition and fast removal of early phase apoptotic cells [2]. However, even cells undergoing secondary necrosis may not necessarily be pro-inflammatory either because of biochemical mechanism sequestering pro-inflammatory molecules inside dying cells or as the result of the anti-inflammatory effects of the coexisting apoptotic cells. Indeed, there is ample evidence to show that apoptotic cells do not only fail to induce but can actively suppress the release of pro-inflammatory cytokines from macrophages which engulf them.

3.2. How apoptotic cells suppress inflammation?

Early studies demonstrated that apoptotic cells actively suppress an inflammatory response, and not just fail to provide inflammatory signals. The presence of thymocyte-derived apoptotic cells during monocyte activation increased their secretion of the anti-inflammatory and immunoregulatory cytokine IL-10 and decreased production of the pro-inflammatory cytokines such as TNF-α, IL-1 and IL-12. [18] Later, these results were reproduced using apoptotic neutrophils instead of apoptotic lymphocytes [19]. Fadok et al. had shown that phagocytosis of apoptotic neutrophils by human macrophages inhibited the production of IL-1β, IL-8, IL-10, GM-CSF, TNF-α, as well as leukotriene C4 and tromboxane B2 trough an autocrine/paracrine mechanism and increased the production of TGF-β1, prostaglandin E2 and platelet activating factor [20]. Moreover, TGF-β neutralizing antibodies largely reversed the inhibitory effect of apoptotic cell uptake and exogenous TGF-β1 down-regulated the synthesis of the chemokines IL-10 and TNF-α [21]. There are convincing in vivo data that TGF-β1 released by macrophages has an anti-inflammatory effect in inflamed peritoneum and lung [22]. Using the LPS lung inflammation model it was demonstrated that surfactant protein A has a role in induction of alveolar macrophage TGF-β1 release thereby promoting the resolution of acute inflammation within alveolar space [23]. All these data led to the conclusion that suppression of inflammatory response by apoptotic cells in macrophages is mediated mainly by an indirect mechanism through the release of TGF-β1 [24]. In contrast to this it was found by Cvetanovic and Ucker that the initiation of anti-inflammatory modulation occurs as a direct consequence of the interaction of apoptotic target cells with macrophages and without involvement of TGFβ [14]. Nevertheless, the high importance of TGFβ in the clearance of apoptotic cells has been strengthened by the observation that lack of TG2, the cross-linking enzyme which is involved in the biochemical maturation of TGFβ, compromises phagocytosis of apoptotic cells and the anti-inflammatory response to the dying cells [9,25].

The signaling mechanisms that determine the anti-inflammatory mediator release are far from being completely understood. The process appears to be related to the appearance of phosphatidylserine on the surface of apoptotic cells and recognition of PS by macrophages through a PS receptor was suggested to be the dominant element in the release of TGF-β [26]. While a PS receptor may exist, the one described by Fadok et al. has turned out not to be a real PS receptor mediating uptake of apoptotic cells [26,27]. Knock out mice with deletion of this putative PS receptor and defects in phagocytosis of apoptotic cells have been shown to be double knock out animals and when only this putative PS receptor was deleted, the phagocytosis defect disappeared. Interestingly, the double knockout mice were impaired in pro- and anti-inflammatory signaling after stimulation with apoptotic cells [28]. Antibody ligation experiments indicate that CD36 and vitronectin receptor, and bridging protein TSP1 can suppress monocyte/macrophage inflammatory responses by stimulating TGF-β release [18,29]. The endogenous ligand, Annexin-I, inducible by glucocorticoids as well, seems to be capable of inducing IL-10 production and IL-12 synthesis inhibition [30]. A direct suppressive signaling could arise through the tyrosine inhibitory domain of CD31 and kinase domain of MER via Gas-6 [3]. Another signaling pathway which does not require soluble factors and can suppress the pro-inflammatory response is the ligation of Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1 (SHPS-1) [31].

It is important to realize that a maturation process must take place before macrophages can recognize and remove dead cells and display a concomitant anti-inflammatory response [32,33]. This suggests that gene expression regulation is involved in preparing macrophages to these tasks. Glucocorticoids are the most effective anti-inflammatory and immunosuppressive agents. They have been shown to inhibit in macrophages the expression of cytokines, adhesion molecules, and enzymes involved in the inflammatory process. Glucocorticoids act by binding to the glucocorticoid receptor, that upon activation, translocates to the nucleus and either transactivates or transrepresses gene expression. Inhibition of pro-inflammatory transcription factors such as adhesion protein (AP-1), nuclear factor of activated T cells (NFAT) and nuclear factor kappa B (NF-κB) is thought of being a major action of glucocorticoids [34]. Although treatment of maturing macrophages with glucoroticoids results in a large increase in the efficiency of macrophages to engulf dying neutrophils [33], there is no evidence that the anti-inflammatory effects of apoptotic cells and glucocorticoids are interrelated. Our recent results have clearly demonstrated the involvement of PPARγ in the regulation of both the engulfment and the anti-inflammatory response (Majai et al., manuscript in preparation).

Not only the phagocyting cells can release anti-inflammatory cytokines, but also the cells that are dying by apoptosis can release cytokines with direct immunosuppressive properties. It was shown that apoptotic T cells release TGF-β1 which is not only latent but also bioactive and is localized within the intracellular membrane bound compartments including mitochondria [35]. Administration to the eye of antigen-bearing lymphocytes showed that the observed tolerance depends on the ability of dying cells to secrete IL-10 [36].

In contrast to previous studies which suggested an anti-inflammatory effect of apoptotic cells, Iyoda and Kobayashi showed that injection of apoptotic cells into the peritoneal cavity induced the expression of an inflammatory chemokine, MIP-2, and infiltration of neutrophils; anti-MIP-2 antibodies suppressed this infiltration significantly [37]. They also showed that macrophage mediated uptake and digestion of apoptotic thymocytes was accelerated upon coculturing them with neutrophils and the latter are recruited for acceleration of apoptotic-cell clearance in tissues with high apoptosis rate [38].

3.3. Loss of the anti-inflammatory response to apoptotic cells leads to diseases

Apoptotic cells also can suppress the inflammatory response elicited by PAMPs through the TLRs. The combination of apoptotic cells and ligands for TLR2, 4, and 9 mount cytokine responses that differ importantly from those elicited by either class of stimulus alone. TLR ligands induced early secretion of TNF-α, MIP-1α, and MIP-2 with later secretion of IL-10, IL-12, TGF-β1; apoptotic cells alone stimulated TGF-β1 secretion only. The combination of apoptotic cells and TLR ligands enhanced early secretion of TNF-α, MIP-1α, and MIP-2 and increased late TGF-β1 secretion, while suppressing late TNF-α, IL-10, IL-12 by a mechanism which could nevertheless be overridden by IFNγ [39]. These results point to the possibility that inflammatory diseases initiated by microbial pathogens are influenced, very likely attenuated, by apoptosis occurring in the infected tissues and lack of this influence may contribute to a serious outcome in the pathologic response. In some pathologic conditions like chronic granulomatous disease (CGD) phagocytes are severely compromised in their ability to produce anti-inflammatory mediators such as PGD2 and TGF-β1 during clearance of apoptotic debris and invading pathogens, contributing to persistence of inflammation in CGD [40].

The clearance of apoptotic cells has a role not only in tissue homeostasis, but provides also a source of antigens for immune tolerance and activation. Macrophages' anti-inflammatory and immunosuppressive tendencies in responding to apoptotic cells make them a potentially powerful regulator of adaptive immune responses, including autoimmune and anti-tumor responses. It is known that immature DCs are capable of engulfing apoptotic cells by a mechanism involving CD36, PS receptor and integrins. This 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-κB 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 [41]. This process can be beneficial in the development of tumor immunity, but severely pathogenic when it targets self. Defective clearance of apoptotic cells is often associated with autoimmune diseases: mice with deleted C1q, MER tyrosine kinase, TG2 or MFG-E8 have high titres of autoantibodies and develop autoimmune syndromes [42–45]. In TG2 knock out mice even neutrophils show up in tissues where a high rate of apoptosis has been initiated [9]. On the other hand, lack of CD14 does not lead to autoimmune disease despite of extensive persistence of apoptotic cells in tissues. It has been suggested that defective apoptotic-cell clearance plays a primary role in autoimmune disease pathogenesis only under circumstances when the clearance deficiency is accompanied by a defect in the regulation of anti-inflammatory response by apoptotic cells as it seems to be the case in C1q, MER tyrosine kinase, TG2 or MFG-E8 knock out mice [2].

4. Clearance of apoptotic cells by non-professional phagocytes

In the early phase of investigations macrophages have been considered of being the main actors capable of engulfing apoptotic cells. However, even in the early papers there are mor-

phological evidences of non-professional phagocytes "eating" adjacent cells dying by apoptosis. In recent years more and more evidences have been collected to support the notion that perhaps the reason why it is very difficult to see apoptotic cells in tissue sections is the efficient and fast removal of the daily formed apoptotic cells by their adjacent neighbors even before they show morphological features of apoptosis. According to this view, macrophages are important in the clearance of dying cells in the circulation and in places where the apoptotic rate is so high that the non-macrophage system cannot cope with the highload of corpses [2]. Mice without macrophages develop normally and are capable of removing dying corpses by the less efficient mesenchymal cells during embryogenesis without inducing an inflammatory response [46]. In spite of the high ongoing rate of apoptosis in several of their organs (just like in normal mice), there is no sign of excess apoptotic cells in tissues. These mice although born alive, rapidly succumb to bacterial infections in the absence of macrophages and neutrophils unless they receive daily antibiotic treatment.

Removal of dying corpses by neighboring viable cells not born to be phagocytes is becoming a well recognized process during tissue remodeling. Many cell types of different dermal origin have been "convicted" of being "guilty" for engulfing apoptotic [24,47,48], necrotic [49] cells as well as those undergoing anoikis or autophagy (our own unpublished findings). The extensive list of non-professional phagocytes includes, but is not limited to fibroblasts, kidney mesangial cells, testis Sertoli cells, ovarian thecal cells, smooth muscle cells, endothelial and epithelial cells, hepatocytes and mesenchymal cells (for citations see [2]). Some widely used cell lines such as 3T3, HeLa, Jurkat, COS7 (see Fig. 2), MCF-7 and HepG2 cells (from our own experiments) have been found to engulf apoptotic cells in culture systems. It is very likely that all cell types can do this to a certain extent if and when the need for "cannibalism" arises.

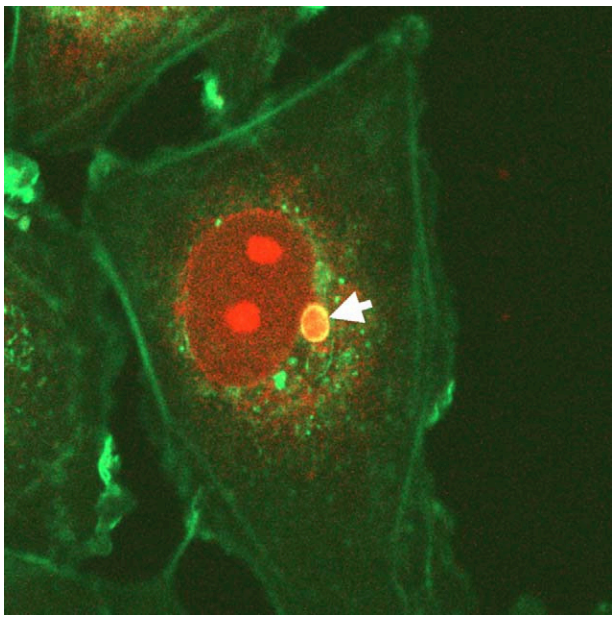

Fig. 2. Attached COS7 cell stained with phalloidin-FITC/Propidium iodide that engulfed remnant of anoikic COS7 cell stained with propidium iodide (24 h coincubation time).

The mechanism of non-professional recognition, signaling and engulfment of apoptotic and necrotic cells is being increasingly studied and at the present not well defined. Naturally, basic results obtained by studying engulfment in the nematode *Caenorhabditis elegans*, which do not have macrophages, can be all considered as part of typical non-macrophage mechanisms. Genetic studies of the removal of apoptotic cell corpses by *C. elegans* have found on the phagocytic cell *ced*-1 encoding a transmembrane receptor similar to the mammalian scavenger receptor of endothelial cells (SREC) involved in tethering and initiation of signaling for uptake of the target cell [50,51]. *Ced*-7 encodes a protein similar to the mammalian ABCA-1 transporter on both the apoptotic and phagocytic cell and is also required for phagocytosis [52]. The description of signaling molecules involved in the engulfment process have also been built upon studies in *C. elegans* (*ced*-2, 5, 7, 10, 12) and by analogy the corresponding mammalian molecules have been found, respectively (CrkII, DOCK180, ABC-1, Rac-1, ELMO) [53–55]. All the latter molecules function in cytoskeletal reorganization, ruffling, membrane extension and fusion of the membrane during the engulfment process irrespective whether they function in macrophages or non-professional phagocytes.

The changes on the surface of apoptotic cells that "flag" recognition and removal are unanimous for both professional and non-professional macrophages. Many groups have studied these including but not limited to the exposure of specific carbohydrates binding to phagocyte lectins [56], thrombospondin [57], collectins [58] or complement products (iC3b) [59] all playing roles in formation of bridges between apoptotic cells and the phagocyte. Loss of phospholipid asymmetry and surface exposure of PS on apoptotic cells is required for their phagocytosis performed by fibroblasts [60]. Gas-6 is one of the soluble proteins able to bind PS on the target cell bridging it to MER, member of the receptor tyrosine kinase family, on the phagocyte [61]. Different non-professional phagocytic cell lines express varying receptors for target interaction: fibroblasts have $\alpha_V\beta_3$, $\alpha_V\beta_5$ and CD36; kidney mesangial cells show $\alpha_V\beta_3$; testis Sertoli and ovarian thecal cells exhibit SR-B1 (scavenger receptor); endothelial cells display lectins; smooth muscle cells and epithelial cells expose PS receptors [2]. Mouse mammary epithelial cells eat apoptotic cells using the same receptors utilized by professional phagocytes as well, including the putative PS receptor, CD36, the vitronectin receptor $\alpha_V\beta_3$ and CD91 [62]. As more and more data are collected, it is becoming increasingly obvious that there are no unique molecular tools available for the non-macrophage system to ingest apoptotic cells; they just take advantage of the same molecular repertoire which is used by the professionals. However, while we know that maturation of monocytes to macrophages is a prerequisite for their recognition of apoptotic cells, it is not clear what regulatory mechanisms make fibroblasts, epithelial and other cells capable of doing the same. Apparent differences also have been observed in the engulfment of apoptotic cells by professional and non-professional phagocytes [63]: the lat-

ter are slower, recognize late stage apoptotic cells more, digest the corpses slower [46]. This may reflect a need for "educating" these cells perhaps by apoptotic cells for the unusual task of phagocytosis.

