Apoptotic cell clearance by macrophages and dendritic cells : immunoregulation in the context of innate immunity

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To my wife and our son,
and to our parents

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ABBREVIATIONS

APCs	antigen presenting cells
CFSE	carboxyfluorescein diacetate succinamidyl ester
C4ds	C4-depleted serum
DCs	dendritic cells
DMA	5-(N, N-Dimethyl)amiloride hydrochloride
ELISA	enzyme-linked immunosorbent assay
GM-CSF	granulocyte/macrophage colony-stimulating factor
LPS	lipopolysaccharide
LY	lucifer yellow
MBL	mannose-binding lectin
M-CSF	macrophage colony-stimulating factor
MØ, Mφ	macrophages
MLR	mixed lymphocyte reaction
MR	mannose receptor
NHS	normal human serum
Pds	properdin-depleted serum
PI	propidium iodide
pMφ	peritoneal macrophages
PS	phosphatidylserine
SLE	systemic lupus erythematosus
UV	ultra violet

1

General Introduction

1. Introduction

Systemic lupus erythematosus (SLE) is an incurable autoimmune disease characterized by a wide array of clinical manifestations and involvement of multiple organs, such as skin, kidneys, and the central nervous system [1]. Despite genetic susceptibility, the actual pathogenesis of SLE remains elusive [2]. To note, SLE is a systemic autoimmune disease and it differs from other autoimmune diseases in such a way that no particular cell type seems to be targeted rather, the response seems to be directed against antigens that are widely expressed [3]. Among these antigens, nuclear components (DNA, histones, ribonucleoproteins) are the major targets [1,3]. Dying cells serve as potential reservoirs of modified forms of autoantigens that may trigger autoantibody responses in susceptible individuals [4]. Therefore, it has been proposed that defective clearance of dying cells breaks peripheral tolerance and predisposes to the development of SLE [5-7]. Several *in vitro* and *in vivo* studies have provided evidence for a link between inappropriate clearance of dying cells and SLE [8,9]. However, it remains unclear how apoptotic cell clearance is regulated by different phagocytes and soluble factors from the innate immune system, and how dying cells ultimately initiate a break of peripheral tolerance.

2. The many modes of cell death

Cell death is an essential and highly orchestrated process, which contributes significantly to normal homeostasis and tissue turnover. There are at least three major modes of cell death: apoptosis, necrosis and autophagy [10]. Apoptosis, coined in 1972 by Kerr *et al.* [11], comes from two Greek words, apo- and -ptosis. “Apo" means "separate from" and "ptosis" means "fall from"--a description of cells that naturally die as part of normal development without any inflammatory flare (cited from Wikipedia). Apoptosis is an active molecular “programmed” process [12-14]. It was called “programmed”, owing to the significant findings by Sulston and Horvitz who elegantly showed that in each worm (*C. elegans*), out of 1090 newborn cells, the same 131 cells die during development, resulting in a nematode of exact 959 cells [15]. During apoptosis, dramatic biochemical and morphological changes take place, including the redistribution of membrane lipids such as phosphatidylserine (PS), and fragmentation of the nucleus [16]. Importantly, the membrane of apoptotic cells remains intact until relatively late in the process [12]. Thus based on the permeability of cell membranes, apoptotic cells can be further divided into two categories, namely early apoptotic and late apoptotic cells.

In contrast to apoptosis, necrosis refers to a distinct mode of death where a cell swells and ruptures during its accidental demise [10], and that sometimes occurs as an alternative form of programmed cell death when apoptosis is blocked [17]. To note,

late apoptotic cells are sometimes referred as post-apoptotic or secondary necrotic cells, behaving like necrotic cells as both of them release intracellular contents [18]. In experimental settings, difference between apoptosis and necrosis can be appreciated at the changes of their sizes and granularities based on the dot plots of forward and side scatter by flow cytometry (Fig. 1).

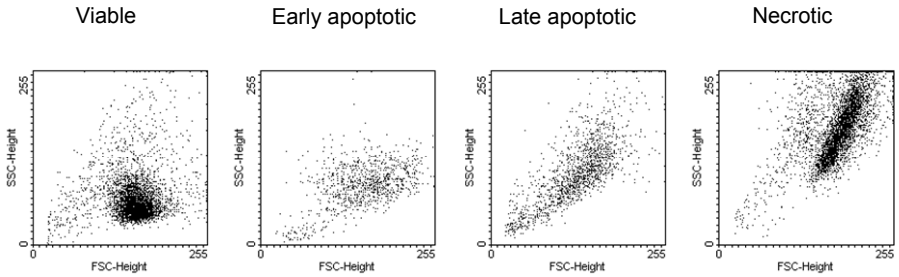

Figure 1. Distinct characteristics of cells at different stages of cell death. Jurkat T cells were treated with UV-C at a dose of 50J/m^2, and cultured in serum-free RPMI culture medium for 3 hours or 30 hours to obtain early apoptotic cells and late apoptotic cells, respectively. Necrosis was induced by incubating cells at 56°C for 1 hour.

As a third major form of cell death, autophagy is a relatively new term and it is a process that a cell recycles cellular products such as cytoplasma and defective organelles [19]. The major difference between apoptosis and autophagy is that apopotic cells are degraded by phagocytic cell lysosomes while autophagic cells do it by their endogeneous lysosomal machinery. It remains unclear whether autophagy directly executes cell death or that it is a secondary effect of apoptosis [19].

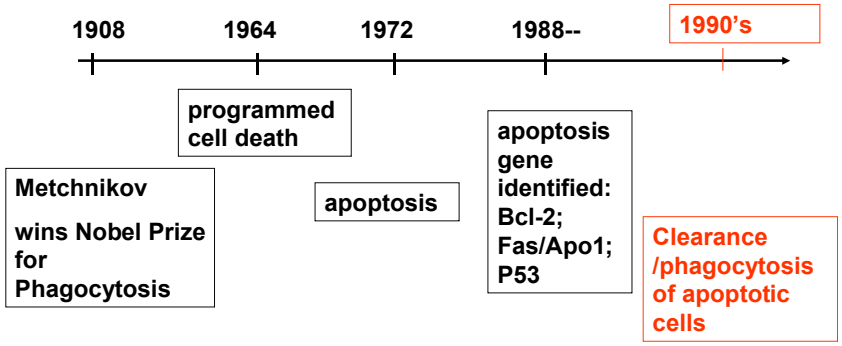

Figure 2. A brief history of apoptosis.

3. The clearance machinery

The theory of "phagocytosis" was formulated by the pioneering biologist Elie Metchnikov in 1880's, who won a Nobel Prize in 1908 (www.nobelprizes.com). Phagocytosis was initially recognized as the first line of internal defence when foreign particles that enter our bodies. Although apoptosis or programmed cell death was discovered in 1960's, not much attention has been paid by scientists to unravel the mechanisms of phagocytosis of apoptotic cells until the 1990's [5,6,20,21] (Fig. 2).

Investigators in the last 15 years have made clear that once a cell undergoes apoptosis, phosphatidyserine (PS) is redistributed onto the outer layer of the cell membrane and serves as the very first "eat me" signal to attract phagocytes and initiation of phagocytosis (Fig.3). There are many cell types that can be involved in the phagocytic process, including professional phagocytes, i.e. immature dendritic cells (iDCs) and macrophages (Mφ), but also non-professional phagocytes, i.e. epithelial cells, fibroblasts or mesangial cells [22]. Even within the family of professional phagocytes, both DCs and Mφ consist of heterogeneous subsets of cells with different functional characteristics [23,24]. Therefore, the nature of a phagocyte defines the complexity and the consequence of phagocytosis. Furthermore, since both DCs and Mφ are professional antigen presenting cells (APCs), processing of self-antigens derived from dying cells by these cells becomes an essential issue in understanding how these dying cells control and regulate immunity.

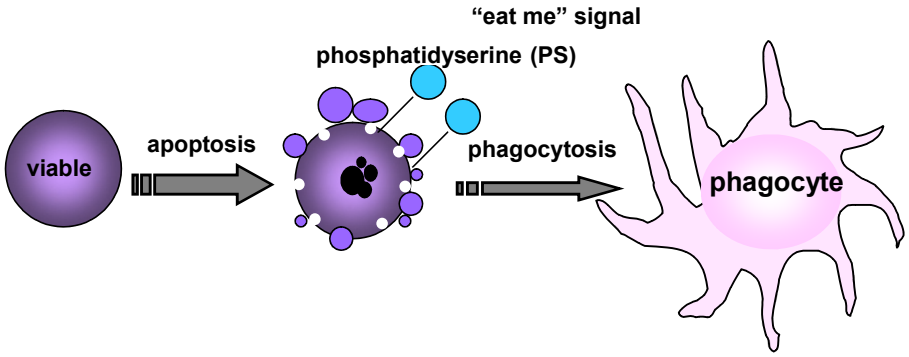

Figure 3. Recognition of apoptotic cells by phagocytes. Viable cells provide "don't eat me" signals, therefore are not recognized by phagocytes. Once cells undergo apoptosis, phosphatidyserine (PS) is redistributed onto the outer layer of the cell membrane, which then serve as "eat me" signals. Phagocytes such as DCs and Mφ are recruited by chemotactic stimuli to phagocytose the dying cells.

4. Linking cell death to autoimmune lupus

Apoptotic cells are a rich source of autoantigens [25]. During daily life, billions of cells undergo apoptosis and then are promptly phagocytosed by different phagocytes and/or APCs and the self-antigens are processed by these APCs or transferred to other professional APCs. Therefore, it is generally thought that presentation of antigens derived from apoptotic cells will contribute to the induction and maintenance of peripheral tolerance or autoimmunity [5,6]. Emerging evidence indicates that the uptake of apoptotic cells is immunosuppressive, as documented by the fact that anti-inflammatory cytokines such as TGF-β are induced whereas pro-inflammatory cytokines are inhibited, by phagocytes that have ingested apoptotic cells [26-29]. This contrasts with the effect of late apoptotic or necrotic cell uptake, which leads to the activation of phagocytes and release of pro-inflammatory cytokines [30,31]. This is most likely due to "danger signals" such as heat shock proteins [32], HMGB1 [33] and uric acid [34], that are released by necrotic cells. Each of these mediators is essential to induce DC maturation and activation of the immune system.

Another challenging recent view proposes that also stimuli for induction of apoptosis may define the immune response upon uptake by phagocytes [35]. Several studies have shown that apoptosis triggered via death receptors results in release of bio-active lipids such as sphingosine-1-phosphate (S1P) [36], or lysophosphatidylcholine (LPC) [37], which then signal through endothelial-derived G-protein-coupled (EDG) receptors. As a consequence, EDG receptors activate nuclear factor-κB (NF-κB), leading to a pro-inflammatory response [35].

A central question is then how apoptotic /necrotic cells ultimately lead to aberrant autoimmunity? The very first evidence from animal studies has shown that injection of large amounts of apoptotic cells into mice [38] or rats [39] led to the production of autoantibodies. In SLE patients, a decreased clearance of apoptotic cells has been documented [9,40], suggesting a strong link of defective clearance to autoimmunity. Furthermore, elevated levels of apoptotic cells have been found in the peripheral blood of SLE patients [41,42]. Together, there is a prevailing belief that delayed or defective clearance of apoptotic cells ultimately leads to a break in self-tolerance and induction of autoimmunity [5,9].

During phagocytosis, soluble factors from the innate immune system enhance the interaction between dying cells and phagocytes and play an important role in this process. Both complement and other innate molecules such as pentraxin family members can opsonize apoptotic cells, and thereby promote their removal by phagocytes [16,43]. In humans, homozygous deficiency of any of the early components of the classical pathway of complement (C1q, C1r, C1s, C4, and C2) predispose to the development of SLE [43], implying that complement is involved in

removal of dying cells. These observations are also supported by animal models showing that C1q knockout mice on certain genetic background develop lupus like disease and exhibit accumulation of apoptotic bodies in the glomeruli [8]. Complement-mediated clearance of apoptotic cells has been well documented both *in vitro* [44] and *in vivo* [45]. Activation of complement by the classical pathway (via C1q) and lectin pathway (via MBL and ficolin) on dying cells seems to be a favorable process [44-48], although complement activation may cause tissue damage and inflammation, suggesting that a balance between the two processes is desirable. Nevertheless, the main product of complement activation, iC3b, was suggested not only to facilitate the removal of dead material, but also to mediate peripheral tolerance [44,49,50].

Therefore, clearance of apoptotic cells is a complex process, involving many factors as discussed so far. Firstly, death stimuli may be important to determine which signal is going to be delivered to phagocytes. Secondly, appropriate opsonization by soluble factors from the innate immune system contributes enormously to the removal of dying cells and the subsequent consequence on the immune response. Thirdly, as discussed earlier, the phagocyte system is largely heterogeneous, therefore the nature of a specific phagocyte that encounters a dying cell, defines the consequences of being eaten. In conclusion, the link between defective clearance of dying cells and autoimmune lupus has a reasonable solid scientific basis, but the exact immunological mechanisms involved remain to be defined.

5. Scope of this thesis

The current thesis was dedicated to understand how different components of the innate immune system contribute to the clearance of apoptotic cells and to the immunological response involved in this process. In **part I** of this thesis, we focus on the biology of phagocyte subsets and their role in the handling of dying cells. This part consists of 4 chapters: **Chapter 2** is a review discussing the latest knowledge on how different subsets of DCs and Mφ handle dying cells, and particularly the immune response evoked by APCs that have eaten dying cells. **Chapter 3** describes the differential contribution of pro-inflammatory Mφ (GM-CSF-driven Mφ1) and anti-inflammatory Mφ (M-CSF-driven Mφ2) in the phagocytosis of early apoptotic, late apoptotic and necrotic cells and the mechanisms that are involved in this process. **Chapter 4** is a follow-up of **Chapter 3,** describing the finding that *in vitro*-polarized pro-inflammatory and anti-inflammatory Mφ seem to have an *in vivo* counterpart as well. We analyzed human peritoneal Mφ (pMφ) freshly isolated from patients on peritoneal dialysis and performed functional comparisons among pMφ, Mφ1 and Mφ2. In **Chapter 5**, it is demonstrated that polarized pro-inflammatory and anti-inflammatory Mφ can be re-differentiated

towards anti-inflammatory and pro-inflammatory cells by switching lineage-determining factors GM-CSF and M-CSF, respectively.

In the latter part of this thesis (**Part II),** the role of serum factors in the handling of dying cells and its association to pathogenesis of SLE are described. **Chapter 6** discusses the importance of the innate immune system, particularly complement, in the clearance of apoptotic cells. **Chapter 7** reports on the role of properdin (an important positive complement regulator) on binding to dying cells and physiological consequences such as complement activation and immune regulation by DCs and Mφ.

Finally, in **Chapter 8** general conclusions are drawn and topics of interest are discussed. We also describe several ongoing studies in the direction of: 1.) elucidation of the role of serum factors in the processing of dying cells in SLE patients; 2.) dissection of how DCs, that are loaded with early, late or necrotic cells, process and present antigens to T cells; 3.) immuno-modulation of Mφ subsets.

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2

Dendritic cell and macrophage subsets in the handling of dying cells

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Summary

Dendritic cells and macrophages are major components of the phagocyte system and are professional antigen presenting cells. In the current review, we discuss the differential contribution of dendritic cell and macrophage subsets in the clearance of dying cells and the consequence of the process of these cells. We hypothesize that under steady-state conditions, the clearance of apoptotic cells is mostly confined to a specialized subset of phagocytes with anti-inflammatory properties.

------ *Immunobiology.* 2006; 211(6-8): 567-575------

Introduction

Phagocytes constantly remove excessive dying cells during normal homeostasis and tissue turnover. Since 1990, significant progress has been made in understanding the immunological meaning of clearance of dying cells [1-4]. The underlying accepted paradigm is that apoptotic cells provide "eat me signals" to phagocytes to promptly and efficiently engulf apoptotic cells during the early stage of cell death, thus preventing them to release noxious substances in the local environment. In contrast, late apoptotic cells or necrotic cells might provide danger signals to activate antigen presenting cells (APCs), potentially breaking self-tolerance [2,3]. One of the well-established examples, both in mice [5,6] and men [7] of a disorder in which apoptotic cell clearance is disturbed is systemic lupus erythematosus (SLE) [8].

There are several checkpoints conceivable which together determine the immunological response towards the safe clearance of dying cells (Figure 1). I.) The different modes of cell death might determine the fate of clearance. There are at least three major types of cell death: apoptosis, necrosis and autophagy [9], which exhibit distinct biochemical and morphological changes [9-11]. Depending on the respective stimuli of death induction, these dying cells might determine phagocyte activation and antigen processing [12]. II.) Opsonization of apoptotic cells by components of the innate immune system, such as complement factors and pentraxin family members, facilitates and modulates the clearance of apoptotic cells [13]. III.) Finally, since the mononuclear phagocyte system is largely heterogeneous [14], the nature of phagocytes that phagocytose apoptotic cells might provide a defined immunological response (Fig. 1).

The concept of differences of dying cells and the differential contribution of opsonins has been extensively reviewed [12,13,15-18]. In the present review, we will concentrate on the differential contribution of phagocyte subsets, particularly subsets of dendritic cells (DCs) and macrophages (MØ) in the clearance of dying cells, as both cell types are professional phagocytes and APCs. Phagocyte subsets might be cells with intrinsic pro-inflammatory or anti-inflammatory characteristics. We suggest that the clearance of apoptotic cells is mostly confined to a specialized subset of phagocytes with anti-inflammatory properties.

DC subsets in the clearance of dying cells

DC subsets

Among the different phagocyte subsets, DCs are the most potent professional APCs [19]. Being an important component of the innate immune system, DCs have been demonstrated in almost all peripheral organs and in lymphoid tissues [20]. The heterogenity of DCs has been extensively studied. In mice, at least six DC subsets

have been described in lymph nodes, derived from two distinct pathways, myeloid and lymphoid [21]. They are distinguished by surface markers such as CD11b, CD8α, and CD11c, as well as by their function [21].

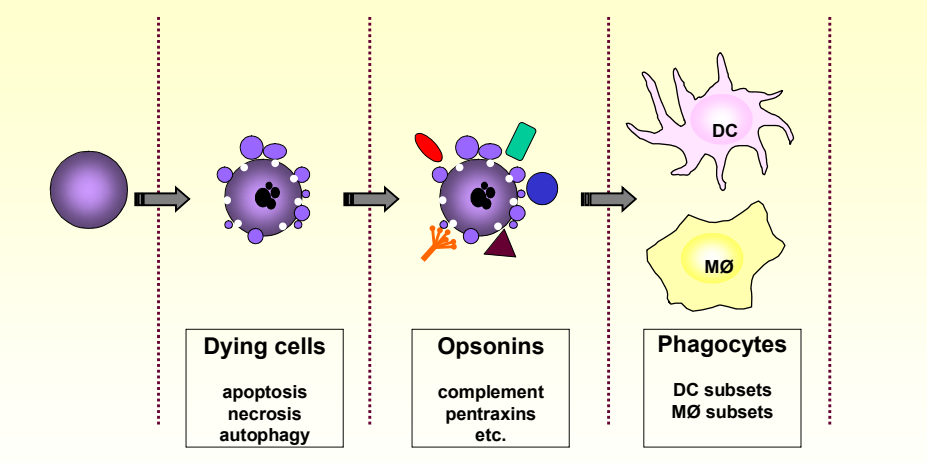

Figure 1. Three checkpoints determine the immunological response during clearance of dying cells. I.) The various modes of cell death such as apoptosis, (secondary) necrosis or autophagy, might determine the fate of dead cells. II.) Opsonization of apoptotic cells by components of the innate immune system, such as complement factors and pentraxin family members, facilitates and modulates the clearance of apoptotic cells. III.) The type of phagocytes that take up apoptotic cells might provide a defined immunological response.

In contrast to well-studied mouse DCs, human DCs have been studied to a relatively lesser degree. Blood is the only readily available source and at least three subtypes of blood-driven DCs have been characterized: Langerhans DCs (LDCs), interstitial DCs (iDCs) and plasmacytoid DCs (pDCs) [21]. Most of the insights into human DC subsets are derived from *in vitro* studies, following the identification of DC precursors, i.e., CD34+ cells from umbilical cord blood or bone marrow, blood monocytes, and plasmacytoid precursors from blood. Migration into non-lymphoid organs, for example the interstitium of peripheral organs, induces differentiation of DC precursors into resident tissue DCs [22]. One of the characteristics of resident DCs is that they are able to capture self (such as cellular debris) and foreign (such as microbial pathogens) antigens by several mechanisms [20]. The maturation status of DCs after antigen-capture determines whether they prime T cells or induce immune tolerance [23]. Taken together, *in vitro* and *in vivo* studies clearly indicate that DCs play a pivotal role in both innate and adaptive immunity.

Clearance of dying cells by DCs

Although heterogeneous populations of DCs have been described in mice and humans (as discussed above), relatively few studies have addressed the question whether DC subsets contribute differentially to the uptake of dying cells. Most of the available studies have investigated dying cell clearance by DCs either using immature monocyte-derived DCs (iMoDCs) in humans [24-26] or splenic $CD8^+$ DCs [27,28] or BM $CD11c^+$ DCs [29] in mice. These data show that DC subsets from different sources can phagocytose dying cells. However, it has been suggested that phagocytosis of dying cells by DCs is restricted to certain types of DCs. For example, although both CD8+ and $CD8^-$ DCs phagocytose latex particles in culture and both DC subsets present soluble ovalbumin captured *in vivo*, only $CD8^+$ DC is specialized in the phagocytosis of dying cells [28]. Another *in vitro* study compared the subsets of splenic CD8 DCs and found that splenic $CD8\alpha^+$ DC are superior to other DC subsets in internalizing dying cells [30].

Also human DC subsets exhibit differential capacities to phagocytose dying cells. A recent study compared side-by-side the phagocytic capacity among three human DC subsets: $CD11c^+$ DCs, iMoDCs and pDCs, and found that iMoDCs are three times better in the phagocytosis of apoptotic cells than $CD11c^+$ DCs, whereas pDCs were hardly able to take up apoptotic cells [31]. This suggests that the uptake of dead material is probably confined to myeloid subsets of DCs.