It is now clear that non-macrophage cellular systems, such as epithelial cells can regulate the function of other cells locally through paracrine actions coupled to the production of cytokines like IL-1 [64,65]. The "amateur" phagocytic cells are important source of cytokines including their pro-inflammatory response to endotoxins. For example, production of the pro-inflammatory cytokines IL-1β, IL-8, TNF-α and MIP-2 has been shown in mammary epithelial cells stimulated by LPS [62]. Therefore, it is important to clarify how apoptotic cells regulate the inflammatory response of non-professional phagocytes while they recognize and engulf these apoptotic cells. The few data available so far show that apoptotic cells, unlike necrotic cells, do not provide pro-inflammatory signals for these cells and can down-regulate the expression of pro-inflammatory cytokines induced by LPS ([62], and personal communication of David Ucker). Furthermore, apoptotic cells can elicit an anti-inflammatory response inducing the secretion of TGFβ in these cells. This means that the non-macrophage cells also posses or can acquire the recognition and signaling mechanisms which are needed to regulate their pro- and anti-inflammatory responses upon meeting apoptotic cells.

Non-professional and professional phagocytes exposed to apoptotic cells are reprogrammed for secretion of growth and survival factors like VEGF and promote the growth of surrounding endothelial and epithelial cells [66]. This is particularly important in the complex processes of wound healing which require the collaborative efforts of several cell lineages, their proliferation, migration, matrix building and contraction capabilities. The wound healing response also involves a regulated inflammatory response. The presence of apoptotic cells and their phagocytosis with the concomitant release of growth and survival factors are crucial elements of the proper healing process. Importantly, mice without macrophages are also to repair wounds with time course similar to wild-type siblings and without raising a pathologic inflammatory response; the dead cells are engulfed by stand-in phagocytic fibroblasts which seem capable of controlling the inflammatory events in wounds [67]. Lack of adequate regulation of the apopto-phagocytic system in some malignancies may lead to unwanted inflammation which promotes tumor growth and this may provide an explanation why chemotherapy-induced tumor cell death sometimes inversely ends up in tumor progression instead of regression [68,69]. In the sequential events of carcinogenesis loss of pro-apoptotic genes (which is very likely reflected in disturbed phagocytosis of dead cells) may be followed by loss of autophagy-based cell death shifting the tumor tissue toward the frequent appearance of necrotic type of cell death and the inflamed tumors often have been considered the best growing tumors. Based on these data restoration or activation of apoptosis and proper phagocytosis of dead cells in tumors may be beneficial in anti-cancer therapy as it would pull the leverage away from necrosis and inflammation towards the anti-inflammatory dominant clearance of apoptotic tumor cells [70].

5. Concluding remarks

For a long time elimination of the high number of apoptotic cells in the body has been considered a passive phenomenon with the sole purpose of getting rid of the cell corpses as fast as possible. It is clear now that naturally dying cells have important duties while passing away—beyond the obvious utilization of their biochemical components for the benefit of the whole organism. They are recognized and engulfed by both professional and non-professional phagocytes and this recognition is not only assisted by molecular elements of the innate immune system but the apoptotic cells influence functions of innate immunity modulating its inflammatory response. In addition, the apoptotic cells have a unique feature: they can induce a direct anti-inflammatory response through a so far unrevealed signaling system which may be considered as an unconventional and ubiquitous innate immunity distinguishing life from effete. It looks that the continuous appearance of apoptotic cells in tissues (cell turnover) dampens inflammatory cytokines and their frequent occurrence in inflamed tissues helps to dissolve the acute inflammatory response to prevent long term tissue damage. Apoptotic cells provide a rich source of antigens for dendritic cells and this way for the adaptive immune response and when these antigens are presented self tolerance develops with the concomitant help of the anti-inflammatory response induced by the dying cells. The balance in the number of apoptotic and necrotic cells is crucial for the development of a responding adaptive immunity including the naturally developing immune response to pathogens and the pathologic conditions in autoimmunity and tumor development. While the significance and major characteristics of the apopto-phagocytic system have been outlined there are many unanswered questions. What is the exact nature of the molecular pattern recognized by phagocytic cells before they respond both by engulfment and regulatory signals for the pro- and anti-inflammatory cytokine genes? Are soluble molecules involved in the regulatory process? Which gene regulatory systems are involved in changing the cytokine pattern secreted by the phagocytic cells? How non-professional phagocytes acquire their capacity to engulf apoptotic cells and to regulate inflammatory process? What is the relative importance of macrophages and non-macrophage phagocytes in the elimination of cell corpses and to keep the inflammatory system quiet? How the adaptive immune system can utilize best the continuous interference of apoptotic cells with pathogens or tumor antigens? The large interface between living and dying cells still have many secrets to reveal and new information to be obtained for better understanding and treatment of major diseases.

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SUPPLEMENT 2.

European Journal of Immunology

PPARγ-dependent regulation of human macrophages in phagocytosis of apoptotic cells

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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 report that addition of PPARγ antagonist during differentiation of human monocytes to macrophages significantly reduced 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 responses.

Key words: Apoptosis · Cytokines · Macrophages · Monocytes · Phagocytosis

Supporting information for this article is available at http://www.wiley-vch.de/contents/jc_2040/2007/36398_s.pdf

Introduction

Clearance of apoptotic cells has an important role in tissue remodeling and resolution of inflammation, protecting tissues from exposure to inflammatory and immunogenic contents of dying cells [1, 2]. Necrotic death leads to the release of toxic substances exacerbating inflammatory responses and triggering leukocyte influx. 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 [3]. The major mechanism to prevent secondary necrosis of apoptotic cells is their recognition and fast removal, not allowing the development of

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Abbreviations: **15d-PGJ$_2$:** 15-deoxy-$\Delta^{12,14}$ prostaglandin J$_2$ · **5(6)-CFDA-SE:** 5(6)-carboxifluorescein diacetate succinimidyl ester · **AXL:** AXL receptor tyrosine kinase · **FABP4:** fatty acid binding protein 4 · **PPARγ:** peroxisome proliferator-activated receptor gamma · **PS:** phosphatidylserine · **PTX3:** prototypic long pentraxin 3 · **Q-PCR:** quantitative RT-PCR · **TG2:** transglutaminase 2 · **TLDA:** TaqMan low-density array

inflammation and autoimmune diseases [4]. There is ample evidence that apoptotic cells do not only fail to induce but can actively suppress the release of pro-inflammatory cytokines from macrophages which engulf them, and potentiate the release of anti-inflammatory cytokines such as TGF-β1, prostaglandin E2 and platelet-activating factor [5].

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 [1, 4, 6]. When human macrophages engulf spontaneously dying neutrophils, cell surface CD31 mediates tethering of the apoptotic cells; it can discriminate between apoptotic and viable cells by selectively imparting detachment signals to viable cells, preventing their ingestion [7]. The important role of the 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 [8–10]. The phagocytic receptor CD14 has been implicated in the ICAM3-mediated interaction between human macrophages and dying neutrophils [11, 12]. We have recently observed that transglutaminase 2 (TG2) is involved in the regulation of the apopto-phagocytic system; its deletion leads to the development of autoimmune disorders [13]. Prototypic long pentraxin 3 (PTX3), an important component of the innate immune response [14], enhances C1q binding and C3 deposition on the surface of apoptotic cells, suggesting a role of PTX3 in complement-mediated phagocytosis of apoptotic cell [15].

Peroxisome proliferator-activated receptors (PPAR) are members of the nuclear receptor superfamily which heterodimerize with the retinoid X receptor [16]. PPARγ was characterized originally as a key regulator of adipocyte differentiation and lipid metabolism and it also plays an important role in glucose metabolism. PPARγ is activated by diverse synthetic and naturally occurring ligands including anti-diabetic thiazolidindione polyunsaturated fatty acids (these days in clinical use), 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2) and components of oxidized low-density lipoproteins [17–23]. Recent studies have suggested that PPARγ may promote monocyte-macrophage differentiation [24]. 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 [25, 26]. 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 [27–29]. It has not been clarified whether PPARγ is required during differentiation of macrophages to prepare them for efficient phagocytosis of apoptotic cells.

In the present study, we demonstrate that treatment of human monocytes with a PPARγ antagonist during their 5-day differentiation period significantly decreases the phagocytic capacity of the matured cells by inhibiting expression of some cell surface receptors and secreted molecules, such as CD36, CD14, TG2 and PTX3, which have been linked to the clearance of apoptotic cells. In combination with the glucocorticoid dexamethasone, which in itself leads to augmentation of the phagocytic capacity, the highly increased uptake of apoptotic neutrophils is strongly reduced. The presence of PPARγ antagonist during macrophage differentiation prevents LPS-dependent IL-10 secretion, suggesting a modulatory role of PPARγ in inflammatory processes.

Results

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 (Fig. 1). 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 (Fig. 2A–C).

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 [26]. 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 2.5 μM Rosiglitasone, a PPARγ agonist. There was no significant difference in the phagocytic capacity of Rosiglitasone-treated macro-

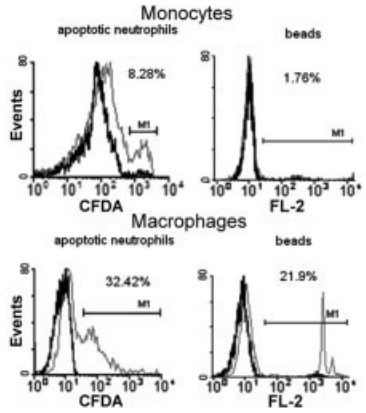

Figure 1. Phagocytic capacity of freshly isolated monocytes compared to 5-day-aged macrophages as determined by flow cytometry. The phagocytosis assay was performed using either apoptotic neutrophils or polystyrene latex beads as targets. Solid line shows the 25-min phagocytosis capacity (percent of macrophages with engulfed apoptotic neutrophils) at 37°C, while the bold line represents controls run at 4°C; the differences between the two were 5.2% (monocytes) and 29.5% (macrophages). M1 represents gating for engulfing cells.

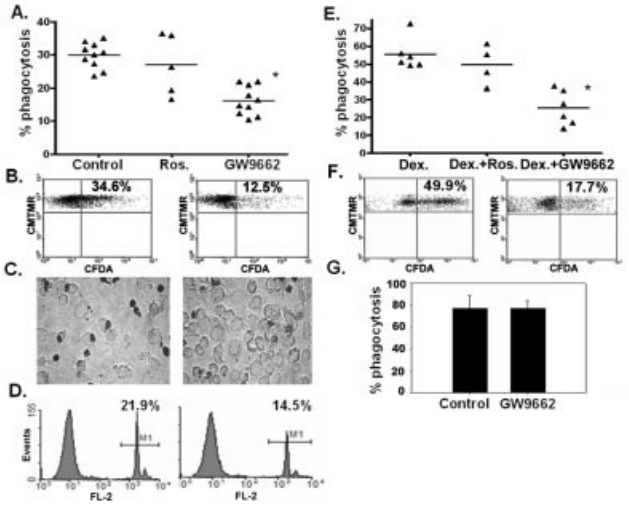

Figure 2. Effect of GW9662 on the uptake of apoptotic neutrophils, polystyrene latex beads and opsonized bacteria by macrophages. (A, E) The phagocytic ability of macrophages in the presence or absence of 10 μM GW9662 or 2.5 μM Rosiglitasone (Ros), 1 μM dexamethasone (Dex.) as determined by flow cytometry. Each triangle (▲) represents the mean of duplicate measurements from one donor; $^*p < 0.01$ considering means of results from different groups of donors. (B, F) Representative flow cytometry data of one donor. (C) Myeloperoxidase staining showing phagocytosis of untreated (left) and GW9662-treated (right) macrophages. (D) Phagocytic capacity of macrophages toward latex beads. (G) Phagocytosis of fluorescence-labeled opsonized *Listeria monocytogenes*. The results were obtained using macrophages from three different donors (means ± SD).

phages as compared to controls (Fig. 2A). 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 [30]. When differentiating monocytes were cultured *in vitro* for 5 days in the presence of 10 μM GW9662, the matured macrophages showed significantly decreased phagocytic capacity as determined by both myeloperoxidase staining and flow cytometry analysis (Fig. 2B, C). The decrease in phagocytosis capacity correlated with increasing GW9662 concentrations (Fig. 3A). Adding T0070907, another selective antagonist of PPARγ, acting by blocking its interaction with co-activators [31], also leads to decreased phagocytosis when added at 10 μM concentration to differentiating cells; in the case of two independent donors, the inhibition was 28.5 and 18.8%, respectively. Reduced phagocytic capacity of GW9662-treated macrophages was also confirmed by using carboxylate-modified polystyrene latex beads (Fig. 2D) which can mimic apoptotic cells [32–34]. GW9662 did not inhibit the engulfment of opsonized *Listeria monocytogenes* (Fig. 2G), suggesting that inhibition of PPARγ-mediated processes specifically suppresses uptake of apoptotic cells.