Consequence of uptake by DCs

Apoptotic cells are a rich source of autoantigens [32]. Upon uptake, DCs acquire antigens from apoptotic cells and (cross-)present these antigens to class I- or class II-restricted T cells [25,33,34]. It remains controversial whether apoptotic cells indeed induce maturation of DCs and whether they stimulate or tolerize T cells. It has been shown that necrotic cells or late apoptotic cells, but not (early) apoptotic cells trigger DC maturation [35,36]. However, others showed that bystander or excessive apoptotic cells induce maturation of DCs, and present antigens in the absence of exogenous danger signals [24,34]. It should be realized that these studies have made use of different sources of DCs, including PBMC [35,36] or D1 cell lines [24,34]. Similarly, various apoptotic targets are used in different studies, such as Jurkat T cells [36], 293 cell lines [35] and OVA-RMA cells [24,34]. Thus different cells used in the studies might explain the substantial difference in the consequence of uptake of apoptotic cells by DCs.

Whether uptake of apoptotic cells leads to DC activation in the context of pro- or anti-inflammatory conditions is also depending on how the death stimuli are applied [12]. Several studies have shown that apoptosis triggered via death receptors could release bio-active lipids such as sphingosine-1-phosphate (S1P) [37], or

lysophosphatidylcholine (LPC) [38]. These agents signal through endothelial-derived G-protein-coupled (EDG) receptors and these receptors are known to signal through nuclear factor-κB (NF-κB), leading to a pro-inflammatory response [12].

To validate the immune regulatory role of apoptotic cells, also *in vivo* studies have been performed. Mouse $CD8^+$ DC were shown to actively capture apoptotic cells and induce immune tolerance [27,28]. These findings are supported by another *in vivo* study showing that a subset of rat DCs, $CD4^-/OX41^-$ DCs, constitutively endocytoses and transports apoptotic cells to the T cell areas of mesenteric lymph nodes [39], suggesting that DCs carrying apoptotic material can silence T cells against self-antigens [40].

If under normal conditions, apoptotic cell removal by DCs is an immunologically silent process, the question remains how apoptotic cell-loaded DCs break peripheral tolerance, as observed in SLE. One possibility is that when early apoptotic cells are not removed promptly, the remaining cells might become late apoptotic (or secondary necrotic), thus converting immune tolerance to autoimmunity. In lupus-prone mice, only DCs loaded with necrotic cells, but not apoptotic cells, induce lupus-like disease [29], suggesting that the intracellular contents released from necrotic cells provide additional danger signals and lead to activation of DCs. A second possibility is that the presentation of autoantigens derived from apoptotic cells by DCs to T cells could be triggered by the presence of ligands for TLRs. A very recent paper showed that only DCs captured apoptotic cells in the presence of TLR4 triggering by LPS could present antigens to CD4 T cells and induce IL-2 production [41]. However, simultaneous phagocytosis of apoptotic cells and microbial pathogens does result in DC maturation, but under these conditions only antigens from bacteria, but not the ones from apoptotic cells, were presented to CD4 T cells [41]. Thus extra danger signals provided by TLR ligands could make a substantial contribution in the initiation of autoimmunity by apoptotic cell-loaded DCs.

Another possible explanation could be that one specialized DC subset contributes exclusively to initiate autoimmunity. Recently, much attention has focused on understanding of how DNAs and RNAs containing autoantigens, that are derived from dying cells, activate DCs in the setting of SLE. Although a previous study showed that *in vitro* pDCs do not take up apoptotic cells [31], they do take up immune complexes (ICs) containing DNAs [42]. It was shown that ICs derived from patients with SLE , upon intracellular delivery via CD32, were able to activate pDCs through toll like receptor 9 (TLR9) [42]. This observation was extended in another study showing that small nuclear RNAs within ribonucleoprotein particles activate pDCs through TLR7 [43], suggesting a link between pDCs and autoimmmunity to both DNA- and RNA-containing autoantigens. These data have provided novel insights in the mechanisms of loss of peripheral tolerance to autoantigens in SLE. The

suggestion that SLE is a pDC-driven disease seems to be confirmed by the identification of IFN-α as an important pathogenic factor [44,45].

MØ subsets in the clearance of dying cells

MØ subsets

It has been proposed that at least two types of MØ exist in vivo: resident (tissue) MØ and inflammatory elicited MØ [46]. Most resident MØ are derived from circulating bone marrow-derived monocytes. Human and mouse studies have indicated that a large heterogeneity exists in MØ populations in lymphoid organs and non-lymphoid organs such as the lung (alveolar MØ), liver (Kupffer cells), spleen (white and red pulp MØ), peritoneum (peritoneal MØ) and nervous system (microglia) [46,47]. These MØ subsets are phenotypically and functionally different, but all of them play broad roles in tissue remodeling and homeostasis [48]. Depending on the cytokine environment, MØ can be activated classically (IFN-γ) or alternatively (IL-4 and IL-13), as reviewed elsewhere [49,50]. Similarly, in mice these subsets have been defined as type 1- (i.e., classically activated) or type 2- (i.e., alternatively activated) activated MØ [51], which are characterized as pro-inflammatory and anti-inflammatory cells, respectively. However, it is not yet fully defined whether resident MØ are anti-inflammatory.

The growth and differentiation of MØ depends on lineage-determining cytokines such as granulocyte/macrophage colony-stimulating factors (GM-CSF) and M-CSF (also termed CSF-1) [50,52]. Mice lacking M-CSF develop a general MØ deficiency [53], whereas GM-CSF-knockout mice have no major deficiency of MØ [54,55]. Importantly, in humans, M-CSF, but not GM-CSF, is a ubiquitous cytokine circulating in the human body [56,57]. Therefore, it is likely that M-CSF is the default cytokine to drive MØ differentiation under steady-state conditions.

Recently it has been shown that human MØ can be polarized *in vitro* into pro-inflammatory (MØ1) and anti-inflammatory cells (MØ2) by GM-CSF and M-CSF, respectively [57-59]. GM-CSF-driven MØ1 are characterized by high production of pro-inflammatory cytokines such as IL-6, IL-12 and IL-23, whereas M-CSF-driven MØ2 are characterized by high production of IL-10 in the absence of pro-inflammatory cytokines. It should be noted that M-CSF-driven MØ2 do not completely resemble alternatively activated MØ or type 2-activated MØ with respect to their surface marker expression and cytokine production [50,60]. For example, alternative activated MØ showed increased expression of mannose receptors (MR) [50], and type 2-activated MØ secrete TNF-α upon stimulation [60]. However, in the case of M-CSF-driven MØ2, low MR expression was found and these cells fail to secrete TNF-α [59,61]. Since MØ2 express the unique surface marker CD163 [61], it is tempting to speculate that MØ2 reflect $CD163^{+}$ highly phagocytic resident MØ *in vivo* [62].

Clearance of dying cells by MØ subsets

The role of MØ in the uptake of apoptotic cells has been studied extensively. In mice, among various MØ subsets, bone marrow-derived MØ [63,64] and peritoneal MØ [65-68] have been widely used. Recently, also microglia [69] and liver macrophages [70,71] have been investigated for their roles in the apoptotic cell removal, suggesting that tissue MØ are active in the removal of dying cells in various anatomical locations. Besides tissue MØ, murine MØ cell lines [72-74] are also able to actively phagocytose apoptotic cells. The data above clearly show that various MØ subsets actively recognize and ingest cells that underwent apoptosis.

In human system, most studies were carried out *in vitro* by using peripheral blood monocyte-derived MØ, differentiated in the presence of GM-CSF [75], M-CSF [76], or in the absence of growth factors [77-80]. These MØ can be activated by various factors. For example, activation of MØ by glucocorticoid augments phagocytosis of apoptotic cells [81,82]. Similarly, IL-10-activated MØ show an enhanced capacity for the uptake of apoptotic cells [83]. These data indicate that the micro-environment influences the differentiation process of MØ and thereby modifies their functions as well.

Recently, we compared the capacities for the uptake of apoptotic cells among three types of phagocytes iMoDCs, MØ1, and MØ2 generated from the same monocyte population, and found that MØ2 have the unique capacity to preferentially take up early apoptotic cells [59]. We found that MØ2 have the capacity to take up early apoptotic cells more efficiently than late apoptotic or necrotic cells (four fold increase), and that this uptake was superior compared to that of MØ1 and iMoDCs. Thus we hypothesize that under steady-state conditions, scavenging of apoptotic cells is largely confined to a specialized subset of phagocytes with anti-inflammatory properties. Other subsets of phagocytes might act as backups, or even be more involved in the resolution of inflammation and /or immune regulation. We propose that in the case of MØ, the anti-inflammatory MØ2 are the default phagocytes that take up early apoptotic cells in a silent manner, whereas excessive apoptotic cells progress to a late stage of cell death and might provide "eat me signals" to the non-resting phagocytes such as the pro-inflammatory MØ1. In the latter case, appropriate opsonization may determine the consequence of the uptake (Fig. 2). It is important to note that most opsonins, including C1q, MBL, PTX3 and SAP, preferentially bind to late apoptotic cells [13].

Consequence of uptake by MØ

A substantial number of *in vitro* studies have shown that MØ that have ingested apoptotic cells are inhibited in their production of pro-inflammatory cytokines [63,65,74,79], consistent with the prevailing believe that apoptotic cells are removed by

MØ in a silent manner. Since both pro-inflammatory and anti-inflammatory MØ exist, a relevant question is then: do they respond to apoptotic cells in different ways? Our studies showed that upon ingestion of apoptotic cells, pro-inflammatory MØ1 down-regulate the production of pro-inflammatory cytokines, whereas anti-inflammatory MØ2 retain high level of IL-10 production in the absence of pro-inflammatory cytokines [59].

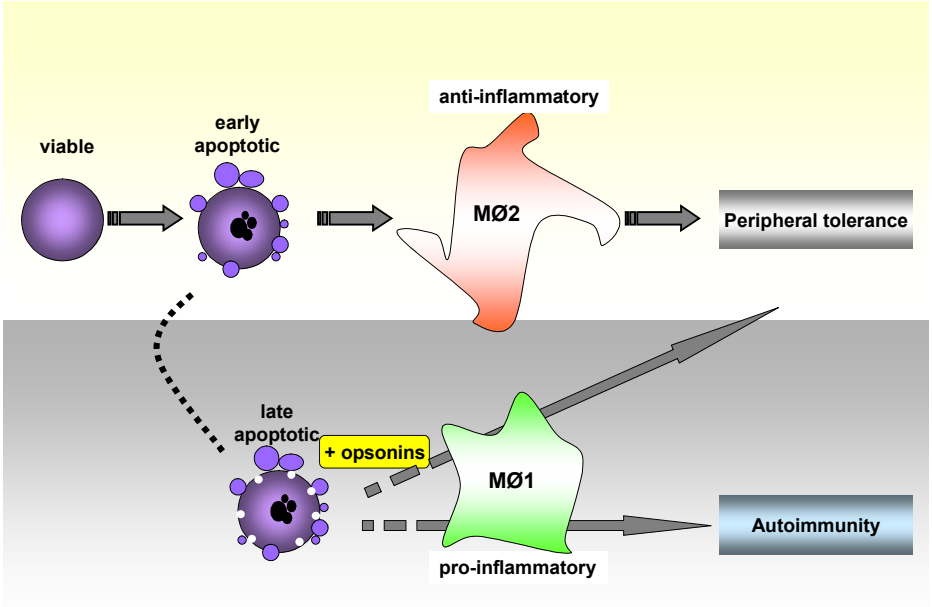

Figure 2. Apoptotic cell uptake by MØ subsets. We propose that concerning MØ, at least two subtypes exist, i.e., the pro-inflammatory MØ1 and the anti-inflammatory MØ2. MØ2 preferentially bind and ingest early apoptotic cells in a non-inflammatory fashion. Excessive apoptotic cells progress to a late stage of cell death and might provide "eat me signals" to the non-resting phagocytes such as pro-inflammatory MØ1. In this case, appropriate opsonization of apoptotic cells may determine the consequence of the uptake.

Similar to DCs, not only apoptotic cells, but also necrotic cells are taken up by MØ [84,85]. Like for apoptotic cells, several studies have addressed the question whether necrotic cells activate MØ to trigger immunity. It has been shown that phagocytosis of necrotic cells by MØ does not induce inflammation [84,85]. However, these findings were challenged in a recent publication showing that exposure of MØ to early and late apoptotic cells induces identical signal transduction in these cells in terms of inhibition of ERK 1/2 and induction of JNK and P38, whereas necrotic cells induced an opposite signal transduction [86]. Taken together, these data provide an

ambiguous image on whether necrotic cells are much more dangerous than apoptotic cells. Further studies will be required to understand the interaction of MØ with the intracellular contents released from necrotic cells such as HSP [87], HMGB1 [88] and uric acid [89].

Like DCs, MØ are also professional APCs [47]. It would be of interest to investigate how these intracellular molecules modulate MØ function including the presentation of autoantigens. Indeed, most available studies have not investigated the role of dying cell-derived antigen presentation on MØ. It has been suggested that MØ fail to cross-present antigenic material contained within the engulfed apoptotic cells [33]. In contrast to DCs that can retain antigen for at least 2 days [90], MØ robustly degrade the ingested antigens, and therefore may fail to promote T cell priming [91]. Thus it remains to be investigated how MØ process antigens derived from dying cells and subsequently present these to Class I- or II-restricted T cells.

Concluding remarks

As discussed so far, there are different professional phagocyte subsets that are actively involved in the clearance of dying cells. Presumably, also neighboring non-professional phagocytes might actively participate in the removal of dying cells. Therefore, especially under steady-state conditions, the fate of dying cells will ultimately be determined by local conditions and the composition of the tissue. Excessive apoptosis leads to the release of intracellular signals and therefore alert the immune system in various aspects. Again, the local environment will determine 1) the composition of attracted phagocytes; 2) the presence of innate molecules that help in opsonization and clearance; or 3) presence of TLR ligands that might activate immunity. The sole presence of high amounts of apoptotic cells as a consequence in deficiencies in clearance, as shown in $CD14^{-/-}$ mice [92] or C1q-/- mice [5] on a non-autoimmune background, does not always lead to a break of immunological tolerance. *In vivo* dissection of phagocyte subsets with distinct functional properties will be of particular importance to understand how the clearance of apoptotic cells by phagocytes is regulated and how this may lead to induction or loss of peripheral tolerance.

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3

IL-10-producing macrophages preferentially clear early apoptotic cells

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Summary

To prevent the development of autoimmunity, professional phagocytes including subsets of dendritic cells (DCs) and macrophages (MØ) promptly and efficiently clear apoptotic cells. Here we identify that M-CSF-driven macrophages (MØ2) are potent phagocytes that have the unique capacity to preferentially bind and ingest early apoptotic cells. This macrophage subset has intrinsic anti-inflammatory properties, characterized by high IL-10 production in the absence of pro-inflammatory cytokines, such as IL-6 and TNF-α. Importantly, whereas the IL-6 and TNF-α production by GM-CSF-driven macrophages (MØ1) is inhibited upon uptake of apoptotic cells, the anti-inflammatory status of MØ2 is retained during phagocytosis. MØ2 were shown to use CD14 to tether apoptotic cells, whereas recognition of phosphatidylserine (PS) contributed to uptake of early apoptotic cells. MØ2 showed more potent macropinocytosis compared to DCs and MØ1, and uptake of apoptotic cells was inhibited by a macropinocytosis inhibitor. Our studies suggest that, under steady-state conditions, IL-10-producing MØ2 are prominently involved in the clearance of early apoptotic cells.

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Introduction

During normal homeostasis and tissue turnover large numbers of cells undergoing apoptosis are promptly removed and replaced. The removal of apoptotic material plays an important role in the suppression of inflammation and the regulation of immune responses [1-3]. Apoptotic cells are a rich source of autoantigens [4], which are involved in the physiologic maintenance of self-tolerance. The uptake and processing of apoptotic cells has been proposed to be a silent process, meaning that release of pro-inflammatory cytokines by phagocytes is prevented [5-8]. Impaired clearance of apoptotic cells, resulting in an accumulation of late apoptotic and secondary necrotic cells, might provide a danger signal to antigen presenting cells (APCs), thus activating autoreactive T cells, and finally leading to the breakdown of peripheral tolerance [3]. Accumulating evidence has been provided that defective clearance of apoptotic cells can lead to exacerbation of inflammation and predisposes to the development of autoimmunity, such as in systemic lupus erythematosus (SLE) [9].

The apoptotic cell clearance machinery includes professional phagocytes, i.e. immature dendritic cells (DCs) and macrophages (MØ), and non-professional phagocytes, including epithelial cells, fibroblasts and mesangial cells [10]. It has become clear that there are various subsets in both DCs and MØ [11-13]. Recent *in vitro* data show that MØ can be polarized into pro-inflammatory (MØ1) and anti-inflammatory cells (MØ2) by granulocyte/macrophage colony-stimulating factors (GM-CSF) and M-CSF (also termed CSF-1), respectively [14,15]. Classically, GM-CSF and M-CSF are thought to be the primary growth factors for the differentiation of macrophages [16]. Mice lacking M-CSF develop a general MØ deficiency [17], whereas GM-CSF-knockout mice showed no major deficiency of MØ [18,19]. In humans, M-CSF, but not GM-CSF, is an ubiquitous cytokine circulating in the human body [14,20]. Thus, M-CSF could be the default cytokine to drive MØ differentiation under steady-state conditions.

The removal of apoptotic cells is an ongoing and constitutive process. Apoptotic cells provide "eat me signals" to phagocytes to promptly and efficiently engulf apoptotic cells during the very early stage of cell death, preventing them to release noxious intracellular contents [21]. Although the silent removal of early apoptotic cells is well appreciated, the immunological mechanisms involved are incompletely defined. There are several checkpoints conceivable which together determine the immunological response towards the clearance of apoptotic cells. First, the process and the stage of cell death and/or the specific death pathways triggered are closely related to the consequence of apoptotic cell clearance [22]. It has been established that late apoptotic or necrotic cells induce phagocyte activation whereas early apoptotic cells do not [23,24]. This is most likely due to differential expression of markers of cell death, such as heat shock proteins [25], HMGB1 [26] and

uric acid [27]. Second, opsonization of apoptotic cells by components of the innate immune system such as complement factors facilitates and modulates the clearance of apoptotic cells (reviewed in [28]). Third, the nature of phagocytes that take up apoptotic cells might provide a defined immunological response.

Due to the large heterogeneity of phagocytes, the contribution of different phagocyte subsets in the clearance of apoptotic cells in terms of cytokine signature and the polarization of immune regulation remains largely unknown. In this respect, we hypothesize that under steady-state conditions, the scavenging of apoptotic cells is largely confined to a specialized phagocyte subset with anti-inflammatory properties. To test this hypothesis, we compared three types of phagocytes, DC, MØ1 and MØ2, with respect to the phagocytosis of apoptotic cells at various stages of cell death. Our results strongly suggest that IL-10-producing MØ2 are prominently involved in the recognition and clearance of early apoptotic cells.

Materials and Methods

Generation of monocyte-derived DCs, MØ1 and MØ2.

Human mononuclear cells were isolated from buffy-coats obtained from healthy donors using Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO), followed by anti-CD14 microbeads magnetic cell sorting, according to the manufacturer's instruction (Miltenybiotec/CLB, Amsterdam, the Netherlands). DCs were generated in 6-well culture plates (Costar, Cambridge, MA) in RPMI culture medium (RPMI 1640 containing 10% heat-inactivated FCS, 90 U/ml penicillin and 90 μg/ml streptomycin) (all from Gibco/Life technologies, Breda, the Netherlands) supplemented with 5 ng/ml GM-CSF (Leucomax, Novartis Pharma BV, Arnhem, the Netherlands) and 10 ng/ml IL-4 (PeproTech, Rocky Hill, NJ) for at least 6 days, as previously described [29]. Two types of macrophages, namely MØ1 and MØ2, were generated in the same RPMI culture medium as DCs but supplemented with 5 ng/ml GM-CSF and 5 ng/ml M-CSF (R&D systems / ITK Diagnostics, Uithoorn, the Netherlands), respectively. In some experiments, MØ2 were generated in the presence of M-CSF and 10 μg/ml neutralizing anti-IL-10-receptor mAb [30]. In all experiments comparing these three types of phagocytes, they were generated in parallel from monocytes of the same donor. Separate unrelated donors were used for each independent experiment.

Analysis of cell surface molecules by flow cytometry.

Cells were harvested and washed in buffer containing 1% BSA, 1% heat-inactivated normal human serum, and 0.02% NaN_3. The following mAbs were used for flow cytometry analysis to analyze the surface molecules of phagocytes: anti-CD1a (Leu-6), anti-CD14 (Leu-M3), anti-CD11b/Mac-1 (all from BD Biosciences, San Jose, CA), anti-DC-SIGN/CD209 (AZN-D1, a gift of Dr. Y. van Kooyk, VU Medical Center, Amsterdam, the Netherlands), and anti-mannose receptor (MR) /CD206 (D547.3, a gift of F. Koning, LUMC, Leiden, the Netherlands). Staining was visualized by PE-conjugated goat anti-mouse Ig (Dako, Glostrup, Denmark) using appropriate isotype controls. Cells were analyzed using FACSCalibur

and CellQuest software (BD Biosciences). Dead cells, identified by propidium iodide (PI) uptake, were excluded from analysis.

Induction of apoptosis and necrosis.

Jurkat T cells were cultured in RPMI culture medium. Apoptosis of Jurkat T cells was induced by culture with 50 μM etoposide (Vepesid, Bristol-Myers Squibb, New York, NY, USA) for 18 h in serum-free RPMI culture medium (RPMI 1640 containing 90 U/ml penicillin and 90 μg/ml streptomycin). Alternatively, Jurkat cells were washed with PBS and exposed to ultra violet (UV)-C light (TUV lamp, predominantly 254 nm, Philips electronic Instruments Inc., Eindhoven, the Netherlands) at a dose of 50 J/m^2. After UV irradiation, cells were cultured for 4 and 30 hours in serum-free RPMI medium to harvest the early and late apoptotic cells, respectively. Necrosis was induced by incubating Jurkat cells at 56°C in a water bath for 1 hour. Both apoptosis and necrosis were confirmed by double staining with fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI, VPS Diagnostics, Hoeven, the Netherlands) according to established methods [31].

Next to apoptotic and necrotic cells, apoptotic blebs were used. Blebs were isolated from Jurkat cells treated with 50 μM etoposide for 48 h, as described before [32]. Blebs from 2.5×10^5 cells were used for the phagocytosis assay (see below) per 1×10^5 phagocytes.

Phagocytosis assay.

Prior to the induction of apoptosis, Jurkat cells were fluorescently labeled with carboxyfluorescein diacetate succinamidyl ester (CFSE, Molecular Probes, Leiden, the Netherlands), according to a previously described method [33]. In brief, Jurkat cells were suspended in PBS at 2×10^7 cells/ml and incubated for 30 min at 37°C with 5 μM CFSE. Cells were washed and resuspended at 2×10^6 cells/ml in serum-free RPMI culture medium, used for apoptosis induction, as described above. For the phagocytosis assay, labeled apoptotic cells (1×10^5) were co-cultured with DCs, MØ1 or MØ2 at 1:1 ratio for different time periods at 37°C or 4°C in 250 μl RPMI culture medium in round-bottom glass tubes. DCs, MØ1 or MØ2 were stained with a PE-conjugated mAb against CD11b or APC-conjugated mAb against CD14 (BD Biosciences) and uptake was analyzed by two-color flow cytometry. The percentage of CD11b-positive cells that stained positive for CFSE was used as a measure for the percentage of DCs, MØ1 and MØ2 that ingested (37°C) and/or bound (4°C) apoptotic cells.

Phagocytosis of apoptotic cells by MØ1 and MØ2 was further investigated by confocal laser scanning microscopy with a BioRad MRC1024 ES krypton–argon ion laser scanning imaging system (Hercules, CA), using appropriate filter settings. Images were visualized using a 40x/0.75 numeric aperture (NA) oil objective, were acquired using Laser Sharp 2000 software (BIO-Rad), and were processed using ImageJ software version 1.33 (NIH Image, Bethesda, MD). For this purpose, CFSE-labeled apoptotic cells (1×10^5) and phagocytes were co-cultured at 1:1 ratio on the Lab-TEK^{TM} chamber slides (NUNC/Sanbio, Uden, the Netherlands) for 2 h at 37°C, followed by washing to remove the non-ingested apoptotic cells, and staining with PE-conjugated mAb against CD11b. Cells were fixed with 4%

paraformaldehyde before analysis. More than 600 single cells of MØ1 or MØ2 were randomly scored. Phagocytosis was presented as phagocytic index (percentage of phagocytosing MØ × average number of apoptotic cells per MØ) [34].

To measure a general phagocytic capacity of MØ1 and MØ2, sheep red blood cells (SRBC, erythrocytes) were obtained and opsonized with rabbit anti-sheep red blood cell IgG (E_{IgG}). MØ1 and MØ2 were pre-cultured on Lab-TEK^{TM} chamber slides at 37°C, followed by the addition of E_{IgG} or non-opsonized erythrocytes at 1:50 ratio for 0.5 h. Unbound erythrocytes were washed away with PBS and uningested erythrocytes were lysed by lysis buffer. Cells were fixed and stained with May-Grünwald /Giemsa. More than 300 single cells of MØ1 or MØ2 were scored by the light microscopy and phagocytosis was presented as phagocytic index.

Cytokine detection.

DCs, MØ1 and MØ2 were stimulated with 200 ng/ml lipopolysaccharide (LPS, *Salmonella Typhosa*, Sigma-Aldrich) for 24 h and supernatants were harvested. Cytokines were detected in the supernatants using ELISA. The measurements of IL-6 and TNF-α were performed as described [35]. The analysis of IL-10 was performed according to the manufacturer's instructions (Sanquin Research, Amsterdam, the Netherlands).

Co-culture was performed by incubating DCs, MØ1 and MØ2 with etoposide-induced apoptotic cells at a 1:1 ratio in RPMI culture medium. After 24 h, supernatants were harvested and tested for IL-6, IL-10 and TNF-α production.

Endocytosis and macropinocytosis assays.

Lectin-mediated endocytosis was examined following co-incubation of phagocytes (1×10^5) with 100 µg/ml Dextran^{FITC} (Molecular Probes) for 30 or 60 min at 37°C in RPMI culture medium. Control experiments were done at 4°C. Blocking experiments were performed by pre-incubation of phagocytes for 20 min with 100 µg/ml mannan, or 50 mM D-mannose (both from Sigma-Aldrich). Incubation with 50 mM L-mannose (Sigma-Aldrich) or culture medium only was used as a negative control. Macropinocytosis was measured as the cellular uptake of 100 µg/ml lucifer yellow (LY) dipotassium salt (Molecular Probes) or 0.2 µg/ml BSA^{FITC} (Sigma-Aldrich). Cells were washed extensively with cold PBS containing 1% FCS and 0.02% NaN_3. Before analysis with flow cytometry, the cell surface fluorescence was quenched with trypan blue (Sigma-Aldrich). To quantify the uptake, mean fluorescence intensity (MFI) values obtained at 4°C were subtracted from MFI values obtained at 37°C.

Inhibition of uptake of apoptotic cells

To assess the role of phosphatidylserine (PS) in the uptake of early apoptotic cells by MØ2, early apoptotic cells were pre-incubated with recombinant annexin V (Sigma-Aldrich) (up to 50 µg/ml) at 4ºC for 20 min to mask PS, before incubation with MØ2. To assess the role of CD14 in the apoptotic cell

clearance, MØ1 and MØ2 were treated with an anti-CD14 blocking mAb (61D3, a kind gift of Dr. C. Gregory, University of Edinburgh, U.K.). MØ were pre-incubated with 61D3 (20 µg/ml) for 20 min at 4ºC before addition of UV-induced early apoptotic cells or etoposide-induced apoptotic cells.

To investigate the role of macropinocytosis in the uptake of apoptotic cells by MØ1 and MØ2, 5-(N, N-Dimethyl)amiloride hydrochloride (DMA, Sigma-Aldrich) was used to inhibit macropinocytosis. MØ1 and MØ2 were pre-incubated with DMA (up to 1 mM) at 37ºC for 20 min before early apoptotic cells or apoptotic blebs were added.

Statistical analysis.

Statistical analysis was performed by two-way ANOVA, Chi-square or one sample *t* test using GraphPad Prism (GraphPad software, San Diego, CA). Differences were considered statistically significant when p values were less than 0.05.

Results

Characterization of DCs, MØ1 and MØ2.

Three types of phagocytes were generated from peripheral blood monocytes by different cytokines, i.e. IL-4 and GM-CSF for DCs, GM-CSF for MØ1, and M-CSF for MØ2, respectively. MØ2 were less-adherent cells, with irregular shapes compared with MØ1 (Fig. 1A). In contrast to DCs, MØ1 and MØ2 shared the typical macrophage phenotype, being positive for CD14, but negative for CD1a and DC-SIGN (Fig. 1B). Notably, MØ2 expressed higher levels of CD14, but much lower level of mannose receptors (MR) than MØ1. All three cell types showed expression of CD11b.

Uptake of apoptotic cells by DCs, MØ1 and MØ2.

To investigate the phagocytosis of these three phagocyte subsets, apoptosis was induced in Jurkat cells using etoposide for 18 hours at 37°C in serum-free RPMI culture medium, as described before [32]. According to annexin V and PI staining, early apoptotic cells ($Annexin^+PI^-$ populations) were routinely 27.52 ± 6.08 % and late apoptotic cells (PI^+ populations) were 48.61 ± 10.76 % (n=8). Apoptosis was confirmed by nuclear fragmentation using Hoechst staining (data not shown). Exposure of apoptotic cells to DCs, MØ1 and MØ2 resulted in a time- and temperature-dependent binding and ingestion, as shown by the appearance of CFSE- and CD11b-double positive populations (Fig. 2A). Notably, from the three types of cells, MØ2 demonstrated the highest capacity for binding of apoptotic cells at 4°C, and for uptake of apoptotic cells at 37°C (Fig. 2A). MØ2 have a 1.5 to 2-fold

higher capacity for both binding and uptake of apoptotic cells compared to DCs and MØ1 (Fig. 2B; ANOVA, P<0.001). Similar results were obtained when different

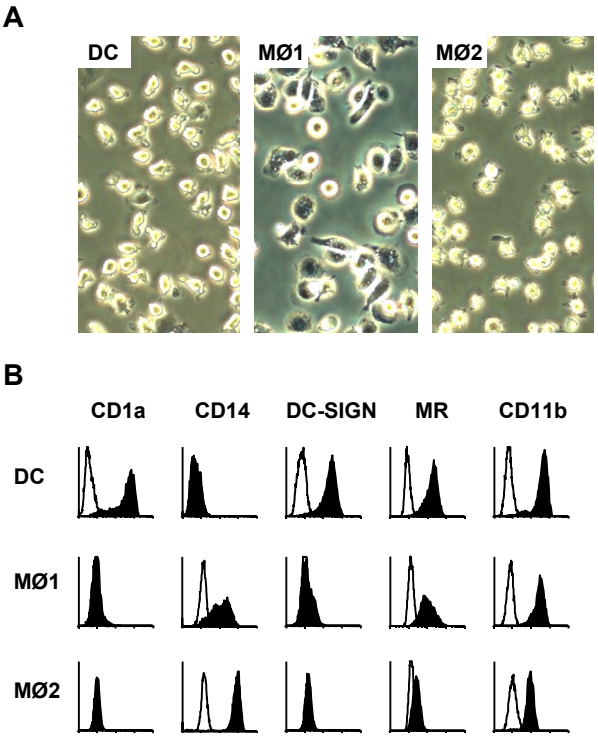

Figure 1. Characterization of DCs, MØ1 and MØ2. (A) DCs, MØ1 and MØ2 were generated in parallel from the same donor cultured for 6 or 7 days. Pictures show the morphology of these 3 different types of cells. Images were obtained using an Axiovert 25 inverted microscope (Carl Zeiss, Sliedrecht, the Netherlands) with a 20 x/0.3 NA objective and Zeiss Axiovision software version 3.1. Magnification 200×. (B) Surface expression of CD1a, CD14, DC-SIGN, mannose receptor (MR) and CD11b on DCs, MØ1 and MØ2 was determined by flow cytometry after 6 days of culture (closed histograms). Open histograms represent matched isotype controls. Data are representative of 3 to 8 independent experiments. Separate unrelated donors are used for each independent experiment.

ratios of phagocytes:apoptotic cells (4:1, 16:1, and 64:1) were applied during co-culture (data not shown). The uptake was significantly increased in time at 37°C (ANOVA, p<0.05 for all three type of phagocytes), but the binding at 4°C was stable (Fig. 2B).

To confirm and quantify the uptake of apoptotic cells by MØ1 and MØ2, confocal microscopy was used. This analysis clearly showed the presence of CFSE-labeled apoptotic material inside the cells (Fig. 2C). Quantification of phagocytic index confirmed our FACS data that MØ2 were more potent in the uptake of apoptotic cells than MØ1 (Fig. 2D). The higher phagocytic index in MØ2 was due to a significantly higher percentage of MØ2 contributing to the uptake as compared to MØ1 (Chi-square test, p<0.0001), whereas the number of apoptotic cells taken up per macrophage between MØ1 and MØ2 were similar (2.33 vs 2.55, respectively). We realize that the difference of uptake between the quantification measured by FACS and by confocal microscopy is large, which is probably due to several factors. First of all, data presented are from separate sets of experiments with cells derived

from different donors. We have not performed a side-by-side comparison of both techniques. Second, for FACS analysis, we were using the cells in solution, which might affect their phagocytic behavior. However, in all cases MØ1 and MØ2 were generated in parallel from the same donor. Therefore we think we can draw firm conclusions from the comparisons between MØ1 and MØ2.

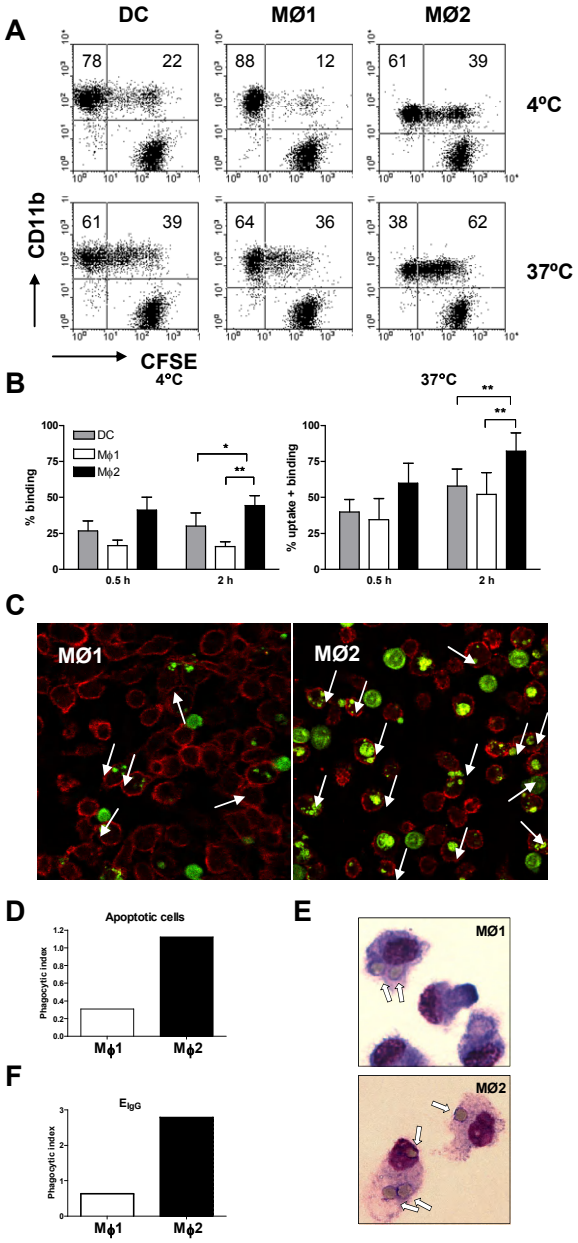

Figure 2. Uptake of apoptotic cells by DCs, MØ1 and MØ2. (A) Jurkat T cells were labeled with CFSE and induced into apoptosis by treating cells with etoposide for 18 hours. Apoptotic cells (0.1×10^6 cells) were co-incubated with DCs, MØ1 or MØ2 at 1:1 ratio, for 0.5 h and 2 h at 37°C or 4°C. Prior to flow cytometry analysis, cells were stained with PE-conjugated mAb against CD11b. CFSE- and CD11b-double positive populations represent the phagocytes that have bound and/or ingested apoptotic cells. Dot-plots represent the phagocytosis of apoptotic cells at 2 h. (B) The percentage of uptake and binding (at 37°C) or binding (at 4°C) were calculated as 100% × ($(CD11b^+CFSE^+)/CD11b^+$). Data indicate the mean ± SD from 4 independent experiments performed in duplicate. Statistics were performed with two-way ANOVA. *, $p<0.01$, **, $p<0.001$. (C) Confocal microscopy images show the uptake of apoptotic cells by MØ1 and MØ2 (see arrows). Red cells represent the CD11b-PE positive MØ and green ones are the CFSE-labeled apoptotic cells. (D) Based on the confocal images, more than 600 cells of MØ1 and MØ2 were scored. Data are presented as phagocytic index (percentage of phagocytosing MØ × average number of apoptotic cells per MØ). A Chi-square test was performed to evaluate the difference in the capacity of apoptotic cell uptake between MØ1 and MØ2 ($p<0.0001$). (E) Sheep erythrocytes were opsonized with rabbit anti-sheep red blood cell IgG (E_{IgG}) and co-cultured with MØ1 and MØ2 on Lab-TEK™ chamber slides at 37°C for 0.5 h, followed by May-

To determine whether the observed highly phagocytic capacity of MØ2 represent a generalized increase on phagocytosis, uptake of E_{IgG} was investigated. We found that the phagocytic index of MØ2 is 6.84 fold higher than that of MØ1 (mean of 3 independent experiments) (Fig. 2E, F). This is especially explained by the fact the percentage of MØ2 contributing to the uptake was significantly higher than that of MØ1 (Chi-square test, $p<0.0001$ for all three independent experiments), whereas the number of erythrocytes taken up per macrophage between MØ1 (1.83 ± 0.17) and MØ2 (2.23 ± 0.72) showed no difference (paired *t* test, $p=0.40$). The non-opsonized erythrocytes were not ingested by MØ1, whereas the uptake by MØ2 was of low efficiency (phagocytic index <0.1, data not shown). Together, these data clearly show that MØ2 have a more generalized higher capacity of phagocytosis than MØ1.

MØ2 retain their anti-inflammatory status after uptake of apoptotic cells.

It has been proposed that clearance of apoptotic material is a non-inflammatory process [1-3]. Therefore we investigated the functional consequences of uptake of apoptotic cells by MØ1 or MØ2 by measuring the cytokine release in supernatants. The capacity of cytokine production by the three phagocyte subsets was first investigated using LPS stimulation (Fig. 3A-C). LPS-stimulated MØ2 produced large amounts of IL-10, but failed to secrete IL-6 and TNF-α. In contrast, MØ1 produced high amounts of IL-6 and TNF-α after LPS stimulation, whereas IL-10 was hardly detectable. DCs derived from the same donor were able to produce IL-10, IL-6 and TNF-α following LPS stimulation.

MØ2 showed intrinsic IL-10 production (Fig. 3A, D), which was retained after uptake of apoptotic cells (Fig. 3D), whereas production of IL-6 or TNF-α was not induced (Fig. 3E-F). Importantly, uptake of apoptotic cells by MØ1 resulted in a down-regulation of IL-6 and TNF-α production, but no induction of IL-10 (Fig. 3D-F). Upon exposure to apoptotic cells, DCs did not secrete IL-10, IL-6 or TNF-α.

MØ2 preferentially take up early apoptotic cells.

To assess whether there are differences among these three types of phagocytes in the uptake of apoptotic cells in different stages of cell death, Jurkat cells were exposed to UV light. Four hours after UV irradiation, around 50-70% early apoptotic cells (annexin V^+ / PI^-) were obtained (Fig. 4A). Thirty hours after UV irradiation, around 70-95% of cells were late apoptotic (PI^+). Necrotic cells generated by heat shock were all PI-positive. Viable cells were used as a negative control. All three types of phagocytes were unable to take up viable cells (Fig. 4B). Importantly, MØ2 were the only phagocytes that were able to distinguish early apoptotic cells from late apoptotic and necrotic cells. MØ2 have a preferential uptake of early apoptotic cells, compared to late apoptotic or necrotic cells (Fig. 4B). In contrast, DCs and

MØ1 show an equal efficiency in the uptake of early apoptotic, late apoptotic or necrotic cells. Furthermore, the efficiency of taking up early apoptotic cells by MØ2 is at least 2-fold higher than that by DCs and MØ1 (mean ± SD of phagocytosis of MØ2: MØ1 from 5 independent experiments is: 2.65 ± 0.62, p<0.0001, two-way ANOVA).

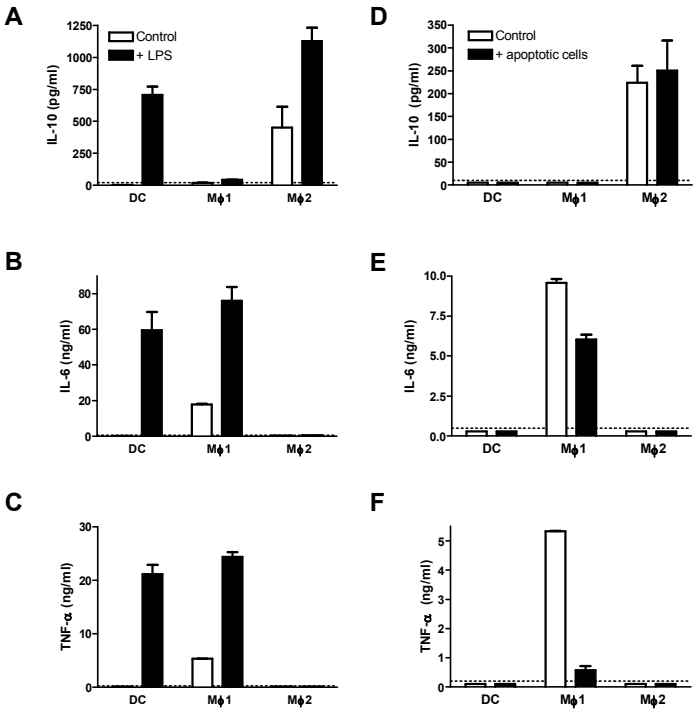

Figure 3. Cytokine production by DCs, MØ1 and MØ2 after LPS stimulation or uptake of apoptotic cells. (A-C) Day-6 DCs, MØ1 and MØ2 were extensively washed, and subsequently stimulated with or without LPS for 24h. (D-F) DCs, MØ1 or MØ2 (1×10^5 cells) were co-cultured with etoposide-induced apoptotic cells at a 1:1 ratio for 24 h in RPMI culture medium. Supernatants were harvested and measured by ELISA for IL-10, IL-6, and TNF-α. Data are presented as mean ± SD from duplicate cultures and are representative of at least 3 independent experiments where cells are generated from separate unrelated donors. Dashed lines represent the detection limits of ELISA.