It has been shown that dexamethasone can reprogram monocyte differentiation toward a pro-resolution phenotype, exhibiting increased phagocytosis of apoptotic cells [35]. To decide whether this phenomenon is linked to PPARγ-dependent processes, monocytes were cultured *in vitro* for 5 days in the presence of 1 μM dexamethasone, alone or in combination with either

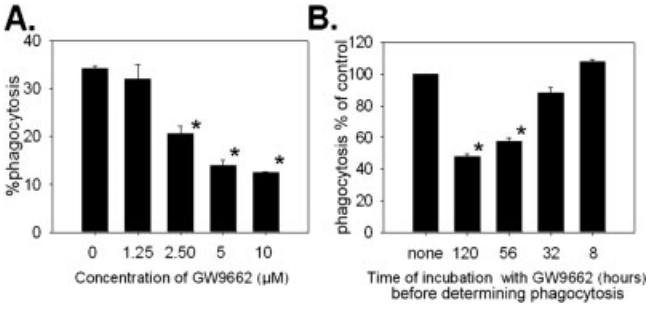

Figure 3. Time and concentration dependence of the effect of GW9662. (A) Phagocytic capacity of monocyte-derived macrophages treated for 5 days with different concentrations of GW9662 before assessment of phagocytosis. (B) Phagocytic capacity of monocyte-derived macrophages that were incubated with 10 μM GW9662 for the indicated time periods during the 5-day differentiation. Data represent results of three experiments using different donors (means ± SD).

Rosiglitasone or GW9662. Macrophages treated with Rosiglitasone in combination with dexamethasone displayed a phagocytic capacity similar to those treated with dexamethasone. However, the stimulatory effect of dexamethasone could be reversed by co-incubation of macrophages with GW9662 (Fig. 2E, F).

Next, we wanted to determine when during the differentiation period GW9662 can interfere with development of the full capacity of engulfing apoptotic cells. During the 5-day maturation period, monocyte-derived macrophages were incubated with 10 μM GW9662 for various time periods prior to the assessment of phagocytosis. GW9662 reduced phagocytosis of apoptotic neutrophils in a time-dependent manner, and our data indicate that significant inhibition of phagocytosis by PPARγ antagonist requires its presence from the early phase of macrophage maturation (Fig. 3B). When GW9662 was added to the macrophages for the duration of the phagocytosis assay itself, it did not influence the uptake of apoptotic neutrophils (data not shown).

Deletion of PPARγ leads to defective phagocytosis of apoptotic cells

To get more evidence for the involvement of PPARγ in the regulation of the phagocytic capacity of differentiated macrophages toward apoptotic cells, we used PPARγ$^{fl/-}$ Lys-Cre mice (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 [36]. 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 (data not shown). Differentiated bone marrow-derived and peritoneal macrophages from PPARγ$^{fl/-}$ Lys-Cre mice showed reduced uptake of apoptotic thymocytes as compared to controls (Fig. 4A, B).

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 expression of the known PPARγ target gene fatty acid binding protein 4 (FABP4) [37]. A 3.5-fold induction of PPARγ and a 14-fold induction of FABP4 were observed by quantitative RT-PCR (Q-PCR) analysis during the differentiation period (Fig. 5A). Cells cultured for 5 days in the presence of 10 μM GW9662 showed a strong inhibition of FABP4 expression, clearly showing that the PPARγ antagonist functions as expected in

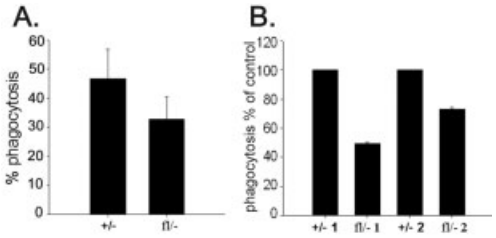

Figure 4. Clearance of apoptotic thymocytes by mouse macrophages with deleted PPARγ. Bone marrow-derived (A) and peritoneal (B) macrophages from PPARγ$^{fl/-}$ Lys-Cre mice present decreased phagocytic capacity as compared to control PPARγ$^{+/-}$ Lys-Cre mice. The experiment was performed on four PPARγ$^{fl/-}$ Lys-Cre and four PPARγ$^{+/-}$ Lys-Cre mice in the case of bone marrow-differentiated macrophages (results show means ± SD; $p < 0.1$). In the experiments with peritoneal macrophages, results (means ± SD of three separate assays in each) were obtained from two PPARγ$^{fl/-}$ Lys-Cre and two PPARγ$^{+/-}$ Lys-Cre mice.

differentiating macrophages. CD36, TG2, AXL receptor tyrosine kinase (AXL) and PTX3, genes that are involved in the phagocytosis of apoptotic cells [2, 8–10, 13, 14], were down-regulated at the transcription level in these cells as compared with control macrophages. While the mRNA levels 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 (TLDA), which in parallel determine the expression levels of all the so far described genes involved in the phagocytosis of apoptotic cells. The list of genes, which includes phagocytosis receptors, cell surface regulatory molecules, bridging molecules, signal transducers, engulfment proteins, effector molecules, transcription factors, inflammatory regulators and cytokines, is provided as Supporting Information to this manuscript. Apart from CD36, TG2, AXL and PTX3, the presence of PPARγ antagonist did not alter significantly (more than two times) the level of expression of other pro-phagocytic genes; there was a small increase in mRNA of the ABCA1 pro-phagocytic gene [38] while we did not observe changes in the level of others not listed but exampled here by showing data of CD14 transcripts by Q-PCR (Fig. 5A).

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 twofold increase of ABCA1 as well as CD14 mRNA (Fig. 5A, B). 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.

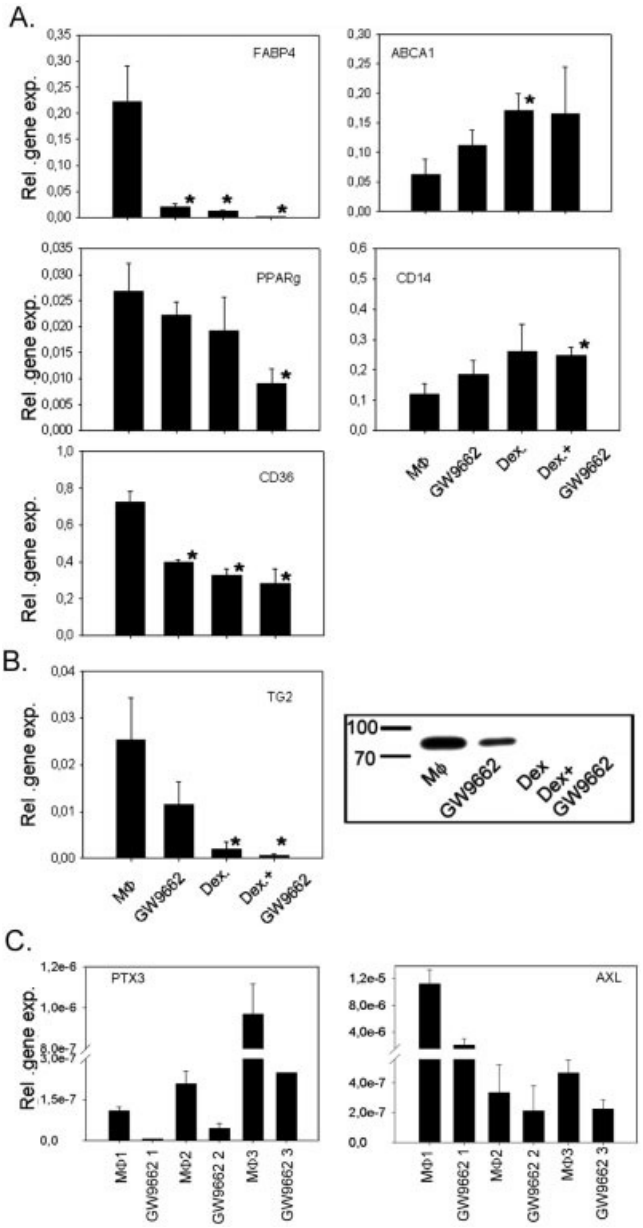

Figure 5. The effect of GW9662 on the expression of genes involved in phagocytosis of apoptotic cells. (A) Q-PCR assays: Data show the means ± SD of triplicate measurements from three different donors; $^*p < 0.05$. (B) The effect of GW9662 on TG2 expression at mRNA (left, RT-PCR) and protein level (right) as detected by Western blotting; $^*p < 0.05$. (C) TLDA assays: The results represent the means ± SD of triplicate measurements obtained. TLDA from three donors.

Flow cytometric analysis was also used to determine whether the effect of GW9662 on phagocytosis was associated with changes of CD36 and CD14 expression at the protein level. As it is seen in Table 1, addition of GW9662 led to down-regulation of CD36 as well as CD14 on the surface of macrophages. In accordance with the mRNA levels, an increase of TG2 protein content was found in differentiated macrophages, which was partially prevented by GW9662 treatment (Fig. 5B). 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 three- or fourfold higher, respectively, as compared to monocytes, though not reaching the ones observed on control macrophages (Table 1). Dexamethasone treatment, added to macrophages either alone or in combination with GW9662, did not affect the expression of these differentiation markers.

Cytokine profile of LPS-stimulated macrophages treated with PPARγ antagonist

Macrophages aged 5 days in the presence of various agents were stimulated with LPS for 30 min, and then incubated in fresh media without serum for 20 h. Following stimulation with LPS, a portion of macrophages was incubated for 25 min with apoptotic neutrophils; then, the non-ingested neutrophils were removed and the macrophages were incubated in fresh media without serum for 18–20 h. The collected supernatants were used for the measurement of cytokine concentrations.

Cells differentiated in the presence of GW9662 did not show significant differences in secretion of TNF-α, IL-6, IL-8 and TGF-β upon LPS stimulation but had much reduced IL-10 secretion (Fig. 6). 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 cells treated with dexamethasone only, excluding IL-10 which was secreted at a lower level (Fig. 6).

Several studies have demonstrated that contact with apoptotic cells leads to a potent anti-inflammatory and immunosuppressive response in macrophages through the production of anti-inflammatory cytokines such as TGF-β and suppression of inflammatory mediators like IL-8 and TNF-α [5, 39]. In accordance with these data, apoptotic cells up-regulated TGF-β secretion and down-regulated IL-6, TNF-α and IL-8 in LPS-stimulated macrophages in our study (Fig. 6). 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 LPS stimulation. Apoptotic cells neither abolished nor enhanced this macrophage response – except in the case of TGF-β where apoptotic cells could enhance the effect

Table 1. The effect of GW9662 on expression of different cell surface receptors. Data show one representative flow cytometry experiment.

	Expression (mean fluorescence)				
	Monocyte	Control macrophage	GW9662-treated macrophage	Dex.-treated macrophage	Dex. + GW9662-treated macrophage
CD206	8.06	47.37	31.19	59.08	52.65
CD16	12.56	78.41	40.06	71.79	39.48
CD36	435.53	200.355	100.235	42.68	39.09
CD14	251.58	283.92	203.02	280.88	228.99

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 (Fig. 6).

We did not observe changes in the induction of the inflammatory cytokines IL-1β (Fig. 6) and IL-12p70 or GM-CSF (not shown) following either GW9669 treatment or incubation of stimulated macrophages with apoptotic cells. Normally, macrophages do not secret the cytokines IL-3, IL-4, IL-7, and IL-5. Using the PPARγ antagonist GW9662 during the differentiation period, we could not induce the secretion of these cytokines either by LPS or by apoptotic cells (data not shown), suggesting that PPARγ does not play a role in blocking the production of these cytokines.

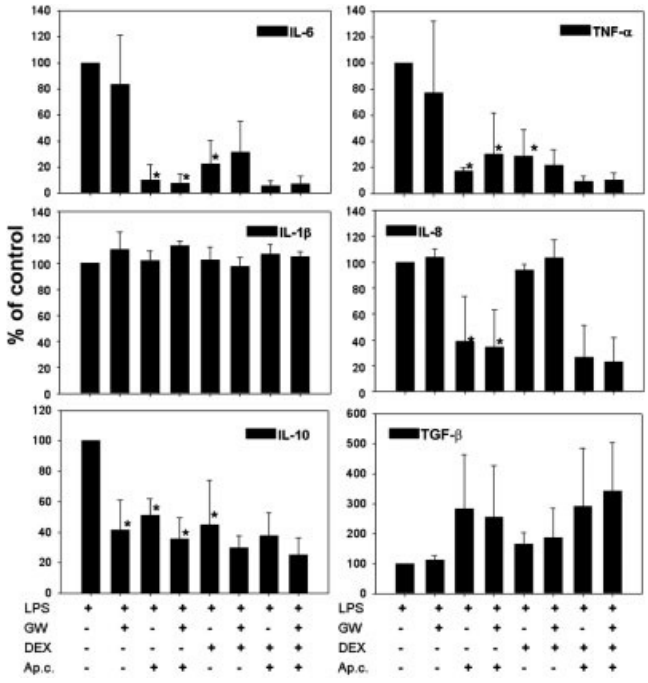

Figure 6. Cytokine profiles of monocyte-derived macrophages treated with GW9662, dexamethasone, or GW9662 in combination with dexamethasone for 5 days. "Control" as 100% represents the level of cytokines produced by 5-day-aged untreated macrophages stimulated on the 5th day with 0.5 μg/mL LPS for 30 min. The levels of basal cytokine secretion (unstimulated *vs.* stimulated) were the following: IL-6 (34.29 *vs.* 4329.31 pg/mL), TNF-α (8.53 *vs.* 799.56 pg/mL), IL-1β (78.96 *vs.* 74.48 pg/mL), IL-8 (4256.71 *vs.* 35592.78 pg/mL), IL-10 (3.5 *vs.* 254 pg/mL), TGF-β1 (864.61 *vs.* 673.2 pg/mL). All data are represented as means ± SD from four independent experiments using different donors. *$p < 0.05$.