It has been show that IL-10-activated MØ have increased phagocytic capacity for the uptake of apoptotic cells [36]. To investigate whether IL-10-producing MØ2 become highly phagocytic to early apoptotic cells as a consequence of the IL-10 environment created by themselves, we generated MØ2 in the presence of neutralizing anti-IL-10-receptor mAb. Although the anti-IL-10-receptor mAb inhibited the endogenous IL-10 production of MØ2 (data not shown), it did not

inhibit phagocytosis of early apoptotic cells as compared to MØ2 generated in the absence of anti-IL-10-receptor mAb (Fig. 4C). We conclude that endogenous IL-10 produced by MØ2 is not involved in driving the development of high capacity for phagocytosis of early apoptotic cells.

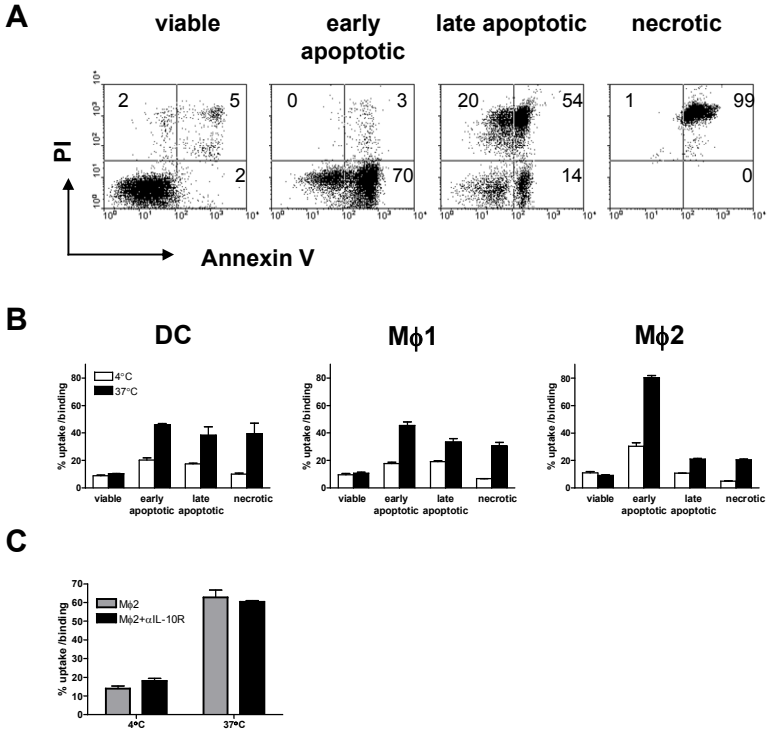

Figure 4. Uptake of viable, early apoptotic, late apoptotic, and necrotic cells by DCs, MØ1 and MØ2. (A) Early and late apoptotic cells were induced by UV-C light at a dose of 50 J/m^2, then cultured for another 4 and 30 h, respectively. Necrosis was induced by incubating Jurkat cells at 56°C for 1 hour. The untreated viable cells were used a control. Apoptosis was confirmed by double staining with FITC-labeled annexin V and propidium iodide (PI). (B) These CFSE-labeled cells were co-cultured with DCs, MØ1 or MØ2 (1×10^5 cells) at 1:1 ratio, in the same way as described at Fig. 2. The percentages of uptake and binding (at 37°C) or binding (at 4°C) were quantified similarly as described at Fig. 2. Data represent at least 3 independent experiments performed in duplicate. (C) Uptake of early apoptotic cells by MØ2 generated in the presence or absence of 10 μg/ml neutralizing anti-IL-10-receptor mAb.

Role of PS and CD14 in the uptake of apoptotic cells by MØ1 and MØ2.

A major feature of early apoptosis is the redistribution of PS from the inner layer of the cell membrane to the outside. Therefore we next investigated whether PS is involved in the phagocytosis by MØ2. Pre-incubation of early apoptotic cells with non-labeled annexin V prevented binding of FITC-labeled annexin V, confirming an effective masking of PS (data not shown), as described before [32,37]. The uptake of

early apoptotic cells by MØ2 was partially inhibited after PS masking (inhibition was between 6.5% to 23.2% in three independent experiments), and this effect could not be improved by applying a higher concentration of annexin V (Fig. 5A).

CD14 has been shown to be involved in the recognition of apoptotic cells by MØ [38]. As a possible explanation for their efficient uptake of apoptotic cells, we noticed that MØ2 express a significantly higher level of CD14 compared to MØ1 (Fig. 1B and Fig. 5B). Surface expression of CD14 on MØ1 is heterogeneous (Fig. 1B). Based on the FACS dot-plots, MØ1 were equally divided into two populations: $CD14^{high}$ and $CD14^{low}$ (Fig. 5C). Phagocytosis experiments showed that $CD14^{high}$ cells were twice as efficient in binding and uptake of apoptotic cells compared to $CD14^{low}$ cells (Fig. 5C). Thus we further investigated the role of CD14 in the engulfment of early apoptotic cells by using a blocking antibody against CD14, mAb 61D3 [38]. The uptake of early apoptotic cells by both MØ1 and MØ2 was not significantly inhibited by this mAb. We did observe that the binding of early apoptotic cells by MØ2 at 4ºC was inhibited ($p<0.001$) (Fig. 5D). Similar data were obtained when apoptosis was induced with either UV or etoposide. Together, these data indicate that CD14 is involved in tethering but not engulfing apoptotic cells.

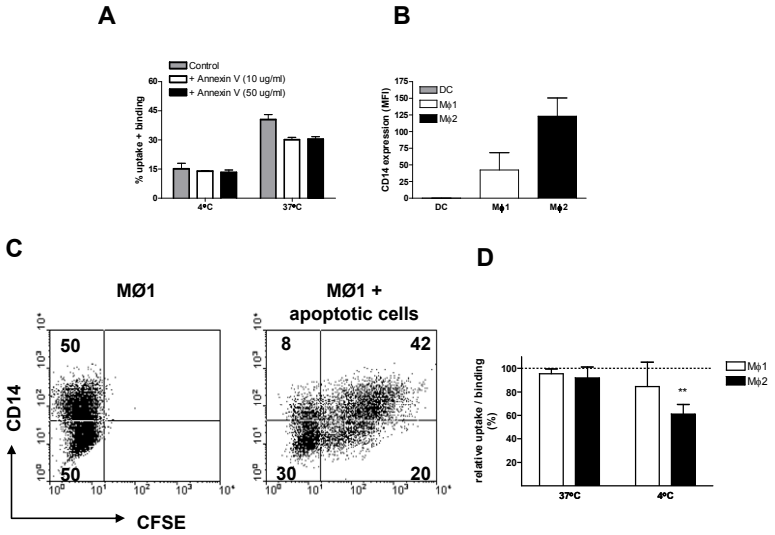

Figure 5. Role of PS and CD14 in the uptake of early apoptotic cells by MØ1 and MØ2. (A) CFSE-labeled early apoptotic cells were pre-incubated with 10 μg/ml or 50 μg/ml unlabeled annexin V or medium as control at 4°C for 20 min, before the co-incubation with MØ2. The percentage of uptake and/or binding was calculated as described in Figure 2. Data shown are representative of 3 independent experiments performed in duplicate. (B) CD14 expression on DCs, MØ1, and MØ2 generated in parallel from the same donor. Bars show the mean fluorescence intensity (MFI). Data shown are the mean ± SD from 4 independent experiments. (C) After co-incubation of apoptotic cells with MØ1, cells were stained with a APC-conjugated anti-CD14 mAb, instead of anti-CD11b. CD14 expression was divided equally into two populations: $CD14^{high}$ and $CD14^{low}$. Dot-plots of the FACS showed the distinct uptake of apoptotic cells by $CD14^{high}$ and $CD14^{low}$ cells. (D) MØ1 and MØ2 were pre-incubated with or without a blocking anti-CD14 antibody

(mAb 61D3) at 4°C for 20 min., before the co-incubation with early apoptotic cells for 2 h. Relative uptake / binding was calculated as 100% × (% in the presence of anti-CD14) / (% in the absence of anti-CD14). Data are shown as mean ± SD from 4 (MØ1) to 6 (MØ2) independent experiments performed in duplicate. Similar results were obtained when etoposide-induced apoptotic cells were applied. **, $p<0.001$, one sample *t* test. Separate unrelated donors are used for each independent experiment. Dashed line indicates 100% of relative uptake.

Uptake of early apoptotic cells by MØ2 is mediated by macropinocytosis.

Phagocytes take up antigens via receptor-mediated endocytosis mostly by C-type lectins, such as mannose receptors (MR), or via macropinocytosis (fluid phase endocytosis) [39,40]. Therefore, we investigated lectin-mediated endocytosis of soluble antigens using Dextran^{FITC}. Despite the fact that MØ2 showed a significantly lower expression of MR (Fig. 1B and Fig. 6A), the uptake of Dextran^{FITC} by MØ1 and MØ2 was comparable, whereas uptake by DCs was more efficient (Fig. 6B). The uptake of Dextran^{FITC} by DCs and MØ1 was completely inhibited by mannan or D-mannose, whereas L-mannose, used as a negative control, did not inhibit the uptake (Fig. 6C). In contrast, pre-treatment of MØ2 by mannan or D-mannose inhibited the uptake of Dextran^{FITC} only by 50% (Fig. 6C), suggesting the involvement of lectin-independent mechanisms such as macropinocytosis. Indeed, for the uptake of both BSA^{FITC} and LY, MØ2 were shown to be 2- to 3-fold more efficient compared to MØ1 and DCs (ANOVA, $p<0.001$) (Fig. 6D, 6E).

To investigate a possible contribution of macropinocytosis in the uptake of early apoptotic cells by MØ2, DMA, a Na^+/H^+ antiporter blocker, was used to inhibit the fluid phase uptake by macropinocytosis [39]. DMA treatment of MØ2 not only inhibited the uptake of both BSA^{FITC} and LY (Fig. 7A), but also inhibited the uptake of early apoptotic cells in a dose-dependent fashion (Fig. 7B), without interfering with the viability of the cells. Similarly, DMA also inhibited the uptake of early apoptotic cells by CD14^{high} MØ1 (Fig. 7C).

We next investigated the uptake of microparticles derived from apoptotic cells (apoptotic blebs). MØ2 were able to take up apoptotic blebs twice as efficiently as MØ1 (Fig. 7D). DMA inhibited the uptake of apoptotic blebs by MØ2 dose-dependently (Fig. 7E), and in concentrations that are lower than needed for the inhibition of uptake of early apoptotic cells.

Discussion

Over the last few years, it has become clear that both DCs and MØ show a large heterogeneity [11,13]. We hypothesized that distinct phagocyte subsets contribute differentially to the clearance of apoptotic cells. Here we identify that the IL-10-producing MØ2 are potent phagocytes that have the unique capacity to preferentially bind and ingest early apoptotic cells. This macrophage subset has

intrinsic anti-inflammatory properties, characterized by high IL-10 production in the absence of pro-inflammatory cytokines, such as IL-6 and TNF-α. IL-10 is an important anti-inflammatory cytokine, which is able to limit and ultimately terminate inflammatory responses and plays a role in differentiation of regulatory T cells [41].

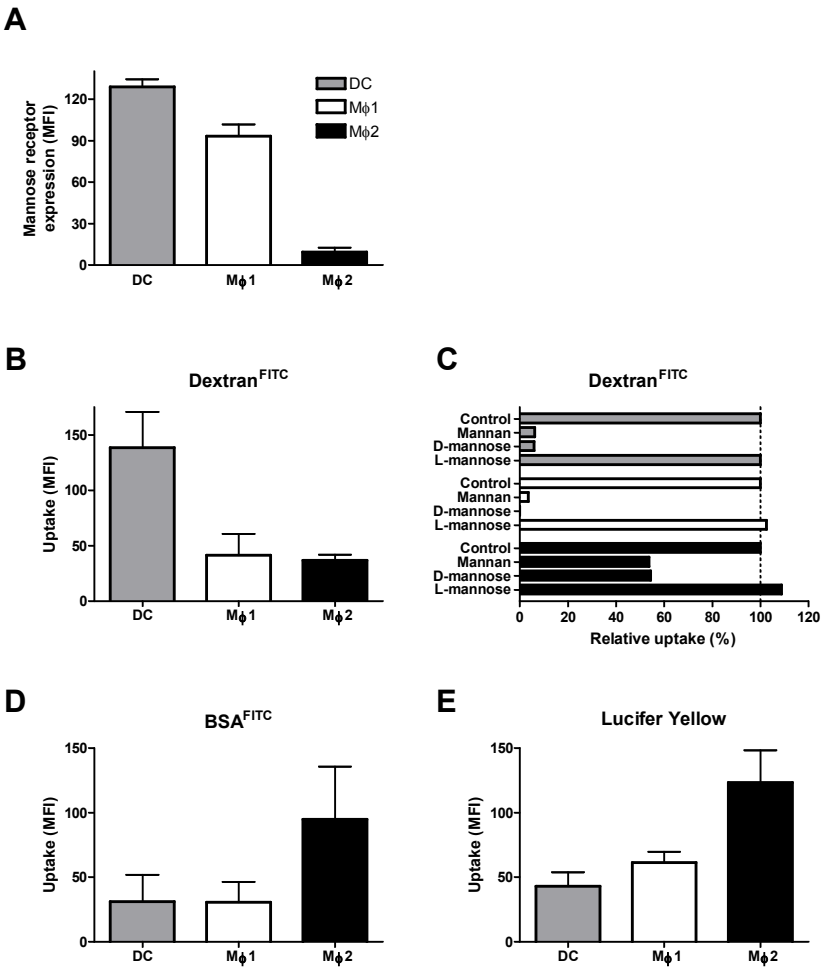

Figure 6. Lectin-mediated endocytosis and macropinocytosis by DCs, MØ1 and MØ2. (A) Mannose receptor (MR)/CD206 expression on DCs, MØ1, and MØ2. Bars show the mean fluorescence intensity (MFI). Data shown are the mean (± SD) from 2 to 3 independent experiments. (B) The uptake of Dextran^{FITC} (100 µg/ml) by DCs, MØ1, and MØ2 was measured at 1 h. Data shown are the mean ± SD from 3 independent experiments. (C) The uptake of Dextran^{FITC} was measured by different phagocytes pre-incubated with 100 µg/ml mannan, or 50 mM D-mannose or L-mannose. The uptake by untreated phagocytes (control) is considered as 100%, and data show the relative uptake against the control. Data represent 3 independent experiments. (D) The uptake of BSA^{FITC} (0.2 µg/ml) by DC, MØ1, and MØ2 at 1 h. Data shown are the mean ± SD from 5 independent experiments. (E) The uptake of lucifer yellow (LY) (100 µg/ml) by DCs, MØ1, and MØ2 at 1 h. Data shown are the mean ± SD from 3 independent experiments (**, $p<0.001$, ANOVA).

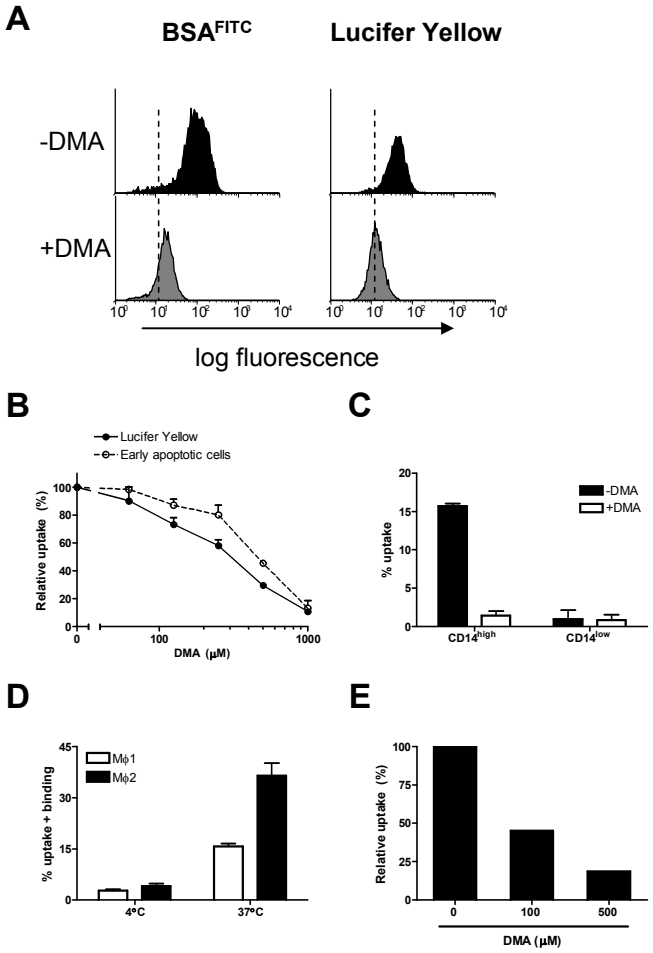

Figure 7. Uptake of early apoptotic cells and apoptotic blebs by MØ2 is prevented by an inhibitor of macropinocytosis. MØ2 were pre-incubated with or without DMA at 37°C for 20 min. before the co-culture with BSA^{FITC} (0.2 μg/ml), LY (100 μg/ml), or early apoptotic cells. (A) Data show the uptake of BSA^{FITC} and LY at 1 h by MØ2 in the presence (gray histogram) or absence (black histogram) of 500 μM DMA. The dashed lines represent the background mean fluorescence of MØ2. (B) Data show the dose-dependent effect of DMA on uptake (37°C-4°C) of apoptotic cells and LY by MØ2 and represent the relative uptake of DMA-treated MØ2 against the controls (untreated cells). Data represent 4 independent experiments. (C) MØ1 were treated with or without 500 μM DMA at 37°C for 20 min before the co-incubation with early apoptotic cells for 30 min. Data show the quantification of uptake (37°C-4°C) by $CD14^{high}$ and $CD14^{low}$ cells. Data represent 3 independent experiments. (D) Apoptotic blebs were isolated from CFSE-labeled Jurkat cells and were used for the phagocytosis assay with MØ1 and MØ2. Data represent 4 independent experiments. (E) MØ2 were pre-treated with or without DMA (up to 500 μM). Relative uptake is shown. Similar results were obtained from 2 independent experiments. Separate unrelated donors are used for each independent experiment.

The generation of apoptotic cells is a continuous process. Although it is generally accepted that clearance of apoptotic cells is a non-inflammatory process, this most likely happens only when apoptotic cells are removed during early stages of apoptosis progression. Late apoptotic or necrotic cells activate APCs, promoting them to present self-antigen to T cells, potentially resulting in autoimmunity [23,24]. Thus under normal conditions, apoptotic cells should be cleared before they

progress into late stages. Our findings that MØ2 have the capacity to preferentially take up early apoptotic cells indicate that these IL-10-producing MØ subsets might be unique safeguards to promptly clear apoptotic cells in a silent way. To note, increased capacity of phgocytosis of apoptotic cells by MØ2 is not unique to apoptotic cells, but also to IgG-opsonized erytherocytes, suggesting MØ2 have a more generalized higher capacity of phagocytosis than MØ1. It remains to be established what the *in vivo* contribution is to apoptotic cell clearance by different myeloid subsets and which mechanisms underlie the specific clearance of early apoptotic cells.

It has been shown that IL-10-activated MØ obtain enhanced capacity for the uptake of apoptotic cells [36]. However, phagocytosis experiments using MØ2 generated in the presence of neutralizing anti-IL-10-receptor mAb suggested that endogenous IL-10 produced by MØ2 is not involved in driving the development of high capacity for phagocytosis of early apoptotic cells. This inconsistence could be due to the fact that Ogden *et al.* [36] generated MØ in the presence of IL-10 during differentiation process, whereas MØ produced low level of IL-10 during MCS-driven differentiation. It is surprising that neutralizing anti-IL-10-receptor mAb inhibited the IL-10 production during differentiation of MØ2. It remains unclear whether endogenour IL-10 is required to signal to IL-10R on MØ2, thus stimulating MØ2 to produce IL-10.

Both GM-CSF and M-CSF are key growth factors, not only for MØ generation *in vitro*, but also for MØ differentiation *in vivo*. *Op/op* mice lacking M-CSF develop a profound macrophage deficiency, and this could be partially corrected by the implantation of diffusion chambers containing M-CSF-producing cells [17], confirming that M-CSF is crucial in MØ differentiation. GM-CSF knockout mice did not show major deficiency of MØ, although the MØ are smaller than normal [18,19]. Under steady-state conditions, M-CSF is the only primary MØ growth factor detectable in peripheral blood [20]. In contrast, GM-CSF is generally viewed as a pro-inflammatory cytokine involved in inflammation and is hardly detectable in circulation [42]. Thus under steady-state conditions, M-CSF could be the default cytokine driving MØ differentiation.

Cell-cell contact with apoptotic cells is sufficient to induce profound inhibition of IL-12 production by activated macrophages, thus preventing them to mount an immune response [8]. We show here that, upon uptake of apoptotic cells, MØ2 do not produce pro-inflammatory cytokines such as IL-6 and TNF-α, while their IL-10 production was not inhibited. Importantly, and in line with previous results, uptake of apoptotic cells by MØ1 resulted in a down-regulation of IL-6 and TNF-α production. It has been shown that monocyte-derived MØ differentiated with M-CSF acquire the ability to selectively induce T cell apoptosis in an activation-specific fashion [43], leading to the speculation that peripheral tolerance can be helped by selectively deleting autoreactive T cells that respond to self-antigen.

Therefore, at least two mechanisms might be operational to prevent that uptake of apoptotic cells leads to immune activation.

Recently, CD14 has been reported to play an important role in the recognition of apoptotic cells [38] and was further suggested as a tethering molecule for MØ to recognize apoptotic cells [44]. CD14-/- mice showed a persistence of apoptotic cells, supporting that CD14 plays a broad tethering role in the apoptotic cell clearance *in vivo* [45]. In our study, CD14 expression is remarkably different between MØ1 ($CD14^{+}$) and MØ2 ($CD14^{+++}$). Notably, MØ2 have a strong interaction with apoptotic cells already at 4°C. Blocking studies using anti-CD14 antibody showed that CD14 is mainly involved in the surface binding, but hardly in the uptake of apoptotic cells, supporting the notion that CD14 is a tethering molecule rather than an engulfing molecule [38].