Discussion

For the greater benefit and less risk of PPARγ ligands in clinical use, the tissue-specific biology of this nuclear receptor should be fully understood. It has been shown that macrophages derived by *in vitro* differentiation of PPARγ-deficient mouse embryonic stem cells were comparable to wild-type similarly derived macrophages in their expression of macrophage-specific cell surface markers, phagocytic activity toward microbial agents and their inflammatory responses [40]. In our study, typical cell surface markers of macrophages could be detected when PPARγ antagonist was present during their differentiation (Table 1). This means that, similarly to the mouse system [40], differentiation of human macrophages, at least in terms of commonly used parameters, also can take place without the presence of functioning PPARγ. Maturation of macrophages from monocytes is required before they can recognize and remove dead cells and display a concomitant anti-inflammatory response [36, 41]. 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 that are compromised in their ability to engulf apoptotic cells. Furthermore, macrophage-specific deletion of PPARγ in mice led to decreased phagocytosis of apoptotic cells by either differentiated bone marrow cells or peritoneal macrophages. 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.

Our results clearly suggest that a naturally formed ligand of PPARγ acts during macrophage differentiation driving expression of apopto–phagocytic genes. We could demonstrate that macrophages that differentiate in the presence of PPARγ antagonist express significantly less CD36, TG2, AXL and PTX3, suggesting their regulation by PPARγ. Expression of other so far described pro-phagocytic genes was not in influenced by PPARγ antagonist. Cell surface expression of CD36 was decreased and the TG2 contents of antagonist-treated cells were reduced. Interestingly, the concentration of detectable CD14 was also decreased on the cell surface in antagonist-treated cells, although the level of CD14 mRNA did not change. PPARγ was earlier found to modulate CD36 gene expression through direct interaction with the proximal promoter of CD36 *via* its specific response element [24, 42]. The TG2 gene is under retinoid regulation in macrophages, and it has been recently shown that PPARγ can induce retinoid-producing enzymes, leading to indirect activation of TG2 [43]. There is no data available that may link regulation of AXL or PTX expression to PPARγ ligation. PTX3 production is mainly induced by inflammatory signals [44]; the human PTX3 proximal promoter contains Pu1, AP-1, NF-κB, SP-1 and NF-IL-6 sites [14].

Are the observed changes in gene expression upon blocking PPARγ activity sufficient to explain the decreased phagocytosis 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 the activation of Rac, which is obligatory for the uptake of dead cells [2]. In the case of phosphatidylserine (PS) recognition – which is a dominant "eat me" signal when neutrophils undergo apoptosis [10] – 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. TG2 facilitates the process of PS exposure and TGF-β activation needed for efficient phagocytosis [13]. With TLDA, we could determine that all the necessary molecular elements of PS-dependent recognition and engulfment are expressed in differentiated human macrophages (manuscript "in press"). Although 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-regulation of 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 [10]. Exposed calreticulin on the apoptotic cell surface is recognized by collectins and ficolins (such as C1QA, PTX) serving as bridging molecules; then, the opsonized dead cells are bound to phagocytosis receptors on the surface, including calreticulin in conjunction with LRP1 [2]. 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 [45]. 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 [14, 15]; by blocking PTX3 expression, PPARγ antagonist may significantly reduce the efficiency of phagocytosis.

In our system, addition of the synthetic PPARγ ligand Rosiglitasone to maturing macrophages could not increase either their phagocytic capacity toward apoptotic cells (Fig. 2) or CD36 expression (not shown). It has been reported that PPARγ ligand-treated alveolar macrophages present both enhanced phagocytic capacity and increased CD36 expression [46]. It should be noted that alveolar macrophages are highly specialized phagocytic cells which, unlike the M-CSF-differentiated macrophages in our study, may become sensitive to PPARγ ligands while they mature in the lung.

There are several studies that have suggested that PPARγ-specific ligands can regulate inflammation by inhibiting the expression of various pro-inflammatory proteins. On the other hand, several investigators have proposed that PPARγ activation has no anti-inflammatory effects. PPARγ agonists have been found to inhibit the secretion of the pro-inflammatory mediators such as gelatinase B, IL-6, TNF-α and IL-1β in monocytes and also reduced the expression of inducible NOS, scavenger receptor A, metalloproteinase 9, IL-10 and IL-12 in macrophages [27, 47–49]. In contrast, it has been reported by others that the PPARγ ligand 15d-PGJ_2 induced IL-8 gene expression, suppressed monocyte

chemoattractant protein-1 and did not affect the secretion of the chemokine RANTES in human monocyte/macrophages. The PPARγ agonist Rosiglitasone failed to modulate LPS-induced IL-8 secretion [50]. It has been also shown in macrophages with deleted PPARγ that PPARγ expression is not essential for PPARγ ligands to exert their anti-inflammatory effects [51]. From these studies, one may conclude that the effects of PPARγ ligands on macrophage inflammatory responses may depend on the type of PPARγ ligand used, the mode by which the macrophages are activated and the investigated inflammatory response.

In our experiments, inhibition of PPARγ during macrophage differentiation did not influence the basal cytokine production of these cells (data not shown). The most significant consequence of blocking PPARγ-depen--dependent gene expression during macrophage differentiation was the prevention of IL-10 release upon LPS stimulation. IL-10 is an essential anti-inflammatory cytokine that inhibits the synthesis of several pro-inflammatory proteins, including other cytokines. Mice defective in IL-10 expression develop an inflammatory Crohn's-like disease and produce enhanced amounts of TNF-α in response to LPS [52]. Recently, it was found in a mouse model of asthma that administration of the PPARγ agonist may improve the asthmatic features *via* regulation of IL-10 expression/IL-10 receptor activation, suggesting that PPARγ agonists may have therapeutic potential for the treatment of airway inflammation and hyper-responsiveness [53]. Based on our results, one may presume that upon inflammatory stimuli the initial IL-10 secretion from macrophages is driven by a natural PPARγ ligand.

The secretion of the inflammatory IL-1β and IL-8 and the anti-inflammatory TGF-β upon LPS addition was not influenced by treatment of maturing macrophages with PPARγ antagonist. Regarding TNF-α and IL-6, we have not observed significant inhibition of their secretion either – though some studies suggest that they may be controlled by PPARγ ligands. It was found that 15d-PGJ_2 inhibited TNF-α and IL-6 production in peripheral blood monocytes, but in the same study other high-affinity ligands failed to affect cytokine production [54]. We also showed that none of the responses of macrophages to apoptotic cells was altered by blocking PPARγ during macrophage differentiation. This suggests that the so far unrevealed anti-inflammatory signaling pathway elicited by the contact of apoptotic cells with recognition receptors of macrophages does not depend on PPARγ-mediated gene expression – even in the case of IL-10.

Glucocorticoids represent powerful anti-inflammatory compounds due to their capacity to inhibit inflammatory cell recruitment and to down-regulate production of and responsiveness to pro-inflammatory cytokines [55]. Long-term exposure of monocytes to the synthetic glucocorticoid dexamethasone programs monocyte differentiation toward a pro-resolution phenotype, and this includes increased capacity for phagocytosis of apoptotic cells. The pro-resolution macrophage phenotype was associated with loss of actin-containing podosome structures, reduced p130 Cas expression, loss of paxillin/pyk2 phosphorylation and high level of active Rac [36]. 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). The PPARγ-regulated pro-phagocytic genes CD36 and TG2 are down-regulated by dexamethasone itself (while they acquire higher phagocytic capacity), and this cannot be reversed by PPARγ antagonist, suggesting that the presumed natural PPARγ ligands and glucocorticoids exert their pro-phagocytic effect through different pathways. Further work is needed to clarify the details of these two molecular mechanisms, both leading to enhanced clearance of apoptotic cells.

It was reported that glucocorticoid treatment of maturing macrophages can prevent the induction of TNF-α by LPS, and it also inhibits the release of IL-6 and IL-10 during the differentiation period [56, 57]. Accordingly, dexamethasone alone down-regulated LPS-induced secretion of pro-inflammatory cytokines and up-regulated the release of anti-inflammatory TGF-β in differentiated macrophages. Furthermore, dexamethasone treatment of differentiating macrophages does not interfere with the anti-inflammatory action of apoptotic neutrophils, rather potentiates it. 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.

Materials and methods

Materials

Sterile plastics were purchased from BD Biosciences and Corning Costar; IMDM was from Gibco; PBS was from Oxoid; RPMI, dexamethasone, Histopaque1119, Histopaque1077, AB serum, propidium iodide, 3,3′-dimethoxybenzidine, carboxylate-modified fluorescent polystyrene latex beads, brain heart infusion, Ca-Ionophor A23187, and LPS were purchased from Sigma Aldrich. *Listeria monocytogenes* was from LGC Promo-

chem. Ficoll-Paque Plus was obtained from Amersham Bioscences. CD14 microbeads, human specific, were purchased from Miltenyi Biotec, Germany. Human and murine M-CSF were from Peprotech. The PPARγ ligands GW9662 and Rosiglitasone were purchased from Alexis Biochemicals.

FITC-conjugated mouse IgM, PE-conjugated mouse IgG1κ and mouse anti-human CD36, CD16, CD206 monoclonal antibodies were from BD Biosciences Pharmingen; PE-conjugated mouse IgG2a and monoclonal mouse anti-human CD14 antibodies were obtained from Dako Cytomation. Cell Tracker Orange CMTMR and 5(6)-carboxifluorescein diacetate succinimidyl ester [5(6)-CFDA-SE] were purchased from Molecular Probes.

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, 2 mM EDTA. Neutrophils were isolated from the "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, 5 ng/mL M-CSF in the presence or absence of either 1 μM dexamethasone or 10 μM PPARγ antagonist GW9662, or 2.5 μM PPARγ agonist Rosiglitasone. Neutrophils were kept in tissue culture for 24 h in IMDM with 1% AB serum while they underwent apoptosis [36]. The apoptosis of neutrophils was verified by light microscopy using May–Giemsa staining and by flow cytometric analysis of propidium iodide-stained samples, before feeding them to macrophages. Isolated thymocytes from 4-wk-old NMRI mice were cultured in RPMI 1640 medium 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.

Bone marrow and peritoneal cells from mice with macrophage-specific deletion of PPARγ

We obtained the macrophage-specific PPARγ knockout mice from L. Nagy (University of Debrecen). Mice carrying null or floxed alleles of PPARγ were created as described [58–60]. These mice were backcrossed to the C57BL/6J strain for eight generations. The lysosyme promoter was utilized to express the *cre* recombinase specifically in the macrophages. Mice were bred with Lysozyme-Cre (Lys-Cre) transgene animals by L. Nagy *et al.* in order to create the following genotypes: $PPAR\gamma^{+/-}$ Lys-Cre and $PPAR\gamma^{fl/-}$ Lys-Cre. Genotypes were determined by PCR of tail DNA as described [58, 61]. Recombination was analyzed by RT-PCR as described [61]. Lys-Cre animals [62] were obtained from I. Förster (University of Munich). Testing large numbers of animals, the efficacy of the Lys-Cre-mediated recombination was found to be the same in bone marrow and peritoneal macrophages (A. Szanto *et al.*, manuscript in preparation). All animal experiments were carried out under the ethical guidelines established by the 28th Act in 1998 of the Parliament of the Republic of Hungary.

Bone marrow cells were isolated from the femur of $PPAR\gamma^{+/-}$ Lys-Cre and $PPAR\gamma^{fl/-}$ Lys-Cre mice, then washed in saline and cultured in RPMI 1640 supplemented with 10% FBS, 2 mM 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 and assayed after 10 days.

Thioglycolate-elicited macrophages were harvested by peritoneal lavage, 4 days after injection of 3 mL 3% thioglycolate solution. Cells were washed with saline and cultured for 2 days in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 1 mM Na-pyruvate, streptomycin and penicillin at 37°C and 5% CO_2.

Macrophage phagocytosis assays

Dying neutrophils or thymocytes before death stimuli were labeled with 5(6)-CFDA-SE (15 μM, overnight), washed free of conditioned media and resuspended in PBS before their addition to a prewashed Cell Tracker Orange CMTMR-labeled (3.75 μM, overnight) 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 or 4°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. Macrophages were gated on the basis of forward and side scatter properties, and the percentage of macrophages positive for both CMTMR and 5(6)-CFDA-SE was determined subtracting 4°C values from 37°C ones.

Phagocytosis of *Listeria monocytogenes*

The bacterial strain used in this study is derived from ATCC 35152 (LGC Promochem). Cultures of *Listeria monocytogenes* were grown in brain heart infusion at 37°C for 15 h. *Listeria monocytogenes* was labeled with FITC isomer (0.1 mg/mL, overnight at room temperature). Next morning the bacteria were washed with PBS and opsonized with 10% serum for 10 min. After opsonization, the serum was removed by centrifugation and added to human differentiated macrophages for 25 min. The ratio of macrophages to bacteria was 1 : 15. The percentage of macrophages taking up labeled bacteria was determined 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 a 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 1 h at 4°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 25 mM Tris, 1% NP40, 150 mM NaCl, and protease inhibitor cocktail (Sigma). Membrane and nuclear fractions were removed by centrifugation at 14 000 × *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* Q-PCR using TaqMan probes. Transcript levels in the case of PPARγ, FABP4, ABCA1, CD36, CD14, and TG2 were normalized to the expression level of cyclophilin D.

For TLDA (Applied Biosystems, Assay ID: 4342379–18S) pre-designed TaqMan probe and primer sets for target genes were chosen from an online catalogue. The sets were factory-loaded into the 384 wells of the TLDA. Array format was customized with two replicates per target gene, and for each biological sample two parallel analyses were carried out. TLDA were used in a two-step RT-PCR process. First-strand DNA was synthesized from 1 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems). Q-PCR reactions and analysis were then carried out in TLDA using the TaqMan Universal Master Mix and the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Per port (4 ports/sample), 100 μL sample volume was used in 1 ng/μL concentrations. Expression levels of target genes were normalized to 18S rRNA as endogenous control. Gene expression values were calculated based on the ΔΔCt method, where one sample was designated as calibrator, through which all other samples were analyzed. ΔCt represents the threshold cycle (Ct) of the target minus that of 18S rRNA, and ΔΔCt represents the ΔCt of each target minus that of the calibrator. Relative quantities (RQ or fold changes) were determined using the equation where relative quantity equals $2^{-\Delta\Delta Ct}$.

Determination of cytokine release

Macrophages aged for 5 days in the presence of various agents were stimulated with 0.5 μg/mL LPS for 30 min. Part of the samples was incubated for 25 min with apoptotic neutrophils following LPS stimulation. After this period, the non-ingested neutrophils were removed and the macrophages were incubated in fresh media without serum for 20 h. The supernatants were collected and analyzed for the presence of IL-8, IL-6, IL-1β, IL-10, TNF-α, IL-12p70, IL-3, IL-4, IL-5, IL-7, and GM-CSF using the Human Inflammation and Human Allergy Mediators BD Cytometric Bead Assay. TGF-β1 measurements were performed by ELISA using the BD OptEIA Set Human TGF-β1 kit (BD Biosciences).

Statistical analysis

Statistical analysis was performed by using the unpaired Student's *t*-test.

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Note added in proof: The manuscript describing the TLDA data of macrophages referred to the text as "in press" has now been accepted for publication. The reference is

Petrovski, G., Zahuczky, G., Katona, K., Vereb, G., Martinet, W., Nemes, Z., Bursch, W. and Fesus, L., Clearance of dying autophagic cells of different origin by professional and non-professional phagocytes. *Cell Death Differ.* 2007. in press.

SUPPLEMENT 3.

DC-SIGN-mediated uptake and activation of dendritic cells by apoptotic neutrophils

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Abbreviations used in this paper: RSG – rosiglitasone, 5(6)-CFDA-SE - 5(6)-Carboxifluorescein Diacetate Succinimidyl Ester

Abstract

Dendritic cells (DC) and macrophages exhibit overlapping functions as sensors of danger signals as well as professional antigen presenting cells. While macrophages are specialized to the rapid clearance of apoptotic cells, the primary role of DC is to present the captured antigens to T cells and direct the immune system either to tolerance or to activation. Here we show that the phagocytic capacity of human monocyte-derived DC is slow and less efficient than that of macrophages but can be upregulated by the synthetic PPARγ ligand rosiglitasone. We also found that $CD1a^-$ DC with high expression of CD209, are preferentially involved in the engulfment of neutrophils undergoing apoptosis. We also provide evidence that CD209 participates in the uptake of apoptotic neutrophils as demonstrated by antibody blocking experiments. Furthermore, functional studies revealed that the long term interaction of apoptotic neutrophils and/or their released products with DC triggers a pro-inflammatory cytokine response that sensitizes DC for high IL-12 and IL-10 secretion induced by additional inflammatory stimuli. These results show that in contrast to macrophages that remain immunologically silent DC respond to apoptotic neutrophils with an inflammatory response suggesting that the interplay of macrophages and DC has an impact on tolerance induction and immunity.

Introduction

Circulating neutrophils are recruited in large numbers to the site of infection where they exert various effector functions to eliminate pathogens and damaged cells. During the resolution of inflammation neutrophils are dying through apoptosis and thus limit leukocyte-mediated tissue damage. Similar to other cells neutrophils undergoing apoptosis release “find-me” and express “eat-me” signals to their environment to alarm for prompt engulfment by neighboring non-professional or by recruited professional phagocytes such as macrophages and dendritic cells (DC).. These cells act as professional antigen presenting cells (APC) and are able to instruct adaptive immunity for the maintenance of self tolerance or to induce immunity (1).

Macrophages are specialized to the rapid and efficient clearance of pathogens and dying tissue cells, whereas the major function of DC is to transport antigens acquired from engulfed apoptotic cells to draining lymph nodes (LN) together with transferring co-stimulatory signals to T-lymphocytes (2-4). 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 or to engulf the apoptotic cells. Several components of the innate immune system are utilized in this process among them soluble factors which bind to distinct molecular patterns of apoptotic cells (5-8). Multiple studies have demonstrated that the contact of macrophages with apoptotic cells leads to a potent anti-inflammatory and immunosuppressive response, whereas recent experiments indicated the DC-mediated immune stimulatory potential of certain tumor cells undergoing drug- or stress-induced apoptosis (9-13). The uptake of apoptotic cells by DC is restricted to their immature differentiation state and has been attributed to the $\alpha_v\beta_5$ integrin, the scavenger receptor CD36, and the MFGE8 receptor (14, 15). Dendritic cells are

able to present antigens acquired from virus-infected apoptotic cells and stimulate antigen-specific MHC class II-restricted $CD4^+$ as well as MHC class I-restricted $CD8^+$ T cells referred to as 'cross priming'(14). This T-cell stimulatory capacity is highly dependent on activation signals mediated by concomitant inflammatory stimuli (16).

Peroxisome proliferator activated receptors (PPARs) are members of the nuclear hormon receptor superfamily of transcription factors, which heterodimerize with the retinoid X receptor (17). PPARγ is activated by diverse synthetic and naturally occurring lipid ligands including anti-diabetic thiazolidindione and poly-unsaturated fatty acids such as 15-Deoxy-$\Delta^{12,14}$ Prostaglandin J_2 (15d-PGJ_2) and several components of oxidized low density lipoproteins (18-24). PPARγ is highly up-regulated during the monocyte to macrophage maturation program, and has been implicated in the regulation of anti-inflammatory responses upon apoptotic cell uptake. In earlier studies we have developed an *in vitro* phagocytosis system that mimicks *in vivo* inflammation sites, and studied the effects of apoptotic neutrophil engulfment on macrophages. We found that addition of PPARγ antagonist during the differentiation of human monocytes to macrophages significantly reduces the capacity of macrophages to engulf apoptotic neutrophils, suggesting that a naturally formed ligand of PPARγ acts during macrophage differentiation driving the expression of apopto-phagocytic genes (6). It has also been shown that not only monocytes and macrophages, but also DC express PPARγ receptors at high levels (25, 26). We found that monocyte-derived DC represent a heterogeneous population of cells that can be divided to $CD14^{low}CD1a^-$ and $CD14^-CD1a^+$ subsets that correspond to consecutive differentiation stages and differ in the expression of PPARγ and in their functional activities (27). Importantly, the $CD1a^-$ subtype exhibits higher phagocytic and internalizing capacity and induces more tolerogenic immune responses than their $CD1a^+$ counterparts. Furthermore, the ratio of the $CD1a^-$ subset can be increased by the transcriptional activation of the PPARγ receptor gene by its synthetic ligand

rosiglitasone (RSG) or by serum lipids (25, 27). However, the potential of these DC subsets to internalize apoptotic neutrophils and the cellular response to this event has not been investigated so far.

The C-type lectin DC-SIGN (CD209) is widely expressed on the surface of immature tissue DC and *in vitro* generated monocyte-derived DC and acts as a multifunctional receptor for many pathogens. As a pattern recognition receptor, DC-SIGN binds to a variety of viral, bacterial, fungal and parasitic pathogens including human immunodeficiency virus 1 (28). *Mycobacterium tuberculosis* targets DC-SIGN through ManLAM and induces DC maturation with IL-10 secretion thus inducing immune suppression and supporting the survival of bacteria (29). As an adhesion molecule DC-SIGN binds to ICAM-2 (30) on endothelial cells and assists DC migration, whereas the interaction of DC-SIGN with ICAM-3 on T-cells is essential for the initiation of immune responses (28). It has also been shown that DC-SIGN does not bind to ICAM-3 present on the surface of neutrophils, but it is specific for the carbohydrate moieties of polymorphonuclear glycoproteins such as the β_2 integrin Mac1 and the mucin-like structure CEACAM1 (31, 32).

In this study we used a previously established *in vitro* phagocytic system (33) to study the effect of apoptotic neutrophil uptake on DC activation and have shown that i) apoptotic neutrophils are preferentially internalized by the CD1a$^-$DC-SIGN^{high} DC subset, ii) similar to macrophages the activation of PPARγ regulates the capacity of DC to engulf apoptotic neutrophils, iii) DC-SIGN is not only involved in the binding of glycoproteins expressed by neutrophils as described earlier (31, 32) but also mediates the engulfment of apoptotic cells, iv) the long term interaction of apoptotic neutrophils with DC results in pro-inflammatory cytokine secretion, and v) in the presence of additional inflammatory stimuli the uptake of apoptotic neutrophils sensitizes DC for robust inflammatory responses with the concomitant secretion of IL-10.

Results

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

Our previous results (33) showed that 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 DC are also able to internalize apoptotic neutrophils albeit this process is slow and less efficient than that of macrophages. As shown in Fig. 1A the internalization of neutrophils undergoing apoptosis by immature DC requires 8 – 24 hours and results not more than 20 – 35 % of phagocytosing cells (Fig. 2B). Taken the rapid progress of neutrophil apoptosis and to avoid secondary necrosis, in our further experiments we used an *in vitro* phagocytosis system where immature DC were mixed with an excess of neutrophils (1:5 ratio) previously undergoing spontaneous apoptosis for 16 hours (~35% apoptotic cells, Fig 1B), and co-cultured them for an additional 8 hours with DC.

Our previous studies also revealed that PPARγ is up regulated during the differentiation of monocytes towards both macrophages (33, 34) and DC (25). The maturation program and the phagocytic activity of macrophages is down regulated by PPARγ antagonists, whereas the agonistic ligand RSG is able to shift monocyte-derived DC differentiation to the generation of $CD1a^-$ cells previously characterized by high phagocytic and internalizing capacity of dextran sulphate, latex beads and pathogens (27). To assess whether the capacity of immature DC to engulf apoptotic neutrophils could also be influenced by endogenous or exogenous PPARγ ligands, freshly isolated human monocytes were cultured for 5 days in the presence of 2.5μmol RSG and the generated immature DC were tested for their efficacy to internalize apoptotic neutrophils. In line with our previous studies

and as it is shown in Fig 2A, the ratio of $CD1a^-$ cells varied individually, but DC generated in the presence of RSG contained a higher proportion of $CD1a^-$ cells than those differentiated without RSG. This effect was more pronounced when the proportion of $CD1a^-$ cells was low (donors 1, 2 and 5). The agonist-induced activation of PPARγ not only resulted in a shift of $CD1a^-$ ratios but also induced an increased net phagocytosis of apoptotic neutrophils obvious in four out of six donors (Fig. 2B). These results confirmed our previous results on the higher internalizing capacity and the less mature differentiation state of $CD1a^-$ monocyte-derived DC and also identified this DC subtype to be preferentially involved in the engulfment of apoptotic cells (Fig 1C) similar to the engulfment of other particulate or soluble exogenous material (27). These results also suggest that the individually variable ratio of the $CD1a^-$ DC subset regulated by endogenous and/or synthetic PPARγ agonists, determines the efficacy of this important DC function.

Phagocytosis of apoptotic neutrophils by immature dendritic cells down regulates the cell surface expression of CD209 as a result of its participation in the engulfment process

The C-type lectin DC-SIGN/CD209 acts as a pattern recognition, internalizing, signal transducing and adhesion molecule, and is expressed by both $CD1a^-$ and $CD1a^+$ monocyte-derived DC induced by IL-4 (35). When CD209 was used as a DC marker for monitoring the contribution of $CD1a^-$ and $CD1a^+$ DC subsets upon phagocytosis of apoptotic neutrophils (Fig. 2A, B) we observed that DC loaded with apoptotic cells exhibited decreased CD209 expression as compared to non-phagocytosing cells shown at the single cell level by flow cytometry and confocal microscopy (Fig. 3A, B). This effect was observed irrespective of whether the immature DC were generated in the absence or presence of RSG showing that the down regulation of DC-SIGN is not linked to PPARγ-mediated regulation of phagocytosis but is associated with the phagocytic event. This assumption was further studied by experiments

carried out in the presence of a blocking antibody as shown in Fig. 3C. The CD209-specific blocking antibody, present throughout the 8-hour co-cultures of DC and apoptotic neutrophils, resulted in the down regulation of CD209 presumably due to antibody-mediated down regulation of the receptor. However, this effect also resulted in a dramatic reduction of the uptake of apoptotic neutrophils by DC pointing to the direct involvement of CD209 in the internalization of apoptotic neutrophils (Fig. 3D, E). Further analysis of DC that mediate phagocytosis of apoptotic neutrophils by confocal microscopy demonstrated that DC with high CD209 expression were preferentially involved in engulfing apoptotic cells and the membrane expression of CD209 could be synergistically blocked by the presence of apoptotic neutrophils and blocking antibodies (Fig 3E). These results revealed that membrane CD209 of DC does not only interacts with apoptotic neutrophils (32) but also participates in the internalization process preferentially mediated by $CD1a^{-}DC\text{-}SIGN^{high}$ IDC.

Pro-inflammatory cytokine secretion of dendritic cells incubated with apoptotic neutrophils

Uptake of apoptotic cells, unlike necrotic ones, in most cases do not provoke an inflammatory response and can even down regulate signaling events initiated by pro-inflammatory stimuli (2, 36). 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 (33, 37). In order to study the effect of apoptotic neutrophils on the cytokine response of immature DC, apoptotic neutrophils were added to immature DC generated in the absence or presence of PPARγ for 8 hours and the cell culture supernatants were harvested. As shown in Fig. 4A the interaction of apoptotic neutrophils with immature DC resulted in the up regulation of IL-8, IL-6, TNF-α and IL-1β secretion in all the three donors tested, though IL-6, TNF-α, and IL-1β cytokine

concentrations showed high individual variations. To provide evidence that the measured cytokines were secreted by DC we measured the concentration of these cytokines also in the supernatant of apoptotic cells. The culture medium of apoptotic neutrophils, harvested after 16 hours of incubation contained 969-2430 pg/ml IL-8, whereas IL-6, TNF-α, IL-1β, IL-10 or IL-12 were undetectable (data not shown). Incubation of immature DC with fresh neutrophils for 30 minutes resulted in similar or even lower amounts of secreted cytokines than that measured in the DC cultures without neutrophils suggesting that the pro-inflammatory cytokine response is the consequence of co-incubation of immature DC with apoptotic neutrophils (data not shown).