Next to the tethering process, other mechanisms are involved in the engulfment of apoptotic cells. Uptake of antigens (Ag) takes place via at least two different pathways: receptor-mediated endocytosis and macropinocytosis [39,40]. Although macropinocytosis is thought to be a specific characteristic of DCs [39], it can be induced in other cells, including MØ stimulated with M-CSF [46,47]. Here we show in a side-by-side comparison that MØ2, generated in the presence of M-CSF but tested in the absence of M-CSF, exhibit more potent macropinocytosis compared with monocytes-derived DCs. The suggestion that macropinocytosis contributes to the uptake of apoptotic cells has already been put forward [48]. Engagement of MØ surface receptors by apoptotic cells, either directly (via PS) or indirectly (via C1q or mannose binding lectin (MBL) binding to apoptotic cells) actively promotes macropinocytosis [34,49]. Our studies using the inhibitor DMA suggest that the efficient uptake of early apoptotic cells or apoptotic blebs by MØ2 is mediated by Na^{+}/H^{-} antiporter-dependent macropinocytosis. Rac, Rho and Cdc42 have been described as the major regulators of actin-driven macropinocytosis [50]. Whether these regulators are involved in initiating macropinocytosis, particularly in the uptake of early apoptotic cells by MØ2, remains to be established. Our findings are in agreement with the proposal that the uptake of apoptotic cells is a two-step process [3]: first, apoptotic cells are engaged with phagocytes by receptor-dependent tethering processes such as CD14 for MØ2; Second, formation of fluid-filled macropinosomes allows MØ2 to promptly and efficiently engulf early apoptotic cells or microparticles-derived from apoptotic cells.

In conclusion, different subsets of phagocytes, such as DCs, MØ1 and MØ2 recognize and process early and late apoptotic cells in a differential manner. The cytokine environment and the nature of phagocytes at a certain location contribute to the decision whether apoptotic cells are cleared in a silent or inflammatory fashion. In this respect, we demonstrate that IL-10-producing MØ2 have the unique capability to preferentially bind and ingest early apoptotic cells. Our studies suggest that under steady-state conditions, M-CSF-driven MØ2 might be the default

phagocytes to preferentially clear early apoptotic cells in a silent way, thereby contributing to the induction and maintenance of peripheral tolerance after encountering self-antigen.

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4

Human peritoneal macrophages show functional characteristics of M-CSF-driven anti-inflammatory type-2 macrophages

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Summary

We have recently shown that *in vitro*-polarized M-CSF-driven anti-inflammatory macrophages (Mφ2) have the unique capacity to preferentially bind and ingest early apoptotic cells. However, these data are based on *in vitro* polarized cells and it is unclear whether Mφ2-like cells exist *in vivo*. Here we used CD163 as a cell surface marker to distinguish Mφ2 from the pro-inflammatory Mφ1. We show that human peritoneal Mφ (pMφ) freshly isolated from patients on peritoneal dialysis have the phenotypical characteristics of Mφ2, including CD163 surface expression and lack of CD16. Like Mφ2, pMφ have the capacity for endocytosis and macropinocytosis, are able to preferentially bind and ingest early apoptotic cells, and produce large amounts of IL-10 upon stimulation with LPS. Moreover, upon LPS stimulation both pMφ and Mφ2 down-regulate CD86, resulting in a reduced capacity to stimulate proliferation of allogeneic T cells and an inhibition of Th1 cytokine release of these T cells. Our data provide the evidence for the first time that *in vitro*-polarized Mφ2 exist in vivo, and human pMφ resemble the anti-inflammatory Mφ2. We propose that pMφ have the potential to maintain an anti-inflammatory condition in the peritoneal cavity.

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Introduction

The removal of apoptotic cells by phagocytes plays an important role in the suppression of inflammation and the regulation of immune responses [1,2]. Since professional phagocytes including dendritic cells (DCs) and macrophages (Mφ) represent a very heterogeneous population of cells [3,4], the extent of clearance of apoptotic cells and its immunological consequence may depend on the nature of phagocyte subsets in a certain tissue compartment [5]. We and others have recently shown that human Mφ can be polarized *in vitro* into pro-inflammatory (Mφ1) and anti-inflammatory cells (Mφ2) by granulocyte/macrophage colony-stimulating factors (GM-CSF) and M-CSF (also known as CSF-1), respectively [6-8]. Particularly, Mφ2 were shown to have the unique capacity to preferentially bind and ingest early apoptotic cells [8]. This raises the question whether the *in vivo* clearance of apoptotic cells is confined to a specialized subset of phagocytes with anti-inflammatory properties, such as Mφ2 [5,8].

Since the results mentioned above are based on *in vitro* studies, a challenging task is to obtain insight in the physiological relevance of *in vitro*-polarized Mφ1 and Mφ2. Classically, most resident Mφ are derived from circulating bone marrow-derived monocytes *in vivo* [4]. GM-CSF and M-CSF are major growth factors for Mφ differentiation *in vivo* [9]. Peritoneal Mφ (pMφ) represent resident tissue Mφ of the peritoneal cavity, and constitute one of the few human resident Mφ subpopulations that are easily accessible for isolation [10]. It has been shown earlier that in mice, M-CSF, distributed locally, plays a critical role in the differentiation of resident pMφ *in vivo* [11]. In humans, the M-CSF level in the peritoneal fluid is 2.5-fold higher as compared to plasma, and it has been shown that the levels of M-CSF are correlated to pMφ numbers [12]. Considering the importance of M-CSF as a major factor in the differentiation of resident Mφ, we undertook the current study as a further step to identify the *in vivo* counterpart of M-CSF-driven Mφ2. We found that human resident pMφ, freshly isolated from patients on peritoneal dialysis (PD), share several characteristics of Mφ2. Moreover, like Mφ2 and in contrast to Mφ1, resident pMφ down-regulate CD86 upon stimulation with LPS and this is associated with a reduction of their allogeneic T cell stimulatory capacity. We hypothesize that pMφ have the potential to maintain an anti-inflammatory status in the peritoneal cavity.

Materials and Methods

Patients and pMφ

pMφ were isolated from peritoneal dialysate effluents from in total fifteen PD patients at the department of Nephrology of the Leiden University Medical Center (the Netherlands). The underlying primary diseases were renal vascular disease, diabetes, hypertension, cystic nephritis and systemic lupus

erythematosus (SLE). All patients had been free of infection for at least 4 weeks before the collection of dialysate effluents. Effluents were collected from 3 to 4 h dwells. The isolation of pMφ was performed according the method described elsewhere [13]. Briefly, peritoneal cells were collected by centrifugation (RPM 1500, 10 min.) from chilled dialysate effluents. After washing two times with PBS, cells were seeded at 1×10^6/ml in 6-well culture plates (Costar, Cambridge, MA) in RPMI culture medium (RPMI1640 containing 10% heat-inactivated FCS, 90 U/ml penicillin and 90 μg/ml streptomycin). After 2 h incubation at 37°C, non-adherent cells were removed by washing with PBS. Adherent cells (pMφ) were harvested by cell scraper (Costar).

Generation of Mφ1 and Mφ2.

Generation of Mφ1 and Mφ2 from human peripheral blood monocytes was performed using the methods described previously [8]. Briefly, Mφ1 and Mφ2 were generated from $CD14^+$ monocytes in RPMI culture medium in the presence of 5 ng/ml GM-CSF (Leucomax, Novartis Pharma BV, Arnhem, the Netherlands) and 5 ng/ml M-CSF (R&D systems / ITK Diagnostics, Uithoorn, the Netherlands), respectively, for 6 days.

Analysis of cell surface molecules by flow cytometry.

The following mAbs were used for flow cytometry analysis to analyze the surface molecules on different Mφ: anti-DC-SIGN/CD209 (AZN-D1, a gift of Dr. Y. van Kooyk, VU Medical Center, Amsterdam, the Netherlands), anti-CD86 (IT2.2; Pharmingen, San Diego, USA), anti-CD16 (3G8, a gift from Dr. J.G.J. van de Winkel, University of Utrecht, the Netherlands), and anti-CD163 (EDhu1, [14]). Staining was visualized by PE-conjugated goat anti-mouse Ig (Dako, Glostrup, Denmark), and compared to appropriate isotype controls. PE-conjugated anti-CD14 (Leu-M3), and PE-conjugated anti-CD11b/Mac-1 were purchased from BD Biosciences (San Jose, CA). Cells were analyzed using FACSCalibur and CellQuest software (BD Biosciences). Dead cells, identified by propidium iodide (PI) uptake, were excluded from analysis.

Phagocytosis assay.

Phagocytosis of early or late apoptotic cells was assessed using a protocol described previously [8]. Briefly, fluorescently labeled with carboxyfluorescein diacetate succinamidyl ester (CFSE, Molecular Probes, Leiden, the Netherlands) labeled Jurkat cells were rendered into early (Annexin V^+ /PI^-, routinely ± 70%). and late apoptosis (Annexin V^+ /PI^+, routinely ± 95%). by irradiating them with ultra violet (UV)-C light (Philips TUV lamp, predominantly 254 nm) at a dose of 50 J/m^2 and then cultured for 3 and 28 hours in serum-free RPMI medium, respectively. Labeled early or late apoptotic cells (1×10^5) were co-cultured with pMφ in 1:1 ratio at 37°C or 4°C for 0.5 h in 100 μl RPMI culture medium in round-bottom glass tubes. pMφ were stained with a PE-conjugated mAb against CD11b and uptake was analyzed by a two-color flow cytometry. Phagocytosis of early apoptotic cells by pMφ was further investigated by confocal laser scanning microscopy with a LSM 510 (Carl Zeiss AG), as described

previously [8]. pMφ were stained with mAb against CD11b or CD163 followed by Alexa red-conjugated goat-anti-mouse Abs (Molecular Probes) .

Allogeneic mixed lymphocyte reaction.

Responder T cells used for allogeneic mixed lymphocytes reaction (MLR) assays were isolated by sheep erythrocyte rosetting of mononuclear cells obtained from healthy donors. Stimulator cells, i.e. Mφ1, Mφ2 and pMφ, were first cultured with or without 200 ng/ml lipopolysaccharide (LPS, Salmonella Typhosa, Sigma-Aldrich) for 24 h. Prior to MLR, stimulator cells were irradiated (50 Gy) and added in graded doses to 1.5×10^5 allogeneic T cells in 96-well round-bottom tissue culture plates in RPMI culture medium. Cell proliferation was quantified by incubating the cells during the last 8 h of the 6-day cultures with 1 μCi (37 kBq) of [methyl-^{3}H]thymidine (NEN, Boston, MA). Results are presented as the mean cpm ± SD obtained from triplicate cultures.

Cytokine detection.

Mφ1, Mφ2 and pMφ (2×10^5) were stimulated with or without 200 ng/ml LPS for 24 h in 48-well-plate and supernatants were harvested. Cytokines were quantified in the supernatants using ELISA. The measurements of IL-6 and IL-10 were performed as described previously [8]. The supernatants harvested from MLR were measured for interferon-γ (IFN-γ), according the method described before [15].

Statistical analysis.

Statistical analysis was performed by two-way ANOVA or one sample *t* test using GraphPad Prism (GraphPad software, San Diego, CA). Differences were considered statistically significant when p values were less than 0.05.

Results and discussion

pMφ share similar phenotype with Mφ2

The purity of pMφ isolated from the dialysate effluents of PD patients was determined by analysis of morphology and flow cytometry for surface markers. These cells showed typical morphology for monocytes /Mφ (not shown). Flow cytometric analysis revealed a phenotype for human Mφ, i.e. positive for CD14 and CD11b, but negative for DC-SIGN, a marker for DCs (Fig. 1A). The major contaminating cells are $CD3^+$ T lymphocytes which represented approximately 40% of total peritoneal cells before removing non-adherent cells and remained 5-15% thereafter (data not shown).

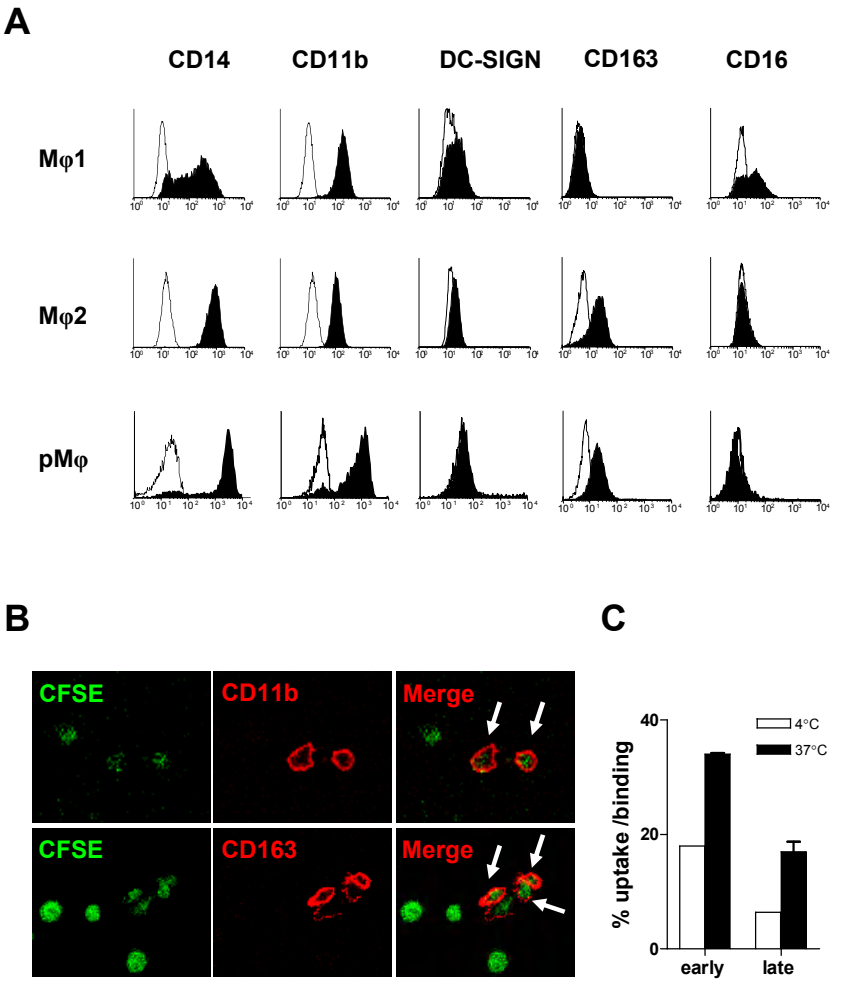

Figure 1. Characterization of Mφ1, Mφ2 and pMφ. Mφ1 and Mφ2 were generated in parallel from the same healthy donor cultured for 6 days. pMφ were freshly isolated from dialysate effluents from PD patients. Surface expression (closed histograms) was determined by flow cytometry. Open histograms represent matched isotype controls. (A) Surface expression of CD14, CD11b, DC-SIGN, CD163 and CD16 (FcγRIII) on Mφ1, Mφ2 and pMφ. Data are representative of at least 3 independent experiments using separate unrelated donors. (B) Confocal microscopy images show the uptake of early apoptotic cells by pMφ (see arrows). Red cells represent the CD11b- or CD163-positive pMφ and green ones are the CFSE-labeled apoptotic cells. (C) The uptake of early and late apoptoic cells was measured by flow cytometry. The percentage of uptake and binding (at 37°C) or binding (at 4°C) were calculated as 100% × $((CD11b^+CFSE^+)/CD11b^+)$. Data are representative of 3 independent experiments. $p<0.01$, one sample *t* test.

Initially, to distinguish Mφ1 and Mφ2, we analyzed an array of surface makers, and found that CD163, a cell surface glycoprotein belonging to the group B cysteine-

rich scavenger receptor family, is exclusively expressed by Mφ2, but not by Mφ1 (Fig. 1A). In contrast, CD16 (Fcγ receptor III) is expressed by Mφ1 or subsets of Mφ1, but not by Mφ2. pMφ are characterized as $CD163^{+}CD16^{-}$, sharing this phenotype with Mφ2 (Fig. 1B). CD163 has been shown to be associated with an anti-inflammatory Mφ phenotype, either alternative activated Mφ or those tissue Mφ isolated from patients with granulomatous disease [16]. Our finding that Mφ2 express CD163 confirms the association of CD163 with anti-inflammatory activities. The underling pathology of the patients used for analysis was diverse, including renal vascular disease, diabetes, hypertension, cystic nephritis and systemic lupus erythematosus. Therefore we feel that it is unlikely that the Mφ2 phenotype of peritoneal macrophages can be attributed to a specific underlying pathology.

pMφ have high capacity for phagocytosis, endocytosis and macropinocytosis

An important functional characteristic of Mφ2 is that they have a higher capacity for antigen uptake, in particular that of early apoptotic cells, as compared to Mφ1 and DCs [8]. Therefore we assessed the capacity for uptake of early and late apoptotic cells by pMφ directly after isolation from dialysate effluents. Uptake of early apoptotic cells was confirmed by confocal microscopy, showing that $CD11b^{+}$ and $CD163^{+}$ pMφ had ingested CFSE-labeled early apoptotic cells (Fig. 1B). Quantification of uptake by flow cytometry shows that pMφ were capable to bind early or late apoptotic cells at 4°C and to engulf them at 37°C (Fig 2B). Importantly, like Mφ2 [8], pMφ exhibited a superior capacity for the binding and uptake of early apoptotic cells, compared with late apoptotic cells (mean fold difference: 1.87±0.17, $p<0.01$, ANOVA) (Fig 1C). Furthermore, pMφ were active in lectin-mediated uptake of $Dextran^{FITC}$ and showed strong macropinocytosis-mediated uptake of luficer yellow (data not shown), which resembles Mφ2. Collectively, these data suggest that pMφ share their strong phagocytic properties with Mφ2.

pMφ produce high amount of IL-10, as do Mφ2

The anti-inflammatory Mφ2 are characterized by high IL-10 production, whereas the pro-inflammatory cytokines such as IL-6, TNF-α are hardly produced [7,8]. We investigated whether freshly isolated pMφ also predominantly produce anti-inflammatory cytokines. Mφ2, but not Mφ1, produced high amounts of IL-10 upon LPS stimulation (ANOVA, $p<0.01$) (Fig. 2A left panel). pMφ produced high amounts of IL-10, and LPS stimulated pMφ to produce significantly more IL-10 (mean fold increase: 5.19 ± 4.67, $p<0.05$, one sample *t* test) (Fig. 2A right panel).

Unlike the Mφ2 which produce little or no IL-6 (Fig. 2B left pannel) [8], pMφ produced considerable amounts of IL-6 after LPS stimulation (ANOVA, $p<0.01$) (Fig. 3D).

However, LPS was unable to induce a further significant increase in IL-6 by these pMφ (mean fold increase: 1.47 ± 0.88, p>0.05) (Fig. 2B right panel). IL-6 production by pMφ has been documented before [17] and this was dramatically elevated in pMφ from peritonitis patients [18]. These data suggest that pMφ may perhaps not be as polarized as *in vitro*-generated Mφ2, but, nevertheless, they do have the high IL-10 producing capacity of typical of Mφ2, suggesting a potential anti-inflammatory activity.

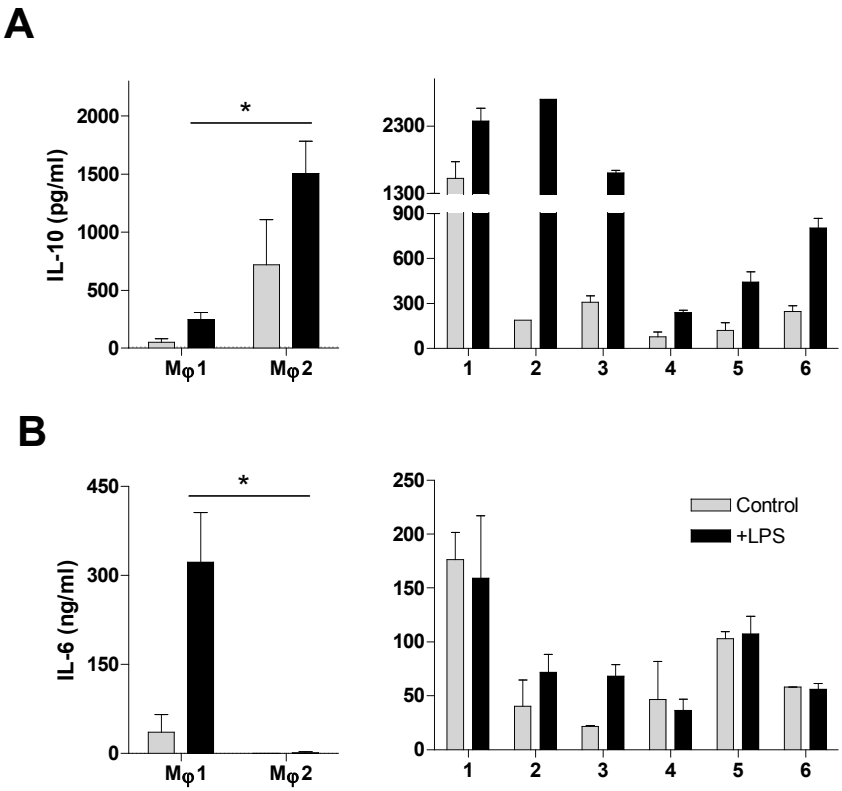

Figure 2. Cytokine production by pMφ after LPS stimulation. Isolated pMφ (2×10^5) were immediately cultured with or without LPS (200 ng/ml) for 24 h in RPMI culture medium. In parallel, Mφ1 and Mφ2 were stimulated by LPS in the same way. Supernatants were harvested and measured by ELISA for IL-10 (A) and IL-6 (B) of Mφ1 and Mφ2 and pMφ. Data shown (Mφ1 and Mφ2) are mean ± SD from 4 independent experiments. *, p<0.01, ANOVA. For pMφ, data shown are from 6 separate unrelated donors. Data represent the mean ± SD from duplicate cultures.