Dendritic cells generated in the presence of RSG in most cases contained more $CD1a^-$ cells (Fig. 1A) and engulfed more apoptotic neutrophils than those generated in the absence of RSG (Fig. 1B). Remarkably, RSG-treated and neutrophil loaded DC secreted less IL-8, IL-6 and IL-1β than DC generated in the absence of PPARγ agonist, however differences in the concentration of secreted cytokines by DC differentiated in the absence or presence of RSG were not statistically significant (Fig. 4A). In accordance with our previous results on the more tolerogenic nature of $CD1a^-$ cells as compared to the $CD1a^+$ subtype (27) these data suggest that increased expression and activation of PPARγ through RSG favours the generations of CD1a- cells and the uptake of apoptotic neutrophils but does not block the inflammatory response of DC.

Up regulation of apoptotic neutrophil-induced inflammatory cytokine secretion of dendritic cells by LPS+IFNγ

To assess how the presence of neutrophils may modulate the cytokine response of immature DC triggered by additional inflammatory stimuli we loaded the immature DC with apoptotic neutrophils for 8 hours and then activated the cells with LPS+IFNγ for a further 16

hours (Fig. 4B). This treatment dramatically and significantly up regulated the secretion of the pro-inflammatory cytokines IL-8, IL-6 and TNF-α, as compared to DC not exposed to apoptotic neutrophils. Most importantly, the activation of DC previously co-cultured with apoptotic neutrophils resulted in the concomitant secretion of both IL-12 and IL-10, an atypical cytokine pattern as compared to TLR4-mediated signalling. Nevertheless, both cytokines are referred to as key polarizing factors of inflammatory and regulatory immune responses, respectively.

Similar to non-activated DC the modulatory effect of RSG resulted in the attenuation of the inflammatory response induced by apoptotic cells and LPS+IFNγ (Fig. 4A, B). These results altogether show that the long term contact and the internalization of apoptotic neutrophils by $CD1a^{-}CD209^{high}$ phagocytic immature DC sensitize the DC for inflammatory IL-12 and also for regulatory IL-10 production combined with the triggering of enhanced pro-inflammatory cytokine secretion.

Dendritic cells incubated with apoptotic neutrophils polarize T-lymphocytes for IFNγ secretion

Based on the inflammatory response of DC induced by apoptotic neutrophils 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 immature DC 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 APC, i.e. monocyte-derived macrophages and dendritic cells derived from the same donor with autologous T-

lymphocytes, the ratio of IFNγ-secreting cells was measured by the ELISPOT method. As it is shown in Fig. 5A, lymphocytes incubated with DC, which previously engulfed apoptotic neutrophils resulted in significantly higher number of IFNγ-secreting T-lymphocytes than DC not sensitized by apoptotic cells. When macrophages fed by apoptotic neutrophils were used as APC instead of DC, no IFNγ-secreting T-lymphocytes could be detected (Fig. 5B). These results suggest that both the cytokine response and the antigen presenting potential of macrophages and DC, loaded by apoptotic neutrophils, is different and may have opposing effects on tolerance induction and immunity.

Discussion

Apoptosis ensures the silent elimination of excessive cells and limits immune responses against the antigenic content of dying cells while renders the immune system tolerogenic to self structures (38). In contrast to apoptosis, necrosis allows the release of cellular content and induces inflammation (39, 40). Macrophages and dendritic cells are tissue resident professional phagocytic cells that derive from common precursors, and exhibit overlapping functions such as engulfing apoptotic cells, sensing self and pathogenic danger signals and acting as professional APC. Here we show that immature monocyte-derived DC that resemble tissue resident migratory DC (41) are able to engulf apoptotic neutrophils but less efficiently than monocyte-derived macrophages. The myeloid C-type lectin DC-SIGN, expressed by tissue resident and monocyte-derived DC has been shown here to participate in the internalization process. Furthermore, the slow uptake of apoptotic neutrophils by DC – in contrast to macrophages – results in the activation of DC for pro-inflammatory cytokine secretion and the potential to induce inflammatory T-lymphocyte responses. As DC-SIGN, besides its pattern recognition function has been considered as an internalizing receptor with the capability to recognize self structures such as Lewis X antigen (Le^x), its down regulation upon apoptotic cell engulfment could be due to the direct involvement of this receptor in the binding and engulfment of apoptotic neutrophils. As DC maturation has been associated with the down regulation of internalizing receptors, this effect can also be a consequence of DC activation (28).

In the present study the kinetics of neutrophil uptake, identification of DC subtypes involved in the engulfement of apoptotic neutrophils and the functional consequences of their interaction with apoptotic neutrophils has been determined. We identified DC-$SIGN^{high}$ immature $CD1a^-$ DC as a cell type preferentially internalizing apoptotic neutrophils. Our

results showed very different kinetics for the uptake of apoptotic neutrophils by macrophages and DC. However, the continuous engulfment of neutrophils undergoing apoptosis resulted in ~20 – 30 % loaded DC and the ratio of phagocytosing cells could be increased up to ~50% by the ligand-induced activation of PPARγ. This treatment was shown to shift monocyte-derived DC differentiation to the generation of the $CD1a^-$ subset characterized by more efficient receptor-mediated endocytosis and phagocytosis of latex beads and pathogens than that of monocyte-derived $CD1a^+$ cells (25, 27).

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 (42, 43). Macrophage activation is controlled by the cytokines IL-10 and TGFβ as well as by arachidonate metabolites such as PGE2. Macrophages that contact with apoptotic cells acquire sensitivity to autocrine TGFβ and suppression of the secretion of inflammatory cytokines (44). Our previous results obtained with an *in vitro* phagocytosis system confirmed this type of regulation and showed that the engulfment of apoptotic neutrophils is able to dampen inflammatory cytokine production induced by LPS (33).

Under physiological conditions saturation of immature tissue DC by metabolites or by engulfed apoptotic cells induces the steady-state migration to draining lymph nodes where DC interact with T-lymphocytes (2). This cell-to-cell communication is translated to tolerance induction due to the lack of co-stimulatory signals and to the induction of regulatory mechanisms that inhibit signaling pathways and cell communication. Phagocytosis of particulate antigens such as microbes or apoptotic cells by DC results in the sorting of engulfed material into distinct intracellular compartments based on their co-expression of TLR ligands (45). Particles carrying both antigenic structures and TLR ligands induce

phagosome maturation and the presentation of antigenic peptides in the context of MHC class II proteins at the DC surface. However, in the absence of coupled TLR signals non-infected apoptotic cells, even internalized together with microbes, will not become immunogenic due to inappropriate phagosome maturation and antigen processing (46). Thus the outcome of apoptotic cell engulfment by DC can lead either to tolerance induction or to priming immune responses, and this decision strongly depends on particle-associated activation signals.

Exogenous lipids transferred by engulfed apoptotic cells to DC may modulate the composition of endogenous lipids and generate 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 and tolerogenic function, we presumed that the loaded DC maintained their anti-inflammatory nature (27). However, in our present study the secretion of pro-inflammatory cytokines induced by the co-culture of apoptotic cells with DC pointed to the co-delivery of both antigenic and co-stimulatory signals by apoptotic neutrophils. This possibility was confirmed by the robust inflammatory cytokine response induced by further activation of the pre-loaded DC by additional stimuli such as LPS+IFNγ. Importantly, the LPS+IFNγ-induced IL-12 secretion of DC was accompanied by IL-10 production, a possible effect of DC-SIGN-mediated signaling event (47), that has been shown to induce immunosuppression through potentiating IL-10 production (48).

When we compared the T-cell polarizing capacity of activated monocyte-derived DC with or without pre-loaded with apoptotic neutrophils we could demonstrate the strong inflammatory potential of DC pre-incubated with apoptotic neutrophils. Furthermore, monocyte-derived macrophages of the same donor saturated by apoptotic neutrophils remained immunologically silent.

Remarkably, DC generated in the presence of RSG, loaded with apoptotic neutrophils and activated by IFNγ+LPS secreted in all cases less inflammatory cytokines than those

differentiated in the absence of PPARγ agonist. Consistent with previous data these results suggest the anti-inflammatory effect of forced PPARγ expression and activation mediated by the relative tolerogenic nature of PPARγ expressing $CD1a^-$ cells involved preferentially in the engulfment of apoptotic neutrophils.

The neutrophils used in our experiments derived from a different donor than the DC. Their antigenic content could be acquired in the host through the uptake by immune complexes readily engulfed by neutrophils. As microbial products were absent in our experimental system the source of danger signals that provoke DC for inducing an inflammatory response remains to be identified. However, neutrophils activated by TNF-α were shown to induce T-lymphocyte proliferation through allogenic DC and resulted in a mixed T-cell response that involved both IL-4- and IFNγ-producing effector T-lymphocytes (32). The Le^x-mediated targeting of DC-SIGN was also shown to enhance T cell immunity and IFNγ production (49). Furthermore, activated autologous peripheral blood mononuclear cells undergoing apoptosis induced the differentiation of IFNγ-producing T-lymphocytes whereas non-actived cells did not (50). Although the allogeneic response induced by neutrophils can not be ruled out, apoptotic neutrophils by themselves may present endogenous danger signals similar to other mimics of tissue destruction such as adenosine triphosphate (ATP), uric acid or hypotonic stress (51, 52). As terminally differentiated short lived effectors, the activated neutrophils rapidly release neutrophil extracellular traps (NET) that involve cytoplasmic proteins bound to intact chromatin (53, 54). This process has been suggested as a unique cell death program that can be induced by PMA, LPS, IL-8 or type I and II IFN (55, 56). As immature DC release high level of IL-8 (27), the induction of NET and the release of cell content emerge as candidate mechanisms to induce DC activation.

Under *in vivo* conditions tissue resident DC are concentrated at mucosal surfaces and similar to macrophages express a wide variety of receptors that are involved in the recognition

and uptake of apoptotic cells (8, 57-60). Microbial invasion recruits neutrophils from the circulation to the site of infection through microbial products, cytokines, chemokines, and factors released by apoptotic cells (61). A recent study also suggested that neutrophils carrying IgG-antigen complexes enter afferent lymphatics, and upon apoptosis they transfer their antigenic content to phagocytic DC residing in the T-cell areas of lymph nodes (62). Inflammatory stimuli also mobilize circulating monocytes and blood DC precursors to the site of infection (63) where cell death through apoptosis, their clearance by macrophages and the presentation of antigens by DC plays a central role in inducing immunological tolerance to self molecules and immunity against foreign or dangerous structures (8). Thus the uptake of neutrophils by macrophages and DC in both peripheral and lymphoid tissues may have multiple effects on the outcome of immune responses including a strong Th1 response. Together with our previous results obtained with macrophages in a similar *in vitro* system our new results suggest that the recruitment of neutrophils to inflammed tissues is able to induce opposing responses in monocyte-derived macrophages and dendritic cells. Hence, the cross talk of macrophages and DC loaded by apoptotic neutrophils through their cytokines and antigen presenting functions may modulate the outcome of immune responses under inflammatory or other pathologic conditions.

Materials and Methods

Cell cultures and reagents

Human peripheral blood mononuclear cells were isolated by density gradient centrifugation with Ficoll–Paque Plus (Amersham Biosciences, Uppsala, Sweden) from "buffy coats" obtained from healthy blood donors. $CD14^+$ cells were separated by magnetic sorting with the Vario-MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany), followed by washing with PBS containing 0.5% BSA and 2mM EDTA. Neutrophils were isolated from freshly drawn peripheral blood by density gradient centrifugation with Histopaque1119 and Histopaque1077 (Sigma-Aldrich, St Louis, MO). Freshly isolated monocytes were seeded into 6-well culture dishes at a density of 2×10^6 cells/ml and cultured in AIMV medium (Invitrogen, Carlsbad, CA) containing 800 U/ml GM-CSF and 500 U/ml IL-4 (Peprotech EC, London, United) in the presence or absence of 2.5 μM PPARγ agonist RSG (Alexis Biochemicals, San Diego, CA). Cells were cultured for 5 days with IL-4 and GM-CSF added at day 0 and day 3. Dendritic cell-depleted autologous lymphocytes were saved for the ELISPOT experiments and were stored in ELISPOT cell freezing medium (CTLe Cellular Technology, Cleveland, OH) at -70 ^oC. Neutrophils were cultured for 16 h in IMDM (Invitrogen, Carlsbad, CA) supplemented with 1% AB serum (Sigma-Aldrich, St Louis, MO) while they underwent apoptosis. The apoptosis of neutrophils was verified by flow cytometry using Annexin and Propidium iodide (Sigma-Aldrich, St Louis, MO) staining.

Dendritic cells phagocytosis assays

Freshly isolated neutrophils were labelled overnight with 15μM 5(6)-CFDA-SE (5(6)-Carboxifluorescein Diacetate Succinimidyl Ester, Invitrogen Carlsbad, CA), washed to remove the conditioned media and resuspended in AIMV before their addition to immature

DC. Dendritic cells and apoptotic neutrophils were mixed at a ratio of 1:5 and co-cultured for 8 hours either at 37 ^{o}C or 4 ^{o}C as a control in 5% CO_2 atmosphere. Following incubation the cells were washed three times with PBS, the DC were labelled by PE-conjugated mouse IgG1κ (control) and anti-human CD209, CD40, CD1a mouse IgG1 monoclonal antibodies (BD Pharmingen, San Diego, CA) and analyzed by flow cytometry. Dendritic cells were gated on the basis of forward- and side-scatter properties and the percentage of DC positive for both PE-conjugated CD40 and CFDA-SE was determined by subtracting mean fluorescence values measured at 4 ^{o}C from those obtained at 37 ^{o}C. For inhibition of membrane CD209 binding to neutrophils the inhibitory anti-CD209 mAb (ab13487 Abcam), was added to the DC at 5 μg/ml concentration 30 min before the phagocytosis assay was started and it was present throughout the whole period of the DC – apoptotic neutrophil co- incubation.

Confocal microscopy

For confocal laser scanning microscopy a Zeiss (Göttingen, Germany) LSM 510 system was used. Fluorescein (CFDA) and phycoerythrin (PE) were excited with 488 nm Ar ion and 543 nm HeNe laser and detected through 505-550 nm and 560-615 nm emission filters, respectively. For high resolution imaging with a Plan-Apochromat 63×/1.4 NA oil immersion objective pinholes were set to obtain 1 μm optical sections. For statistical analysis 75 images of 512×512 pixels (5×10^4 μm^2) were taken from each sample, using a C-Apochromat 40×/1.2 NA water immersion objective at extended depth of focus (8 bit resolution, pixel time 2.5 μs, 2× line-averaging). Image channels were recorded separately and software compensation was used to correct for crosstalk of fluorescence into the PE channel.

Digital Image Processing

Quantitative digital image processing tasks were performed using the C programming environment of SCIL-Image (TNO, Delft, The Netherlands). For each treatment category, a distribution histogram was constructed which collectively clearly indicated two populations, with low and high membrane intensity, respectively and served to determine the cutoff level (60 arbitrary units of fluorescence intensity) for numerating cells expressing high levels of CD209.

Human IFNγ ELISPOT assay

Monocyte-derived macrophages and DC were co-cultured with non-labelled apoptotic neutrophils for 8 hours. After thorough washing procedures (CTL wash medium, Cellular Technology, Cleveland, OH) the harvested cells were seeded to 24 wells plate and were co-cultured with autologous lymphocytes at a ratio of 1:25 for 5 days at 37 ^{o}C in 5% CO_2 atmosphere. On day 5 the cells were collected and subjected to a 24-hour IFNγ Ready Set Go ELISPOT assay (eBioscience, San Diego, CA). The MultiScreen-HTS PVDF plates were purchased from Millipore S.A., Molsheim, France. After 24h at 37 C in 5% CO_2, the cells were removed and the plates were washed with PBS. Detection of cytokine spots was performed by biotinylated IFNγ-specific antibody and avidin-HRPO conjugate (BD Biosciences Pharmingen, San Diego, CA). The plates were washed with tap water to stop the reaction and air-dried. The resulting spots were counted using a computer assisted ELISPOT image analyzer (Series 1 ImmunoSpot Analyzer, ImmunoSpot Version 4.0 Software Academic, Cellular Technology, Cleveland, OH) customized for analyzing ELISPOT to meet objective criteria for size, chromatic density, shape, and color.

Determination of cytokine release

Dendritic cells harvested on day 5 were co-cultured with apoptotic neutrophils for 8 hours and the culture supernatants were harvested and stored until used for cytokine measurements. A fraction of the cells was treated by 0.1 μg/ml LPS and 10 ng/ml IFNγ for another 16 hours and the supernatants were collected. The cytokine (IL-8, IL-6, IL-1β, IL-10, TNF-α, IL-12p70) concentrations of the harvested supernatants were analyzed by using the Human Inflammation Cytometric Bead Assay (BD Pharmingen, San Diego, CA).

Statistical analysis

Statistical analysis was performed by using unpaired Student t test, Kruskal Wallis non-parametric ANOVA and Wilcoxon-Mann-Whitney post tests.

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Figure Legends

Figure 1 Characterization of the internalization process of neutrophils undergoing apoptosis by immature dendritic cells.

Monocyte-derived immature DC were co-cultured with apoptotic neutrophils for the indicated periods of time ***(A)***, and the ratio of apoptotic neutrophils was measured by flow cytometry using Annexin V and PI staining ***(B)***. The preferential uptake of apoptotic neutrophils by the $CD1a^-$ immature DC subsets was demonstrated by flow cytometry ***(C)***.

Figure 2 The effect of PPARγ activation on the phagocytosis of apoptotic neutrophils by monocyte-derived immature dendritic cells.

Monocyte-derived immature DC were generated by the standard method as described in Materials and Methods (■) or with RSG present throughout the 5-day culture (▲). The effect of RSG on the ratio of $CD1a^-$ and $CD1a^+$ immature DC measured on day 5 (n=6) was determined by flow cytometric analysis of membrane CD1a expression shown as percentage of total DC ***(A)***. The phagocytic activity of immature DC, generated in the absence (■) or presence of 2.5 μmol RSG (▲), was measured by flow cytometric analysis of the cells internalizing green fluorescence-labeled neutrophil granulocytes in the same donors (n=6) shown as percentage of phagocytosing cells ***(B)***. Distribution of membrane CD209 expression (pixel average per cell) in immature DC (♦), and in RSG-treated immature DC (■). Data of one representative experiment measured by confocal microscopy are shown ***(C)***.

Figure 3 Phagocytosis of apoptotic neutrophils by monocyte-derived immature dendritic cells down regulates the membrane expression of CD209.

Immature DC harvested on day 5 were incubated with green fluorescent-labeled apoptotic neutrophils at a ratio of 1:5 for 8 hours. Following incubation the immature DC were washed and analyzed by the membrane expression of CD40 and DC-SIGN/CD209 using flow cytometry and confocal microscopy. The expression of membrane CD209 was significantly down regulated in immature DC (n=8) generated either in the presence or absence of RSG and co-cultured with apoptotic neutrophils, as compared to untreated cells. Each symbol represents the mean of duplicate measurements carried out with monocyte-derived immature DC of healthy blood donors ***(A)***. Representative data of flow cytometry and confocal microscopy image showing the down regulation of CD209 receptor as a result of neutrophil phagocytosis. Bold lines show CD209 expression on immature DC as compared to DC incubated with apoptotic neutrophils (grey line). Dotted lines show fluorescent intensities obtained with isotype-matched control antibodies ***(B)***. Membrane expression of CD209 on immature DC in the absence (bold line) and presence (grey line) of blocking CD209-specific mAb. Dotted lines show fluorescent intensities obtained with isotype-matched control antibodies ***(C)***. Comparison of the phagocytic activity of immature DC generated with or without RSG, in the presence or absence of blocking CD209-specific mAb ***(D)***. Percentage of DC with high expression of CD209 in immature DC generated in the presence or absence of RSG and with or without CD209-specific blocking antibody. Data of a representative experiment are shown ***(E)***.

Figure 4 Cytokine profile of monocyte-derived dendritic cells co-cultured with apoptotic neutrophils.

Cytokine secretion of DC differentiated in the absence or presence of 2.5 μmol RSG and incubated with washed neutrophils, undergoing spontaneous apoptosis induced by *in vitro* culture for 16 hours, at a DC:neutrophil ratio of 1:5. After a 8-hour co-culture period the

supernatants of the co-cultured DC were collected and their cytokine contents were measured by the cytokine bead array technique. All data are presented as mean values ± standard errors (SD) of pg/ml concentrations calculated from three independent experiments using neutrophil-loaded dendritic cells derived from different healthy blood donors. * indicates $p<0.05$ ***(A)***. The effect of neutrophil phagocytosis on the cytokine secretion of DC activated by LPS and IFNγ. Immature DC were mixed and incubated with neutrophils undergoing apoptosis and than activated by 100ng/ml LPS in combination with 10ng/ml IFNγ for an additional 16 hours. Cytokine concentrations of the cell culture supernatants were measured by the cytokine bead array and are shown as compared to the cytokine concentrations measured in the culture supernatant of IDC not fed with apoptotic neutrophils. * indicates $p<0.05$ ***(B)***.

Figure 5 Phagocytosis of apoptotic neutrophils by immature dendritic cells induces inflammatory T cell activation.

Immature DC and differentiated macrophages were incubated with neutrophils undergoing apoptosis for 8 hours and 25 minutes, respectively. Following phagocytosis, dendritic cells ***(A)*** and macrophages ***(B)*** were washed, mixed with autologous lymphocytes at a ratio of 1:10 and co-cultured for 5 days. The non-adherent lymphocytes were harvested and subjected to a 24-hour ELISPOT assay to measure the frequency of IFNγ cytokine-secreting cells as described in the Materials and Methods. The number of spots was counted and the area covered by the spots was calculated by a computer assisted ELISPOT image analyzer. The data were given as mean ± SD of 3-5 parallel measurement performed with the cells of three different donors.

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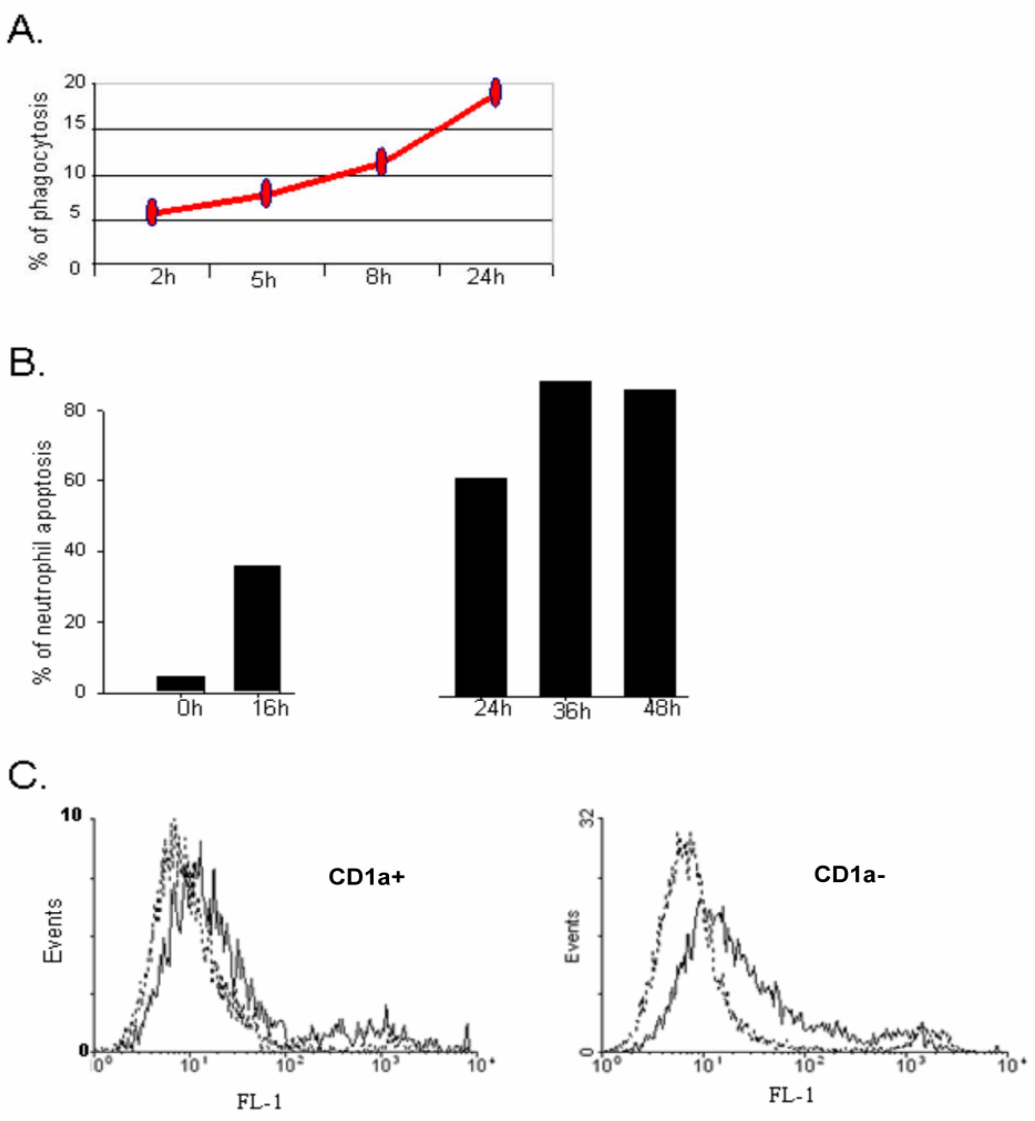
Figure 1.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CD1a+
FL-1
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32
0
CD1a-
FL-1

Figure 2.