Mφ2 and pMφ show hampered T cell stimulatory capacity

We next investigated the expression of costimulatory molecules among these Mφ. Basal levels of CD80 and CD86 were comparable for all three Mφ populations.

LPS stimulation increased CD80 and CD86 expression on Mφ1 but failed to do so on Mφ2 (Fig. 3A). Instead, a significant down-regulation of CD86 was observed on both Mφ2 and pMφ upon LPS stimulation (Fig. 3B, $p<0.01$, one sample *t* test). In line with this, in an allogeneic MLR, activation of Mφ1 with LPS resulted in a strongly increased T cell stimulatory capacity (mean of fold increase: 1.32 ±0.35, $p<0.01$, one sample *t* test) (Fig. 3C). In contrast, LPS activation of both Mφ2 and pMφ resulted in a hampered T cell proliferation (mean of fold increase for Mφ2: 0.90 ±0.42, $p<0.01$, and for pMφ: 0.88 ±0.13, $p<0.01$) (Fig. 3C). Measurement of IFN-γ production by activated T cells showed induction of IFN-γ by Mφ1 and pMφ, but not by Mφ2 (Fig. 3D). However, similar to T cell proliferative responses, LPS activation of Mφ1 increased IFN-γ production (mean fold increase: 2.83, $p<0.01$), whereas LPS activation of pMφ decreased the IFN-γ response (mean fold increase: 0.42, $p=0.012$) (Fig. 3D). Taken together, Mφ2 and pMφ showed a similar pattern to activate allogeneic T cells, in particular with respect to the reduced T cell stimulatory capacity upon LPS exposure.

The antigen presentation activities of pMφ have been studied earlier, showing that pMφ are competent antigen presenting cells [19,20]. Our data confirm that pMφ can stimulate allogeneic T cell proliferation. However, our finding that LPS stimulation hampered the T cell stimulatory capacity and inhibited IFN-γ production by pMφ provides new insight in this type of resident Mφ. It has been well accepted that both *in vitro* and *in vivo* Mφ are heterogeneous in immune activities, i.e. activating versus suppressing [10]. Our data suggests that pMφ might be suppressor Mφ with a role in the maintenance of peripheral tolerance upon scavenging self antigen derived from for instance apoptotic cells. Indeed, the anti-inflammatory and immune suppressive capacities of resident Mφ were also found in other tissue Mφ, such as murine alveolar Mφ [21,22] and human intestinal Mφ [23].

Concluding remarks

In conclusion, we show that human pMφ display a strong resemblance to *in vitro*-generated anti-inflammatory Mφ2, thereby representing an in vivo counterpart of Mφ2. These $CD163^+$ pMφ have similar capacities as Mφ2 for endocytosis, macropinocytosis, and a superior uptake of early apoptotic cells compared to the uptake of late apoptotic cells. We suggest that human resident pMφ are a subset of the primary phagocytes involved in the silent clearance of apoptotic cells. Furthermore, stimulated pMφ show a hampered capacity in allogeneic T cell proliferation and induce inhibition of Th1 cytokines. Thus we propose that pMφ have the potential to maintain an anti-inflammatory condition in the peritoneal cavity.

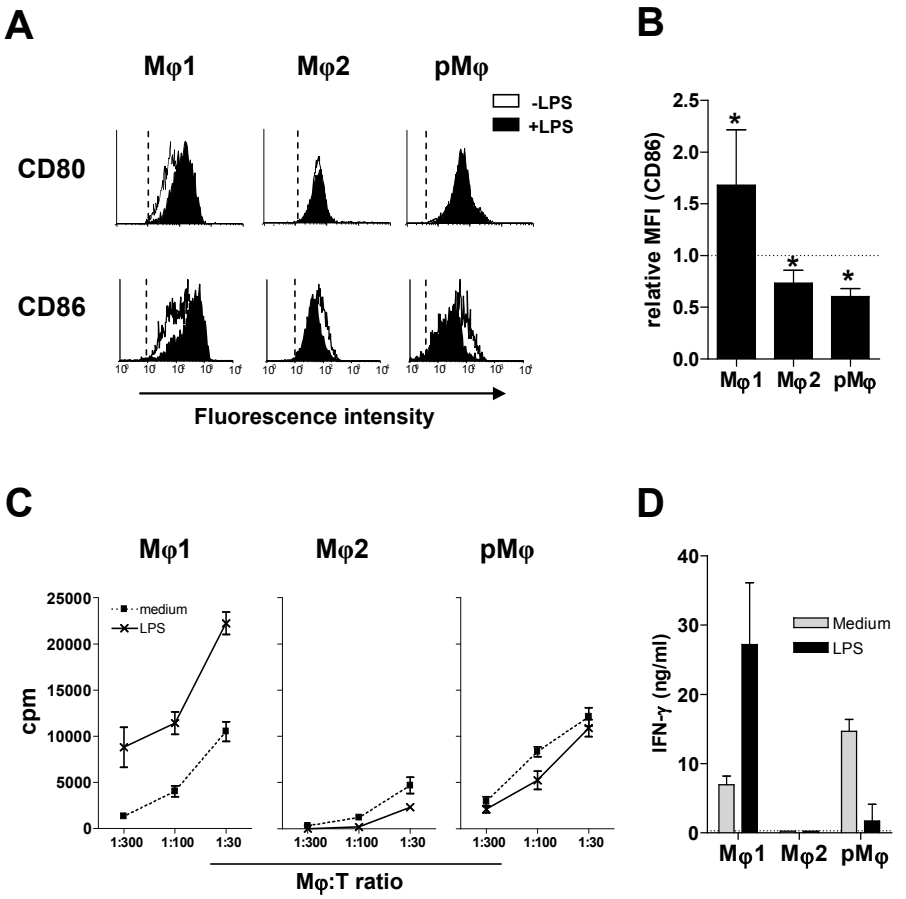

Figure 3. T cell stimulatory capacity of Mφ1, Mφ2 and pMφ. (A) CD80 and CD86 expression was determined by flow cytometry on cells with (closed histograms) and without LPS stimulation (open histograms) for 24 h. Dashed line represents the matched isotype control. (B) Relative expression of CD86 on Mφ1, Mφ2 and pMφ after LPS stimulation compared to the ones without stimulation. Data are the mean ± SD of 3 independent experiments using 3 unrelated donors. *, $p<0.01$, one sample *t* test. (C) Irradiated Mφ1, Mφ2 and pMφ were added in graded dose to 1.5×10^5 allogeneic T cells. T cell proliferation was quantified by incubating cells during the last 8 h of 6-day cultures with [methyl-^{3}H]thymidine. (D) Supernatants of MLR (Mφ:T ratio at 1:10) were harvested and measured by ELISA for IFN-γ. Dashed line indicates the detection limit for ELISA. Data are mean of triplicate cultures and representative of at least 4 independent experiments.

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5

Reversible differentiation of pro- and anti-inflammatory macrophages

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Summary

Macrophages (Mφ) represent dynamic cell populations that develop according to the nature of environmental signals. We and others have recently shown that Mφ can be polarized *in vitro* into pro-inflammatory (Mφ1) and anti-inflammatory cells (Mφ2) by the lineage-determining factors GM-CSF and M-CSF, respectively. Here we show that polarized Mφ1 and Mφ2 are not an end stage of differentiation and are able to reversibly undergo functional re-differentiation into anti-inflammatory and pro-inflammatory Mφ. GM-CSF-driven Mφ1 exposed to M-CSF for 6 days obtained a Mφ2-like phenotype, inhibited the production of pro-inflammatory cytokine IL-6 and TNF-α, and exhibited a reduced T cell stimulatory capacity. *Vice versa*, Mφ2 exposed to GM-CSF exhibited a Mφ1-like phenotype with significant lower production of anti-inflammatory cytokine IL-10 and a higher T cell stimulatory activity, and a decreased capacity for phagocytosis of early apoptotic cells. Our data suggest that polarized macrophages are flexible in modulating their immune functions upon environmental changes, i.e., steady-state versus inflammatory conditions. These observations are important for our understanding of the regulatory role of macrophages in tissue homeostasis and disease pathogenesis.

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Introduction

Macrophages (Mφ), as one of the professional antigen presenting cells with phagocytic capacity, play an essential role in homeostasis as well as in innate and acquired immunity, and as such may be implicated in autoimmunity, inflammation, and immunopathology [1]. In a normal adult, resident tissue Mφ are derived from circulating bone marrow–derived monocytes, and are largely heterogeneous [1,2]. Classically, granulocyte/macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) are the primary growth factors for the differentiation of macrophages from monocytes [3]. Among those two, M-CSF is the only primary Mφ growth factor that is detectable in peripheral blood under steady-state conditions [4]. In contrast, GM-CSF is a pro-inflammatory cytokine, mostly generated during inflammation and hardly detectable in circulation [5]. Compatible with this reasoning, t has been shown that *op/op* mice lacking M-CSF develop a profound macrophage deficiency [6], whereas GM-CSF knockout mice do not show major deficiency of Mφ, except that the Mφ are smaller than normal [7,8], confirming that M-CSF is a crucial growth factor in Mφ differentiation. Thus under steady-state conditions, M-CSF could be the default cytokine in driving Mφ differentiation.

We and others have recently shown that Mφ can be polarized *in vitro* into pro-inflammatory (Mφ1) and anti-inflammatory cells (Mφ2) by GM-CSF and M-CSF (also termed CSF-1), respectively [9-11]. Importantly, several resident tissue Mφ, including alveolar, intestinal and peritoneal Mφ, display anti-inflammatory properties [12-15]. It is tempting to hypothesize that under inflammatory conditions, anti-inflammatory Mφ may undergo functional adaptation when GM-CSF is produced locally. Therefore, in the current study we investigated whether polarized Mφ1 and Mφ2 can be re-differentiated into Mφ2- and Mφ1-like cells when exposed to M-CSF and GM-CSF, respectively. We found that both Mφ1 and Mφ2 can undergo reversibly functional changes, i.e., exposure of Mφ1 to M-CSF resulted in re-differentiation of these cells into Mφ2-like cells, and *vice versa*. Our data reveal the importance of the local cytokine environment in driving Mφ polarization and provide additional options for the modulation of Mφ and therapeutic targeting.

Materials and Methods

Generation of monocyte-derived Mφ1 and Mφ2.

Human mononuclear cells were isolated from buffy-coats obtained from healthy donors using Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO), followed by anti-CD14 microbeads magnetic cell sorting, according to the manufacturer's instruction (Miltenybiotec/CLB, Amsterdam, the Netherlands). Two types of macrophages, namely Mφ1 and Mφ2, were generated in 6-well culture plates (Costar, Cambridge, MA) in RPMI culture medium (RPMI 1640 containing 10% heat-inactivated FCS, 90 U/ml

penicillin and 90 μg/ml streptomycin) (all from Gibco/Life technologies, Breda, the Netherlands) for 6 days supplemented with 5 ng/ml GM-CSF (Leucomax, Novartis Pharma BV, Arnhem, the Netherlands) and 5 ng/ml M-CSF (R&D systems / ITK Diagnostics, Uithoorn, the Netherlands), respectively, as previously described [16]. In all experiments, these two types of Mφ were generated in parallel from monocytes of the same donor. For re-differentiation, day-6 Mφ1 were cultured for another 6 days in the presence of GM-CSF (named as $M\varphi1_{GM}$) or M-CSF (named as $M\varphi1_{M}$). Similarly, day-6 Mφ2 were cultured for another 6 days in the presence of GM-CSF (named as $M\varphi2_{GM}$) or M-CSF (named as $M\varphi2_{M}$).

Analysis of cell surface markers by flow cytometry.

Cells were harvested and washed in buffer containing 1% BSA, 1% heat-inactivated normal human serum, and 0.02% NaN_3. The following mAbs were used for flow cytometric analysis to detect expression of certain surface molecules: PE-conjugated anti-CD14 (Leu-M3), mAb of anti-mannose receptor (MR) /CD206 (D547.3, a gift of F. Koning, LUMC, Leiden, the Netherlands) and anti-CD163 (EDhu1, a gift of Dr. T.K. van den Berg, Department of Molecular Cell Biology and Immunology, VU Medical Center, Amsterdam). Expression was visualized by PE-conjugated goat anti-mouse Ig (Dako, Glostrup, Denmark) using appropriate isotype controls. Cells were analyzed using FACSCalibur and CellQuest software (BD Biosciences). Dead cells, identified by propidium iodide (PI) uptake, were excluded from analysis.

Detection of cytokines.

Day-6 Mφ1 and Mφ2 cultured with either GM-CSF or M-CSF for another 6 days were stimulated with 200 ng/ml lipopolysaccharide (LPS, Salmonella Typhosa, Sigma-Aldrich) for 24 h and the supernatants were harvested for quantification of cytokine release by ELISA. In some experiments, Mφ1 and Mφ2 were cultured with GM-CSF or M-CSF for only one additional day and supernatants were collected. The measurements of IL-6, TNF-α and IL-10 were performed as described before [16,17].

Phagocytosis assay.

Early apoptosis of Jurkat T cells was induced by ultra violet (UV)-C light (Philips TUV lamp, predominantly 254 nm) at a dose of 50 J/m^2, and followed by culture for 3 hours in serum-free RPMI medium. Prior to the induction of apoptosis, Jurkat cells were fluorescently labeled with carboxyfluorescein diacetate succinamidyl ester (CFSE, Molecular Probes, Leiden, the Netherlands), according to a previously described method [18]. Early apoptosis was established by two-color flow cytometry as positive for annexin V but negative for PI. Routinely, about 60% of early apoptotic cells were obtained. For the phagocytosis assay, CFSE-labeled apoptotic cells (1×10^5) were co-cultured with different Mφ at 1:1 ratio 0.5 h at 37°C or 4°C in 100 μl RPMI culture medium in round-bottom glass tubes followed by staining with a PE-conjugated mAb against CD11b. The uptake was analyzed by flow cytometry. The percentage of CD11b-positive cells that stained positive for CFSE was used as a measure for the percentage of Mφ that had ingested (37°C) and/or bound (4°C) apoptotic cells.

Allogeneic mixed lymphocyte reaction.

An allogeneic mixed lymphocyte reaction (MLR) assay was performed as described previously [15]. Briefly, responder T cells were isolated by sheep erythrocyte rosetting of mononuclear cells that were obtained from healthy donors. Stimulator cells, i.e. different Mφ were first irradiated (50 Gy) and then added in graded doses to 1.5×10^5 allogeneic T cells in 96-well round-bottom tissue culture plates in RPMI culture medium. T cell proliferation was quantified by incubation of the cells with 1 µCi (37 kBq) of [methyl-^{3}H]thymidine (NEN, Boston, MA) during the last 8 h of the 6-day cultures. Results are presented as the mean cpm ± SD obtained from triplicate cultures.

Statistical analysis.

Statistical analysis was performed by one sample *t* test or Mann-Whitney U using GraphPad Prism (GraphPad software, San Diego, CA). Differences were considered statistically significant when p values were less than 0.05.

Results

Morphology of re-differentiated Mφ1 and Mφ2.

Mφ1 and Mφ2 were polarized in parallel from peripheral blood monocytes derived from the same donor by GM-CSF and M-CSF, respectively. After 6 days of differentiation, Mφ1 became adherent and mostly showed a “fried-egg” morphology, whereas Mφ2 were less-adherent with irregular shapes as compared to Mφ1 (Fig. 1A). Day-6 Mφ1 that were cultured further in GM-CSF for another 6 days ($M\varphi1_{GM}$) retained their “fried-egg” morphology. Similarly, Mφ2 cultured in M-CSF for additional 6 days ($M\varphi2_M$) retained their Mφ2 morphology (Fig. 1A). By exposure of Mφ1 to M-CSF for 6 days, $M\varphi1_M$ did not show obvious morphological changes. However, culturing of Mφ2 in GM-CSF for another 6 days ($M\varphi2_{GM}$) completely rendered them into Mφ1-like cells, i.e. most of the cells became adherent and showed “fried-egg” morphology (Fig 1A).

To exclude that the observed changes were caused by differential survival of different Mφ subsets during the prolonged cultures, we determined the viable cell counts. Counts of viable cells by exclusion of PI staining by flow cytometric analysis were related to total numbers of monocyte /Mφ harvested. We observed that under all conditions, cell survival was between 79% and 94% (Fig 1B).

Phenotypes of re-differentiated Mφ1 and Mφ2.

Polarized Mφ1 were previously shown to express low levels of CD14 as compared with Mφ2, and had no detectable expression of CD163, whereas Mφ2 expressed

CD163 and high level of CD14 [11,15]. $M\varphi1_{GM}$ conserved their phenotype, i.e. $CD14^{low}CD163^{-}$, whereas $M\varphi1_{M}$ showed significantly increased expression of CD14 and CD163 (p=0.01, Mann-Whitney U) (Fig 2A, B). In contrast, $M\varphi2_{GM}$ showed no major phenotypical changes as compared with $M\varphi2_{M}$ (Fig 2A, B). MR expression on both cells was not significantly influenced by additional cultures (Fig. 2A).

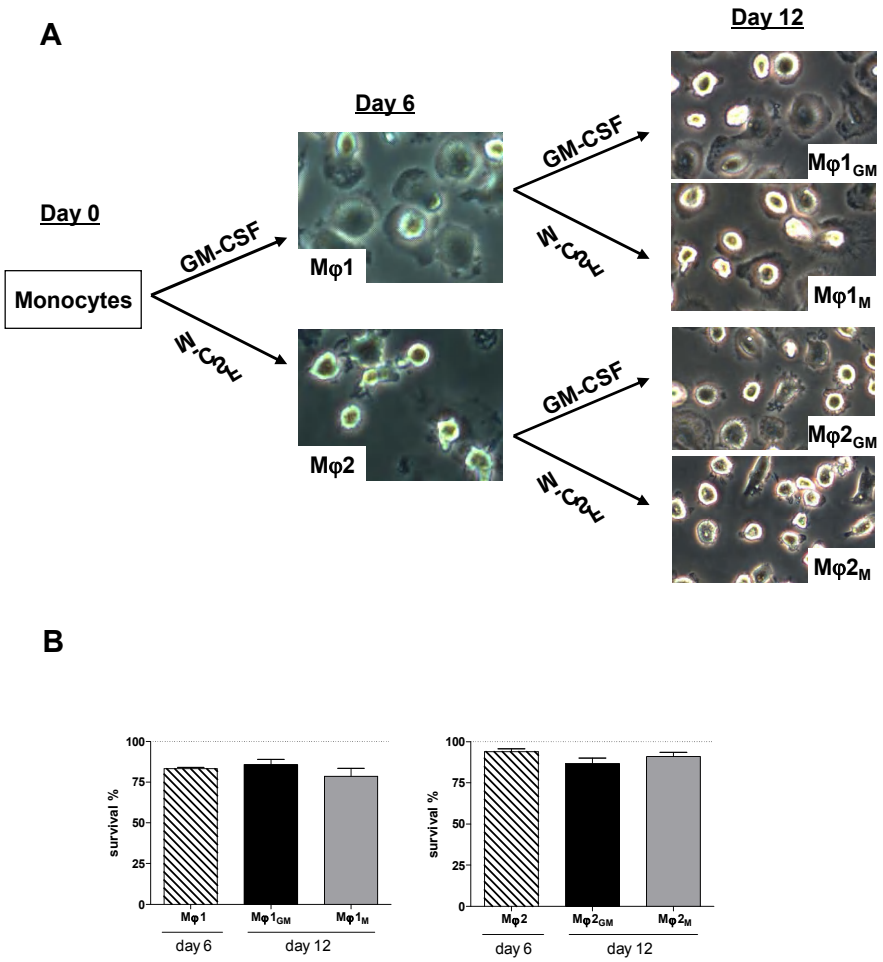

Figure 1. Morphology of Mφ1, Mφ2. Mφ1 and Mφ2 were generated in parallel from the same healthy donor following culture of monocytes for 6 days, and then cultured with GM-CSF ($M\varphi1_{GM}$ or $M\varphi2_{GM}$) or M-CSF ($M\varphi1_{M}$ or $M\varphi2_{M}$) for additional 6 days. (A) Pictures show the morphology of cells at day 6 and day 12. Images were obtained using an Axiovert 25 inverted microscope (Carl Zeiss, Sliedrecht, The Netherlands) with a 20 x /0.3 NA objective and Zeiss Axiovision software version 3.1. Magnification, x 200. (B) After harvesting from day 6 or day 12 cultures, cells were stained with PI for the measurement of cell survival by flow cytometry.

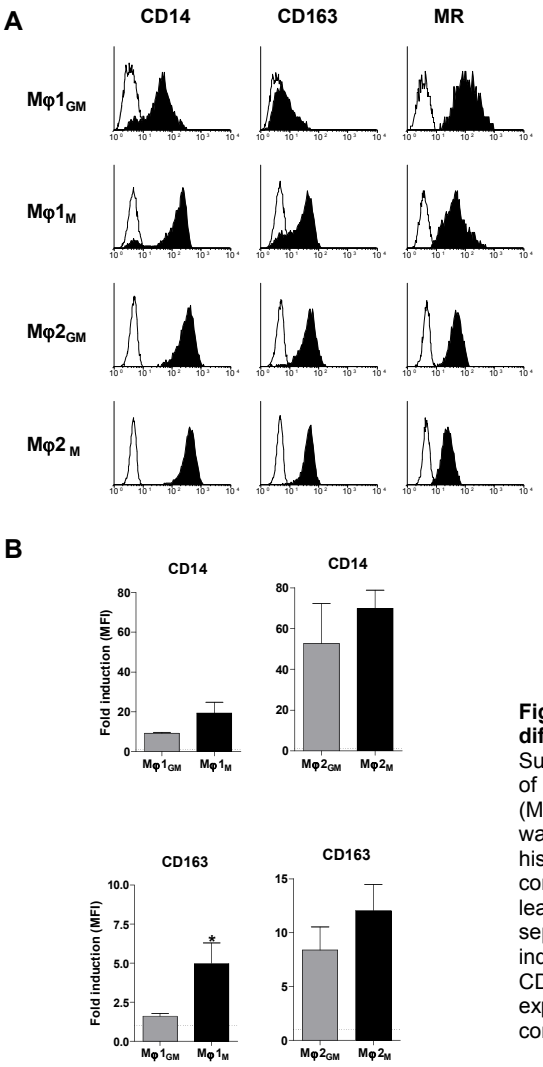

Figure 2. Phenotypes of re-differentiated Mφ1 and Mφ2. (A) Surface expression (closed histograms) of CD14, CD163 and mannose receptor (MR) on $M\varphi1_{GM}$, $M\varphi1_{M}$, $M\varphi2_{GM}$ and $M\varphi2_{M}$ was determined by flow cytometry. Open histograms represent matched isotype controls. Data are representative of at least 3 independent experiments using separate unrelated donors. (B) Fold induction of expression of CD14 and CD163 were calculated MFI of expression divided by MFI of isotype control. *, $p<0.01$, Mann-Whitney U.

Functional reversal in cytokine production of re-differentiated Mφ1 and Mφ2.

We and others have previously shown that Mφ1 are pro-inflammatory cells that produce pro-inflammatory cytokines such as IL-6, IL-23 and TNF-α, whereas Mφ2 have an anti-inflammatory profile as documented by a large production of IL-10 but complete absence of IL-6 and TNF-α [10,11,16]. In the absence of LPS activation, non of the Mφ populations produced IL-6 (Fig. 3A) or TNF-α (Fig. 3B). Upon LPS activation, $M\varphi1_{GM}$ retained their capacity to produce IL-6 and TNF-α. Importantly, under the same conditions, $M\varphi1_{M}$ completely lost their capacity to produce IL-6 and TNF-α. Like Mφ2, $M\varphi2_{M}$ are unable to produce IL-6 or TNF-α upon LPS activation.

However, $M\varphi 2_{GM}$ gained the capacity to produce IL-6 and TNF-α although this production was lower than production by $M\varphi 1_{GM}$.

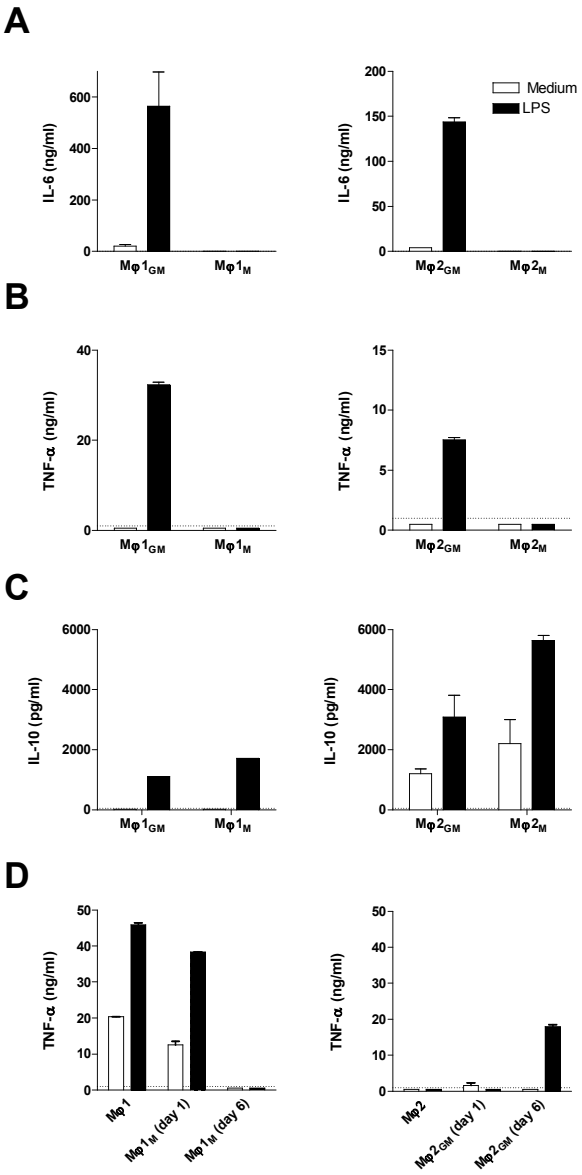

Figure 3. Cytokine production by re-differentiated Mφ1 and Mφ2. Mφ1 and Mφ2 were cultured with GM-CSF or M-CSF for another 6 days. After harvesting, cells were stimulated with or without LPS (200 ng/ml) for 24 h in 48-well plate. Supernatants were harvested and measured by ELISA for IL-6 (A), TNF-α (B) and IL-10 (C). (D) TNF-α production of Mφ1 and Mφ2 cultured with GM-CSF or M-CSF for additional 1 or 6 days. Data shown are mean ± SD of duplicate cultures and represent 4 independent experiments.

We next measured the production of IL-10. Compatible with Mφ1 and Mφ2 data [10,16], $M\varphi 2_M$ produced high levels of IL-10 compared to $M\varphi 1_{GM}$. In all five independent experiments, $M\varphi 2_{GM}$ produced significantly lower levels of IL-10 as

compared with $M\varphi 2_{GM}$ ($p<0.001$, two way ANOVA).However, we found only minor effects when $M\varphi 1$ were exposed to M-CSF as compared with GM-CSF ($p=0.28$).

To rule out the possibility that difference in cytokine production were a direct consequence of the presence of GM-CSF or M-CSF, we performed kinetic experiments. $M\varphi 1$ cultured with M-CSF for one day did not reverse TNF-α production, whereas an inhibition of TNF-α production was observed in 6-days culture of $M\varphi 1_M$ (Fig. 3D). Similarly, TNF-α production by $M\varphi 2$ was only induced when GM-CSF was given for 6 days but not 1 day (Fig. 3D). Together, these data suggest that upon changes of growth factors, pro-inflammatory $M\varphi 1$ and anti-inflammatory $M\varphi 2$ can be re-differentiated into anti-inflammatory and pro-inflammatory $M\varphi$, respectively.

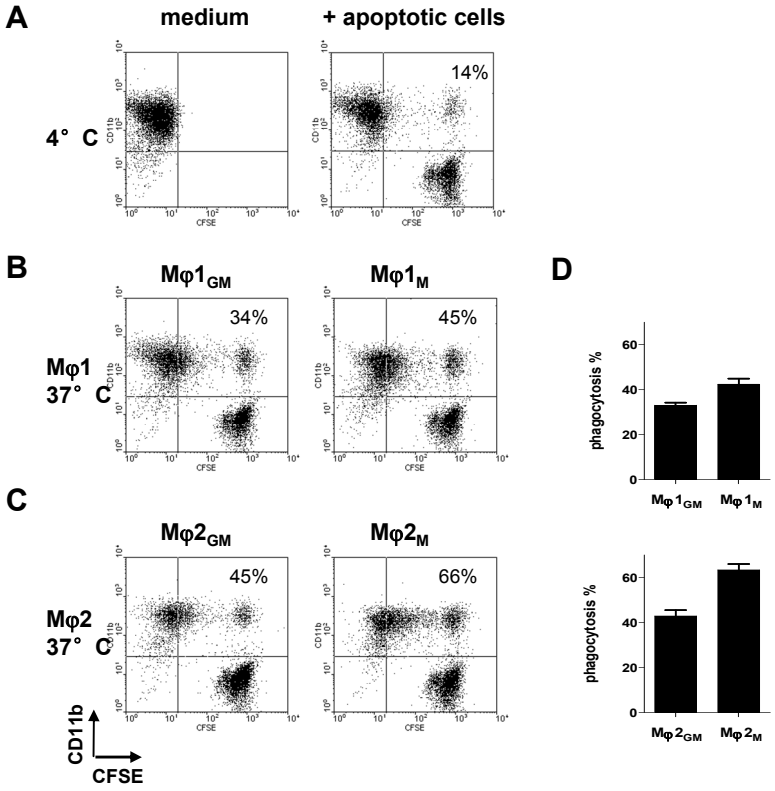

Figure 4. Phagocytosis of early apoptotic cells by re-differentiated Mφ1 and Mφ2. CFSE-labeled Jurkat T cells were induced into early apoptosis by treating cells with UV-C light at a dose of 50 J/m^2 and cultured in serum-free RPMI medium for another 3 h. Early apoptotic cells (1×10^5 cells) were co-incubated with $M\varphi 1_{GM}$, $M\varphi 1_M$, $M\varphi 2_{GM}$ or $M\varphi 2_M$ at 1:1 ratio for 0.5 h at 37°C or 4°C. Prior to flow cytometric analysis, cells were stained with PE-conjugated mAb against CD11b. (A) shows binding of early apoptotic cells to Mφ at 4°C. CD11b+CFSE$^+$ cells were Mφ1 (B) or Mφ2 (C) that have taken up early apoptotic cells at 37°C. (D) Quantification of uptake (at 37°C) was calculated as 100% × ((CD11b$^+$CFSE$^+$)/CD11b$^+$). Data indicate the mean ± SEM of 2 independent experiments where duplicated cultures were performed.

Phagocytosis of early apoptotic cells by re-differentiated Mφ1 and Mφ2.

One important functional difference between Mφ1 and Mφ2 is that Mφ2 is superior in phagocytosis of early apoptotic cells, as compared with Mφ1 [16]. We therefore examined whether by switching growth factors, Mφ1 and Mφ2 can change their capacity to phagocytose early apoptotic cells. As a control, co-incubation was performed at 4°C to measure the binding of apoptotic cells to Mφ (Fig. 4A), whereas the phagocytosis assay was performed at 37°C (Fig. 4B, C) to allow active ingestion. $M\varphi1_M$ slightly increased their capacity to take up early apoptotic cells, as compared with $M\varphi1_{GM}$ (Fig. 4B, D). In contrast, $M\varphi2_{GM}$ decreased their capacity to take up early apoptotic cells, as compared with $M\varphi2_M$ (Fig. 4C, D).

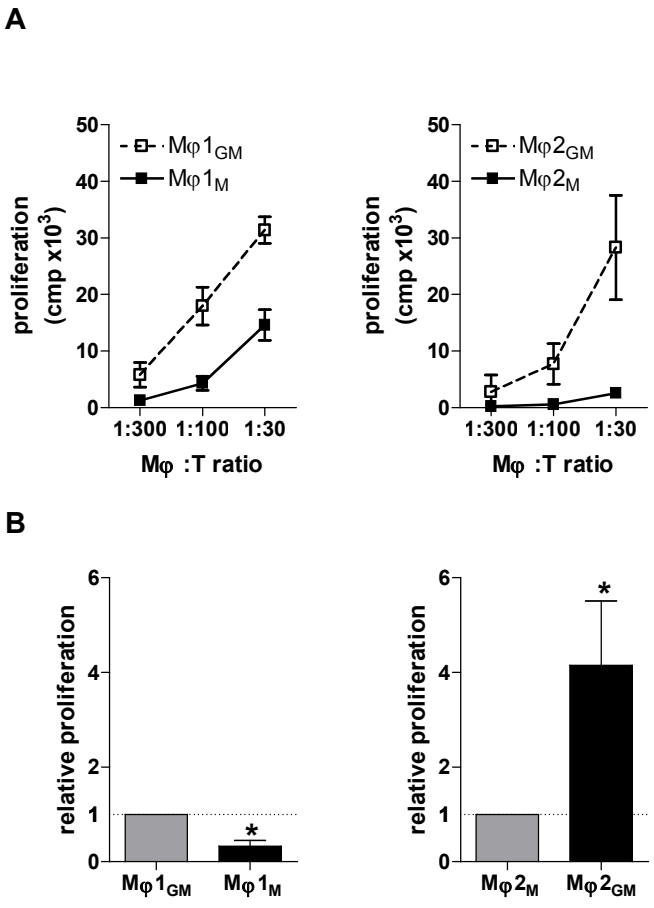

Figure 5. T cell stimulatory capacity of re-differentiated Mφ1 and Mφ2. (A) Irradiated $M\varphi1_{GM}$, $M\varphi1_M$, $M\varphi2_{GM}$ or $M\varphi2_M$ were added in graded dose to 1.5×10^5 allogeneic T cells. T cell proliferation was quantified by incubating cells during the last 8 h of 6-day cultures with [methyl-^{3}H]thymidine. Data show mean ± SD of triplicate cultures, and represent 5 independent experiments. (B) Relative proliferation was calculated as cmp (Mφ:T ratio at 1:100) of $M\varphi1_M$ against those cultured with $M\varphi1_{GM}$, and $M\varphi2_{GM}$ against that $M\varphi2_M$. Dashed line indicates the relative proliferation as 1. Data are mean ± SEM of 5 independent experiments where triplicate cultures were obtained. *, $p<0.01$, one sample *t* test.

T cell stimulatory capacity of re-differentiated Mφ1 and Mφ2.

We have recently shown that Mφ2 exhibit a lower capacity to stimulate allogeneic T cell proliferation, as compared with Mφ1 [15]. Therefore we investigated whether re-differentiated Mφ1 and Mφ2 undergo a conversion in their T cell stimulatory capacity in an allogeneic mixed lymphocyte reaction. $M\varphi1_M$ showed a significantly reduced capacity to stimulate T cell proliferation, as compared with $M\varphi1_{GM}$ (Fig. 5A, B). *Vice versa,* $M\varphi2_{GM}$ significantly increased their capacity to stimulate T cell proliferation, as compared to $M\varphi2_M$, which reached the same level of $M\varphi1_M$ to induce T cell proliferation (Fig. 5A, B).

Discussion

We demonstrate in the current study that polarized pro-inflammatory Mφ1 and anti-inflammatory Mφ2 are able to reversibly undergo functional re-differentiation into anti-inflammatory and pro-inflammatory Mφ when growth factors, i.e. GM-CSF and M-CSF are switched, respectively. This reversal was demonstrated at the level of phenotype, cytokine release, phagocytic capacity and T cells stimulatory capacity. Our data reveal the importance of growth factors in modulating Mφ plasticity, and provide important implications for therapeutic targeting of Mφ.

Mφ represent heterogeneous populations [2,19,20]. Mirroring the Th1/Th2 nomenclature, many researchers refer to classically activated Mφ by IFN-γ as Mφ1 and alternatively activated Mφ by IL-4 and/or IL-13 as Mφ2 [20]. Functional plasticity of Mφ has been documented for those classically and alternatively activated Mφ in human [21,22] and mice [23], showing that polarized Mφ were able to respond to an "opposing" stimulus. In our study, we have used GM-CSF and M-CSF for the generation of Mφ1 and Mφ2, resulting pro-inflammatory and anti-inflammatory Mφ respectively, according to a recent publication of Verreck *et al.* [10]. We realize that the M-CSF-driven Mφ2 might have some resemblance with alternatively activated macrophages by IL-4 and IL-13 [2] or type 2-activated macrophages in the mouse [24]. However alternatively activated macrophages express low CD14 and high MR [2], and mouse type 2-activated macrophages secret high level of TNF-α after stimulation [25]. These characteristics are different from human M-CSF polarized Mφ2, therefore we think that GM-CSF- and M-CSF-driven Mφ are distinct from previously clarified macrophage subsets. Indeed, when GM-CSF-driven Mφ1 or M-CSF-driven Mφ2 were stimulated with IFN-γ or IL-4 for 24 till 48 h, pro-inflammatory cytokine pattern such as IL-6 and TNF-α were not changed (data not shown).

GM-CSF and M-CSF (or CSF-1) are two growth factors which drive Mφ differentiation from monocytes [3,26]. From these, M-CSF is the only primary Mφ growth factor which is detectable in peripheral blood under steady-state conditions [4]. As a pro-inflammatory cytokine, GM-CSF is hardly detectable in circulation, and

much of the production and action of GM-CSF occurs locally at sites of inflammation [5]. For example, allergic patients with late-phase cutaneous reactions show markedly increased levels of GM-CSF mRNA in the skin [27]. Enhancement of GM-CSF levels in circulating are observed in response to endotoxin (LPS) [28]. Interestingly, GM-CSF can induce M-CSF production by monocytes [29,30]. Therefore, it is likely that under inflammatory conditions, both GM-CSF and M-CSF will be present and that this balance might impact the functional differentiation of Mφ. Our data provide insights on how these two growth factors interplay and modify the plasticity of Mφ for a desired immune reaction.

One of the characteristic functions of Mφ2 is that they preferentially recognize and ingest early apoptotic cells, leading to a non-inflammatory removal [16]. We have recently suggested that most resident Mφ such as peritoneal Mφ are anti-inflammatory cells that are the major phagocytes who clear early apoptotic cells [15]. Data in the current paper showed that the pro-inflammatory Mφ1 could acquire higher phagocytic capacity for early apoptotic cells once exposure to M-CSF, supporting the role of M-CSF in modulating phagocytosis of Mφ subsets. We hypothesize that *in vivo* recruitment of Mφ1-like cells locally to modify them into Mφ2-like cells is essential to ensure a silent clearance of apoptotic cells when overloaded apoptosis occurs.

We showed that exposure of pro-inflammatory Mφ1 to GM-CSF completely inhibited their production of IL-6 and TNF-α, and strongly enhanced their capacity to stimulate T cell proliferation, whereas treatment of anti-inflammatory Mφ2 with M-CSF induced their production of IL-6 and TNF-α, and reduced their capacity to stimulate T cell proliferation. In the case of acute inflammation, the early phase is dominated by pro-inflammatory and/or cytotoxic cells, whereas the terminal phase is dominated by anti-inflammatory /tissue regenerative cells [31]. Therefore, our data support the notion that during the due course of an inflammatory reaction, Mφ1 that sustain and stimulate inflammation may convert their function to participate in the healing phase of the reaction [32].

Emerging evidence show that tissue resident Mφ including alveolar [12,13], intestinal [14] and peritoneal Mφ [15] have anti-inflammatory properties. In chronic diseases such as tumors, Mφ have been suggested to have a dual role either in killing tumor or in promoting tumor survival [33]. Tumor associated Mφ acquire CD163 expression and have anti-inflammatory properties [34-36], which potentially promotes immune escape of tumor cells. Our data indicated that GM-CSF treatment on Mφ2 strongly increased their capacity to stimulate T cell proliferation.

In conclusion, *in vitro*-polarized GM-CSF-driven pro-inflammatory Mφ1 and M-CSF-driven pro-inflammatory Mφ2 can undergo functional re-differentiation upon exposure the opposing growth factor. Such plasticity of myeloid cells has been observed in other experimental models including a skewing from dendritic cells

(DCs) to Mφ upon exposure to interferon-gamma (IFN-γ) [37], or IL-6 and M-CSF [29,38], and a transdifferentiation of monocyte-derived DCs into osteolcasts upon culture in M-CSF combined with receptor activator of nuclear factor-κB ligand (RANKL) [39]. Together with the current data, this clearly indicates that myeloid cells have a high plasticity and the local microenvironment will be a determining factor for the functional differentiation of these cells. However, it also indicates that these cells can adapt and functional re-differentiate when there are changes in the environment. These observations are important for our understanding of the regulatory role of Mφ and other myeloid cells in tissue homeostasis and disease pathogenesis.

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A pivotal role for innate immunity in the clearance of apoptotic cells

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Summary

Apoptotic cells can be recognized and taken up by both macrophages and dendritic cells. Phagocytosis of apoptotic cells generally leads to active suppression of cytokine production by professional phagocytes. This is different from the response towards cells died by necrosis, which induce a pro-inflammatory cytokine profile. Uptake of apoptotic cells involves a large number of receptors and opsonins, which bind to cellular ligands exposed during the various stages of apoptotic cell death. Among the opsonins of apoptotic cells, complement factors, including C1q, and complement-activating members of the pentraxin family play an important role. This is indicated by *in vitro* phagocytosis studies and supported by the susceptibility to systemic autoimmunity in carriers of genetic deficiencies for early complement proteins. The present review summarizes the role of molecules of innate immunity in the handling of apoptotic cells by macrophages and dendritic cells. It is 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.

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Introduction

The innate immune system is of key importance in primary recognition of invading pathogens. In this respect, a large number of so-called pattern recognition molecules have been identified, including soluble molecules, such as complement factors, members of the collectin family and the pentraxin family, as well as membrane-bound receptors, such as members of the family of Toll-like receptors (TLR) and the family of C-type lectins. Pattern recognition molecules are not only involved in pathogen elimination but also in the clearance of apoptotic cells. The rapid and early removal of apoptotic cells by phagocytosis is directed by early changes of the apoptotic cell surface that precede the release of its intracellular contents. Apoptotic cells are phagocytosed by professional phagocytes, such as macrophages and immature dendritic cells, but also non-professional phagocytic neighbor cells may participate. Identification of molecules and mechanisms involved has revealed important parallels between innate host defense against pathogenic intruders and clearance of *self* debris, thereby shedding a new light on mechanisms of tolerance and immunity.

The present review summarizes recent data on the role of pattern recognition molecules in the recognition and clearance of apoptotic cells, and will elaborate on the way these molecules might direct differential cellular responses upon apoptotic cell phagocytosis.

Molecules involved in recognition and clearance of apoptotic cells

A well-recognized and early event in the apoptotic process is the loss of phospholipid asymmetry of the cell membrane. This leads to exposure of phospholipids such as phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) on the outside of the cell membrane. Exposure of phospholipids is a major factor in the recognition of apoptotic cells by phagocytes, involving membrane-bound receptors, such as the PS-receptor [2] and several scavenger receptors [3,4], as well as a number of soluble opsonins that can bridge apoptotic cells with phagocytes (Table 1 and references therein). Mice deficient for the membrane receptor for PS show a profound defect in the clearance of apoptotic cells, causing abnormal development and neonatal death [5].