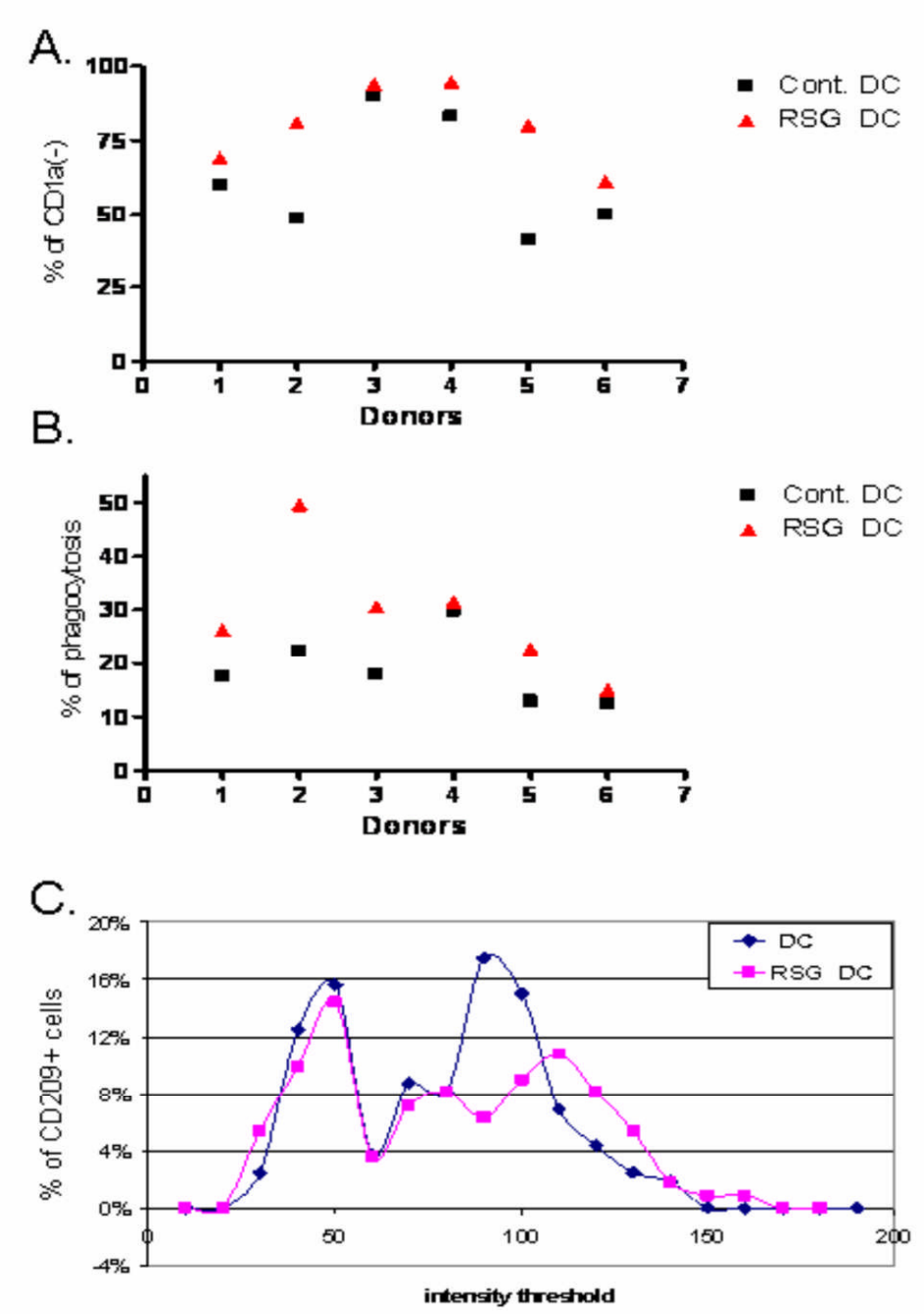

A.
% of CD1a(-)
100
75
50
25
0
0 1 2 3 4 5 6 7
Donors
Cont. DC
RSG DC
B.
% of phagocytosis
50
40
30
20
10
0
0 1 2 3 4 5 6 7
Donors
Cont. DC
RSG DC
C.
% of CD209+ cells
20%
16%
12%
8%
4%
0%
-4%
0 50 100 150 200
intensity threshold
DC
RSG DC

Figure 3.

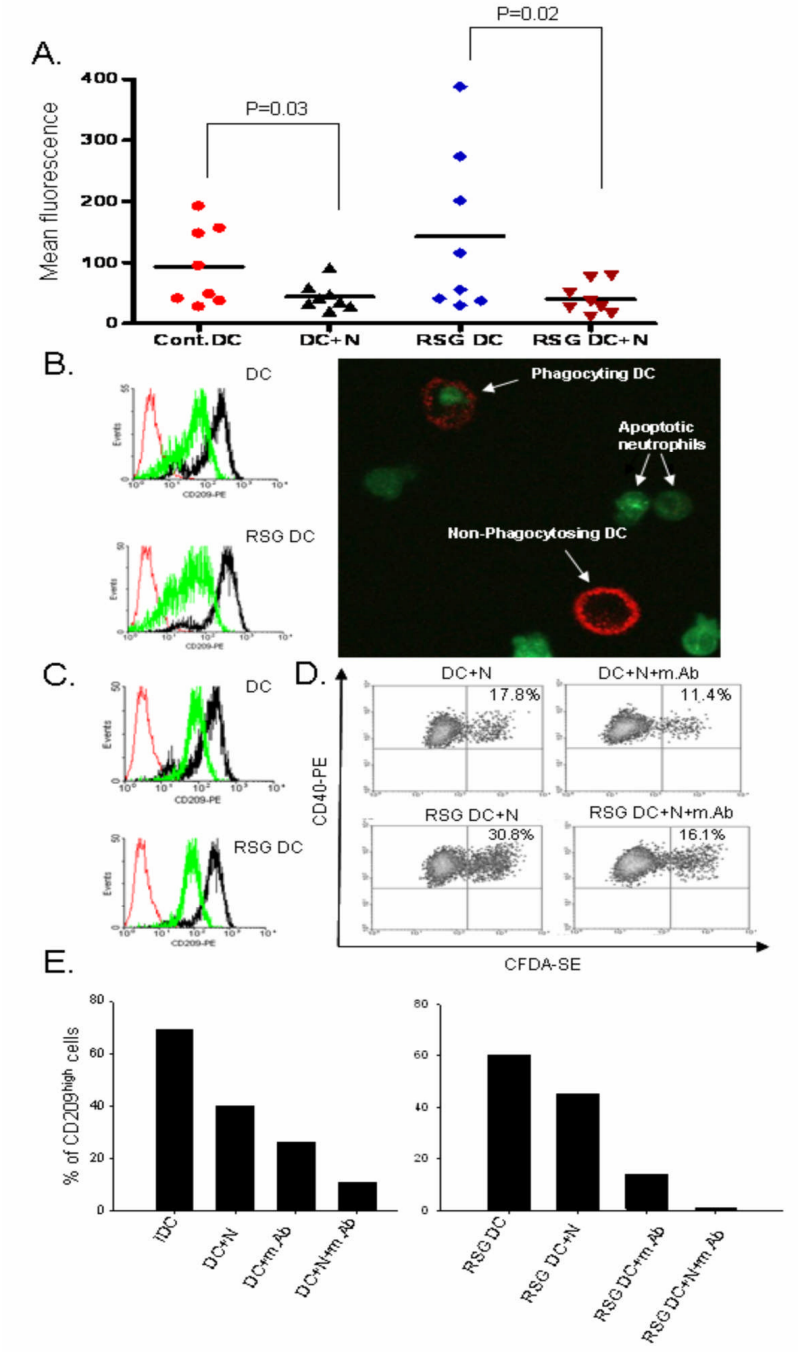

A.
P=0.02
P=0.03
Mean fluorescence
400
300
200
100
0
Cont.DC
DC+N
RSG DC
RSG DC+N
B.
DC
RSG DC
Phagocyting DC
Apoptotic neutrophils
Non-Phagocytosing DC
C.
DC
RSG DC
D.
DC+N
DC+N+m.Ab
17.8%
11.4%
RSG DC+N
RSG DC+N+m.Ab
30.8%
16.1%
CD40-PE
CFDA-SE
E.
% of CD209high cells
80
60
40
20
0
iDC
DC+N
DC+m.Ab
DC+N+m.Ab
RSG DC
RSG DC+N
RSG DC+m.Ab
RSG DC+N+m.Ab

Figure 4.

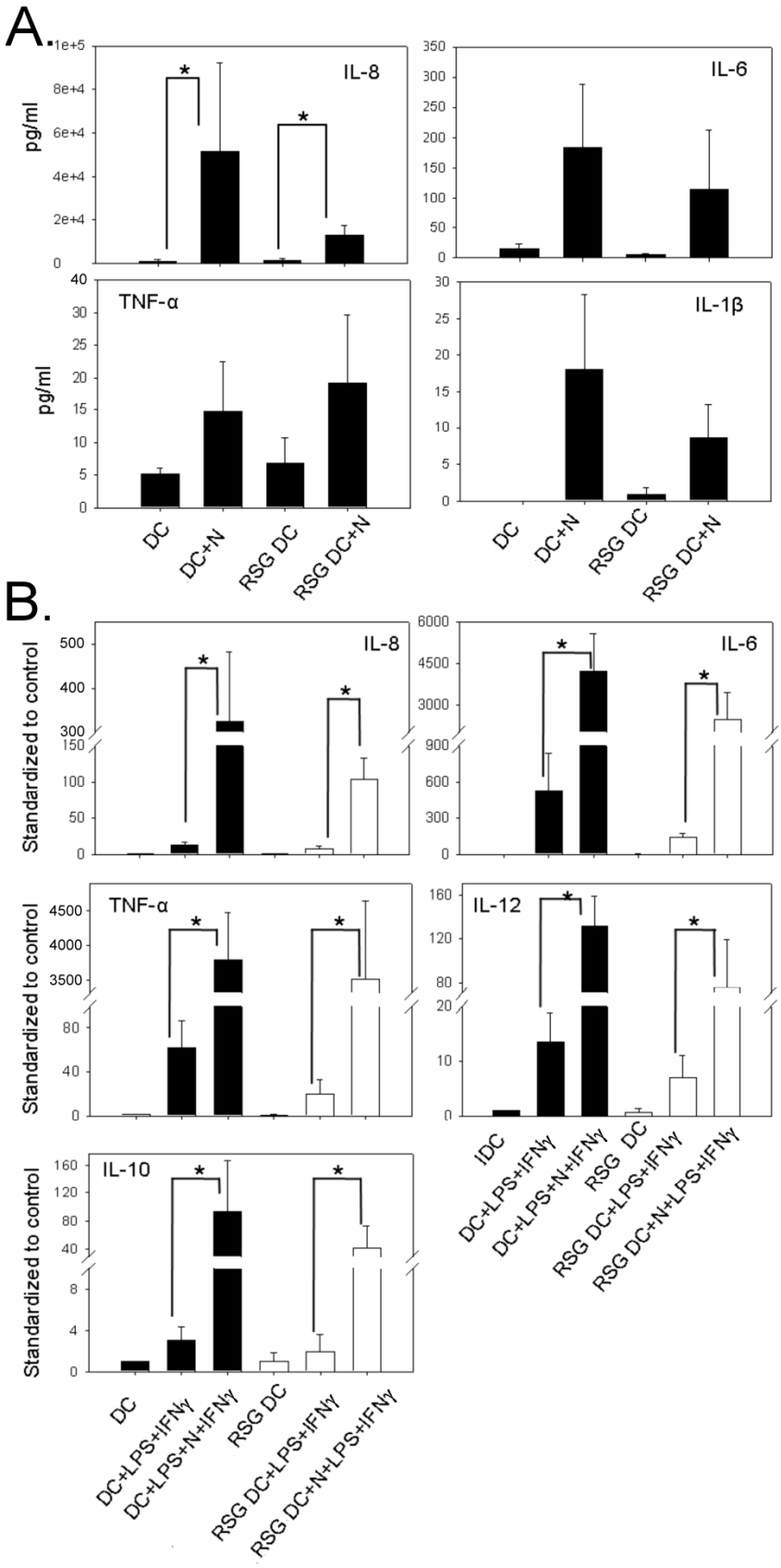
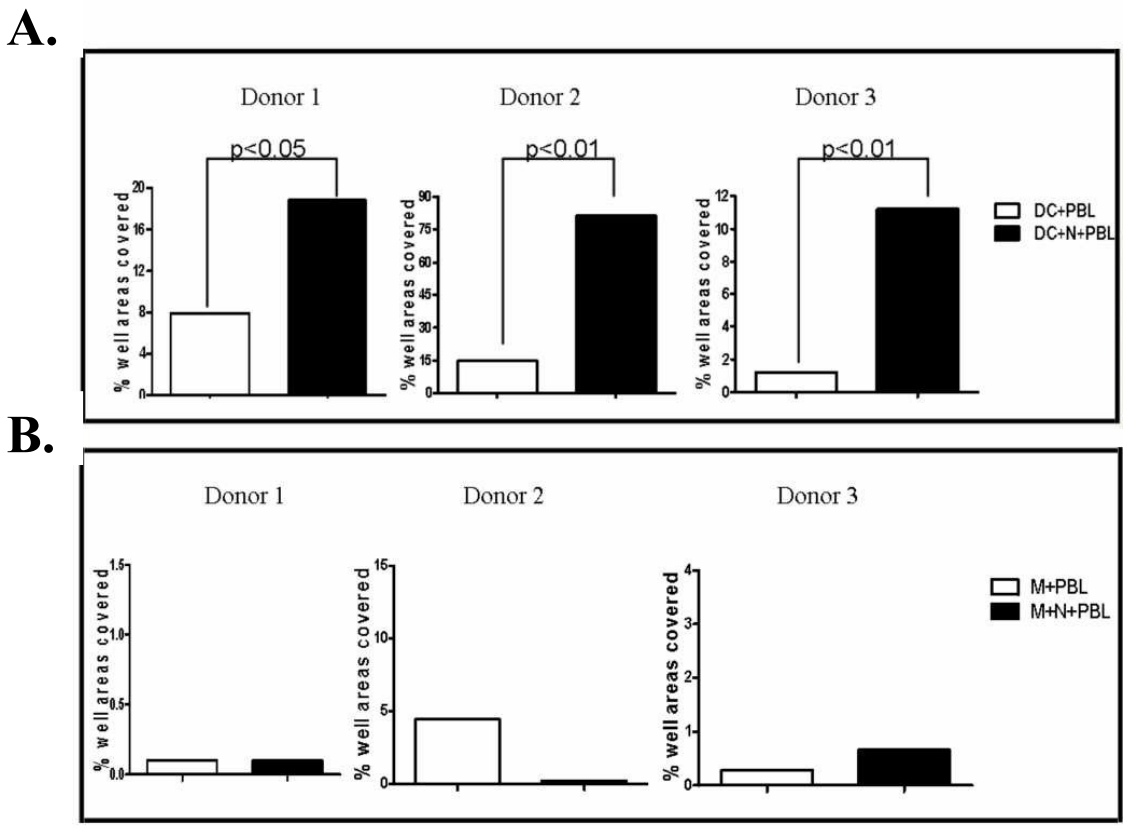

A.
IL-8
IL-6
TNF-α
IL-1β
pg/ml
DC
DC+N
RSG DC
RSG DC+N
B.
IL-8
IL-6
TNF-α
IL-12
IL-10
Standardized to control
IDC
DC+LPS+IFNγ
DC+LPS+N+IFNγ
RSG DC
RSG DC+LPS+IFNγ
RSG DC+N+LPS+IFNγ

Figure 5.


A.
Donor 1
Donor 2
Donor 3
p<0.05
p<0.01
p<0.01
% well areas covered
DC+PBL
DC+N+PBL
B.
Donor 1
Donor 2
Donor 3
% well areas covered
M+PBL
M+N+PBL