Important insight into mechanisms involved in removal of apoptotic cells has been generated by studies in *Caenorhabditis elegans* (reviewed in [6,7]). In this organism, two genetic pathways have been defined, involving the molecules CED-1, CED-6 and CED-7 in the first pathway, and CED-2, CED-5, CED-10 and CED-12 in the second pathway, which are involved in the engulfment of dying cells. A worm homolog of the PS receptor, *psr1*, appeared to be involved in the recognition of cell corpses, and was shown to mediate its function via interaction with the signaling

proteins CED-5 and CED-12 [8]. However, in contrast to observations in humans and mice, disruption of cell engulfment pathways in worms did not lead to overt disease.

Table 1. Opsonins involved in bridging apoptotic cells with phagocytes

Cell surface receptors involved	Opsonin	Ligand on apoptotic cell	Ref.
CD91/calreticulin	C1q	?	1,9-12
Scavenger receptor-A/calreticulin ?	MBL	?	12,13
	SP-A	?	14,15
Other collectin receptors	SP-D	?	15
Via C3b receptors?[a]	C4b	Covalent binding upon activation[b]	10,16
Complement receptor-3 Complement receptor-4	(i)C3b	Covalent binding upon activation[b]	16,17
Pentraxin receptor(s)?	CRP	PC	18,19
Via collectin receptors?[a]	SAP component	PE, chromatin, DNA	20
Via C3b receptors?[a]	Pentraxin 3	?	1,21,22
Via collectin receptors?[a] Via C3b receptors?[a]	IgM	PC	23,24
c-Mer	growth arrest-specific gene 6[c]	PS	25,26
Tyro3? [d]	Protein S	PS	27
$\alpha_v\beta_3$	milk fat globule-EGF-factor 8	PS, PE	28
$\alpha_v\beta_3$ / CD36	thrombospondin-1	?	29,30
?	β2 glycoprotein	PS	31,32

a. Collectin receptors and C3b receptors can mediate phagocytosis following C1q binding and activation of the classical complement pathway, respectively
b. C3b and C4b can bind to complement-activating surfaces via a covalent thiol-ester linkage
c. GAS6 can also bind to other receptors related to c-Mer (Tyro-3, Axl)
d. It is not sure whether human protein S may bind to human Tyro-3 [33,34]

In mice and humans, impaired clearance of apoptotic cells and material derived from these cells is associated with the development of a systemic SLE-like autoimmune disease. Systemic autoimmunity was observed in mice deficient for serum amyloid P-component (SAP), c-Mer, C1q, C4, and IgM, all molecules potentially involved in such clearance processes (Table 2 and references therein).

Furthermore, there is a striking association between genetic deficiencies of the components of the classical complement pathway (C1q, C1r, C1s, C4, C2) and SLE in humans [35,35]. This association is hierarchical, and the association of C1q deficiency with SLE is almost 100 %. Several groups have established that complement components (C1q, mannose-binding lectin (MBL), C4, C3) directly and indirectly interact with apoptotic cells (Table 1), as is further discussed below.

Involvement of the complement system in the opsonization and clearance of apoptotic cells

The complement system can be activated via three different pathways, namely the classical pathway (CP), the alternative pathway (AP), and the lectin pathway (LP), which converge at the level of C3 activation (Figure 1). Whereas the LP and the AP primarily use a direct target recognition mechanism, the CP is mainly activated via binding of the initiating factor C1q to target-bound IgG or IgM antibodies. C1q is able to directly bind to apoptotic cells via its globular head domain [1,11], which may induce complement activation with subsequent deposition of C4b and C3b [1]. Moreover, C1q can also indirectly bind to apoptotic cells via several intermediates. Binding of IgM autoantibodies to apoptotic cells may be responsible for the major part of C1q binding and complement activation by apoptotic cells exposed to normal human serum [23,24]. The pentraxin family members C-reactive protein (CRP), pentraxin-3 (PTX3), and SAP bind to apoptotic cells [18,20,21], which can lead to a direct interaction with phagocyte receptors [4], as well as to secondary C1q binding and complement activation [18,22].

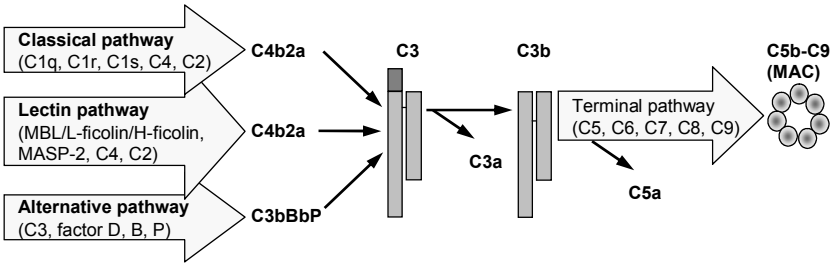

Figure 1. The three pathways of complement activation. Each complement activation pathway generates a C3 convertase (C4b2a / C3bBbP), which mediate cleavage of C3, followed by activation of the terminal complement pathway and formation of the membrane attack complex (MAC).

C1q, being the recognition molecule of the CP, is functionally and structurally related to MBL, a major recognition molecule of the LP [36]. MBL belongs to the family of collectins, soluble multimeric pattern recognition molecules, characterized by C-type lectin domains that serve for ligand recognition and collagenous domains

that interact with receptors. MBL can bind to apoptotic cells via its lectin domains [12,13]. However, this interaction does not lead to complement activation [13]. Not only MBL but also L-ficolin, a member of the ficolin family that is able to activate the LP [37], is able to bind to apoptotic cells (M. Lund Jensen and P. Garred, unpublished observation). Other members of the collectin family include the lung surfactant proteins A and D (SP-A, SP-D). These molecules mediate phagocytosis of apoptotic cells by human and rat macrophages [14,15]. A direct interaction with apoptotic cells has been demonstrated for SP-A [14], which was reported to be lectin domain-independent [14], suggesting that the mechanism involved is different from that used by MBL.

The cellular receptors for C1q have been a matter of debate for a long time. Via peptide sequencing, the endothelial receptor for C1q, called cC1qR, was shown to be identical to calreticulin (CRT). CRT can not only bind to C1q, but also to MBL, SP-A, and SP-D [38]. CRT, as well as several host-derived heat shock proteins (gp96, HSP70, HSP90), can bind to the α2-macroglobulin receptor CD91 [39]. CD91 is a transmembrane receptor that can mediate phagocytosis, and is homologous to the *C. elegans* protein CED-1. Indeed, a complex of CD91 and CRT serves as a collectin receptor on the surface of macrophages and can mediate the phagocytosis of apoptotic cells opsonized with C1q, MBL, SP-A or SP-D [12,15]. Furthermore, scavenger receptor A has now been reported as a second receptor involved in binding and internalization of CRT and gp96 [40]. Scavenger receptor A is prominently expressed not only on macrophages but also on dendritic cells and therefore might also be involved in the uptake of apoptotic material opsonized with C1q and collectins.

Opsonization in relation to the stage of cell death

Depending on the ligand on the apoptotic cell, opsonins may support clearance of apoptotic cells in early or later stages of the apoptotic process. Molecules that bind to exposed phospholipids, such as the PS receptor, several scavenger receptors, as well as a number of opsonins for apoptotic cells (Table 1) presumably interact with early apoptotic cells. For other opsonins, such as C1q, MBL, SP-A, and PTX3, the ligand on the apoptotic cell surface is currently unknown, but, in case of C1q and MBL [1,13], probably distinct from PS. Accordingly, C1q and MBL binding to apoptotic cells are rather late events in the cell death process: C1q binding to early apoptotic cells is much weaker than to late apoptotic cells and binding of MBL and PTX3 was exclusively demonstrated to late apoptotic cells [1,13,21]. Furthermore, SAP, an opsonin binding to PE, is able to bind to early apoptotic cells, but again the binding to late apoptotic cells is of much higher affinity [20]. Also deposition of C4 and C3 on apoptotic cells, as a consequence of activation of the complement cascade, is a late event during apoptosis [24,41].

C1q, MBL and PTX3 do not only bind to intact apoptotic cells, but also to microparticles that are released from the cell during apoptosis [1,13]. Noteworthy, whereas we found that C1q, MBL and PTX3 always bind to a subpopulation of these microparticles, SAP showed strong binding to the complete population of microparticles collected from the supernatant of apoptotic Jurkat cells (Fig. 2), suggesting that this molecule, which is constitutively present in human serum in a high concentration, may play a prominent role in the clearance of such apoptotic debris.

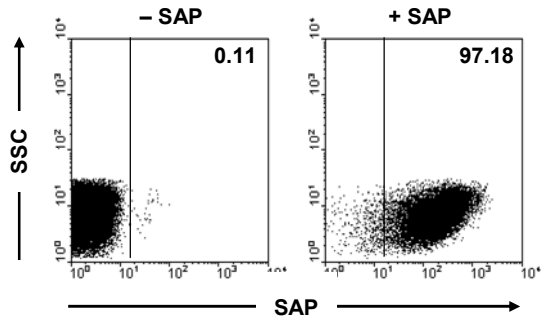

Figure 2. Binding of SAP to cell-derived microparticles generated during induction of apoptosis. Jurkat cells were induced to apoptosis by treatment with 40 µM etoposide during 48 hours in the presence or absence of purified human SAP (20 µg/ml). Microparticles were isolated from the culture supernatant as described [1] and stained for the binding of SAP using a monoclonal antibody directed against SAP, followed by PE-conjugated goat anti-mouse antibodies for detection. SAP and mAb anti-SAP were a kind gift of Dr. Hack and Dr. Familian.

Together, these data suggest that C1q, MBL and the pentraxins are primarily important in the clearance of apoptotic cells in later stages of the cell death process, after they have become leaky and/or have fallen apart in membrane-enclosed apoptotic bodies. Therefore, in the normal clearance of early apoptotic cells, other clearance mechanisms may prevail, involving molecules such as the PS receptor. It is difficult to estimate how often late apoptotic cells do occur *in vivo*. Furthermore, *in vitro* and *in vivo* phagocytosis experiments use a mixture of early and late apoptotic cells, since these cells progress into late stages during handling. Therefore, the role of opsonins in the uptake of early apoptotic cells remains to be established. However, since C1q-deficient mice show impaired clearance of apoptotic cells [42], and since both C1q-deficient mice and humans are strongly susceptible to autoimmunity, the contribution of C1q to the clearance system is apparently important. Complement-dependent clearance mechanisms could be especially relevant during e.g. trauma or inflammation, resulting into an increased load of apoptotic material.

Noteworthy, MBL [13] and C1q (W. Xu et al. unpublished) do not only directly bind to late apoptotic cells but also to cells dying via necrosis. Necrotic cells activate the classical complement pathway, largely via an IgM-dependent mechanism [Ciurana et al. submitted for publication, [41]]. Also necrotic cells expose phospholipids that may serve as ligands for receptors and opsonins. Therefore, the clearance mechanisms for apoptotic cells are presumably also involved in the clearance of

necrotic material and therefore these molecules may function as general scavengers of cellular debris.

Opsonization modulates the uptake of apoptotic cells

Opsonization generally increases the efficiency of the interaction between phagocyte and apoptotic cell and induce receptor-mediated phagocytosis, which accelerates the kinetics of the phagocytosis process and thus enhances the capacity of the phagocytic system. Most of the molecules implicated in apoptotic cell clearance have been shown to contribute to the uptake of apoptotic cells by macrophages *in vitro*. *In vivo* support for their role in clearance of self debris is in a number of cases available by studies in knockout mice, as presented in table 2.

Table 2. In vivo support for a role of receptors and opsonins in the clearance of self debris

Molecule	Phenotype of knockout mice	Ref.
C1q	Systemic autoimmunity with autoantibodies and glomerulonephritis Increased numbers of apoptotic cells in glomeruli Delayed clearance of apoptotic cells from the peritoneal cavity	10,42
SP-D	Delayed clearance of apoptotic cells from the lung	15
C4	Systemic autoimmunity with autoantibodies and glomerulonephritis Delayed clearance of apoptotic cells from the peritoneal cavity	10,43
SAP	Systemic autoimmunity with autoantibodies and glomerulonephritis Accelerated chromatin degradation, enhanced immune response against chromatin	44
IgM	Autoimmunity to nuclear antigens, renal IgG and complement deposition	45,46
c-Mer	Autoimmunity to nuclear antigens, renal IgG and complement deposition	25,47
PS receptor	Defect in clearance of apoptotic cells in the thymus and spleen Accumulation of apoptotic cells in brain and lungs Abnormal development and neonatal lethality	5

Opsonins can contribute to the clearance of apoptotic cells in a tissue-specific way. Mice deficient for SP-D show decreased uptake of apoptotic cells that are administered into the lung [15]. Mice deficient for C1q and C4 show a delayed clearance of apoptotic cells that are injected into the peritoneal cavity [10]. However, $C1q^{-/-}$ mice show a normal uptake of apoptotic cells in the lung [15]. Similarly, despite the fact that C1q is able to bind to apoptotic keratinocytes [9], it does not seem to contribute to the uptake of apoptotic cells in the skin [48]. C1q supports the uptake of apoptotic cells by macrophages but not by renal mesangial cells [49], indicating that a defect in clearance by mesangial cells cannot explain the increased numbers of apoptotic cells in glomeruli from C1q-deficient mice [42].

The data available indicate that deficiency of apoptotic cell opsonins such as complement factors delays the *in vivo* uptake of apoptotic material by professional phagocytes [10]. This may result into a prolonged exposure of the immune system to apoptotic cells and to a progression of these apoptotic cells into later stages of cell death, thereby increasing the risk of induction of autoimmunity. Apoptotic cells are a rich source of autoantigens that are known targets of autoantibodies in systemic autoimmune diseases such as SLE. Uptake of these autoantigens by dendritic cells (DC) may lead to professional presentation to naïve T cells and thereby to a loss of tolerance. DCs are professional antigen presenting cells that are responsible for induction of primary immune responses. It is now clear that DCs, depending on their state of maturation, also play a critical role in induction and maintenance of tolerance [50]. Walport and colleagues have introduced the waste disposal hypothesis to explain the role of C1q in the prevention of systemic autoimmunity [35]. In this hypothesis, the main role of C1q is to promote the uptake of apoptotic debris by macrophages. Without C1q, the efficiency of this process is too low, leading to an overload of apoptotic cells which will then be taken up also by immature dendritic cells.

Recent data obtained by our group indicate that C1q and MBL do not only enhance the uptake of apoptotic material by macrophages, but also by immature dendritic cells (A.J. Nauta et al., submitted for publication). Therefore, it seems that these apoptotic cell opsonins do not make a distinction between macrophages and immature DCs, but allow uptake of apoptotic cells in both cell types. A potential role for C1q in antigen uptake by immature DCs is also strongly supported by our recent observation that immature DCs, but not mature DCs, are strong producers of C1q, even stronger than macrophages [51].

Not only C1q and MBL, but also complement activation products promote the uptake of apoptotic cells by DC, involving the complement receptors CR3 and CR4 [17,52]. Data obtained from complement-deficient mice and humans indicate that C1q and complement are involved in maintenance of tolerance. Since C1q and complement components promote apoptotic cell uptake by both macrophages and immature DC, we hypothesize that these apoptotic cell opsonins have important immunomodulatory effects.

Opsonization may direct the cellular response to apoptotic cells

Phagocytosis of apoptotic cells by professional phagocytes not only prevents the release of their harmful intracellular contents, but also actively modulates phagocyte function [4,53]. Phagocytosis of apoptotic neutrophils by human macrophages was shown to inhibit production of cytokines and chemokines, whereas production of TGF-β1 was increased [54]. The addition of apoptotic cells to macrophages also modulates cytokine production induced by TLR ligands [54,55],

which partially involves production of TGF-β1. Apoptotic cell-induced release of TGF-β1 *in vivo* was shown to require exposure of PS [56].

With respect to dendritic cells, it has been shown that DC can engulf both apoptotic and necrotic cells [57-59]. Similarly to macrophages, DC show suppressed cytokine production upon their exposure to apoptotic cells [17,59]. Furthermore, antigens delivered to DC via apoptotic cells induced tolerance *in vivo* [60]. However, under certain circumstances, exposure of DC to apoptotic cells can also induce DC maturation and immunity [61,62]. This might be dependent on the amount of apoptotic cells, as well as on the presence of pro-inflammatory signals and opsonizing antibodies.

At this moment, there is only little information available about how the known apoptotic cell opsonins interfere with the modulation of phagocytic responses by apoptotic cells. Apoptotic cells opsonized with CRP and complement induced expression of TGF-β by macrophages [18]. Furthermore, stimulation of the iC3b receptor was shown to inhibit cytokine production by DC [17]. In contrast, the interaction of SP-A and C1q with the CD91/CRT complex on macrophages was recently demonstrated to be a pro-inflammatory interaction [63]. However, this has not been studied in the context of these opsonins present on apoptotic cells, which could make a crucial difference.

Contrary to the phagocytosis of apoptotic cells, which is in most cases described as a non-inflammatory process, phagocytosis of necrotic cells leads to DC maturation and macrophage activation [57,60,64]. The pro-inflammatory effects of necrotic cells have been linked to the release of heat shock proteins [65], the chromosomal protein HMBG1 (high mobility group box chromosomal protein 1) [66], and high concentrations of uric acid [67]. Strikingly, heat shock proteins and HMBG1 were not released from cells dying by apoptosis, also not in a late stage of cell death.

Heat shock proteins are now described as endogenous ligands of several members of the TLR family, including TLR2 and TLR4 [68]. Interestingly, SP-A was recently also shown to bind to TLR4 [69]. Furthermore, heat shock proteins can signal towards phagocytic cells via CD91 and via scavenger receptor A [39,40]. This involves an interaction between heat shock proteins and these receptors in a similar way as described for calreticulin, the molecule proposed to be involved in the binding of C1q and collectins [12,63]. Therefore, it is presently unclear whether and how phagocytic cells may distinguish between signals coming from heat shock proteins, derived from necrotic cells, and signals coming from calreticulin bound to C1q, present on the surface of apoptotic cells. It has been proposed that the PS receptor is an important "molecular switch" in this respect, determining whether or not the phagocytic cell should mount a pro-inflammatory or a tolerogenic response

[70]. This hypothesis includes the premise that necrotic cells are somehow able to prevent signaling via the PS receptor.

Concluding remarks

Opsonization of apoptotic cells is required for their safe elimination. Whereas appropriate opsonization will direct a rapid and anti-inflammatory clearance of apoptotic material by macrophages and DC, inappropriate opsonization, either in a quantitative or qualitative way, may lead to disturbed uptake and phagocyte modulation, finally resulting in a loss of self tolerance (Figure 3).

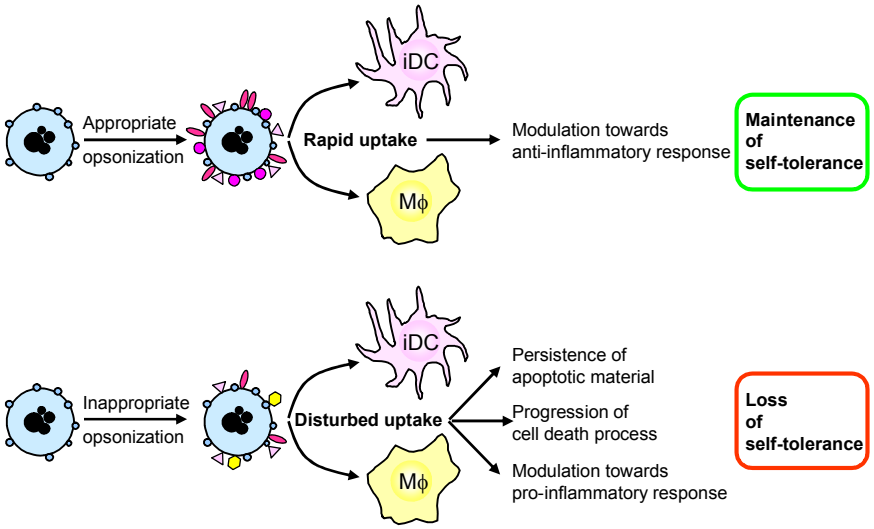

Figure 3. The role of opsonins in the handling of apoptotic cells. Appropriate opsonization of apoptotic cells leads to their rapid uptake by macrophages (Mϕ) or immature dendritic cells (iDC), and to a modulation of these phagocytic cells, leading to an anti-inflammatory response and maintenance of tolerance. In contrast, quantitatively or qualitatively inappropriate opsonization may disturb the apoptotic cell uptake, leading to a loss of tolerance, as illustrated in the lower panel.

Dendritic cells are well recognized as the central mediators of both immunity and tolerance. Recent studies have revealed that also for the induction of tolerance immature DC need to reach a certain stage of maturation [71]. In this respect we hypothesize that a combination of stimuli, such as opsonins, membrane-associated ligands, cytokines and TLR ligands, will determine whether the DC will induce immunity or tolerance upon encountering dying cells. Further definition of these signals and their effect on phagocyte responses towards apoptotic cells are required to understand why deficiencies in molecules involved in the handling of apoptotic cells are associated with systemic autoimmunity.

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7

Properdin regulates alternative pathway complement activation on late apoptotic and necrotic cells

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Summary

Cells that undergo apoptosis or necrosis are promptly removed by phagocytes. Soluble opsonins such as complement can opsonize dying cells, thereby promoting their removal by phagocytes and modulating the immune response. However, the pivotal role of the complement system in the handling of dying cell has been mainly linked to the classical pathway (via C1q) and lectin pathway (via MBL and ficolin). Here we report that the only known naturally occurring positive regulator of complement, properdin, binds predominantly to late apoptotic and necrotic cells, but not to early apoptotic cells. This binding is independent of C3b, and we show that properdin becomes a focus point for local amplification of alternative pathway complement activation. Properdin does not compete with C1q and MBL for binding to late apoptotic and necrotic cells, but exhibits a strong interaction with DNA which is released and exposed on these cells. Although purified properdin does not enhance phagocytosis, it limits the pro-inflammatory potential of necrotic cells by reducing TNF-α release by dendritic cells and macrophages, whereas IL-10 production remains unchanged. Our data indicate that recognition of dying cells by properdin is essential to drive alternative pathway complement activation and actively promotes an anti-inflammatory response.

------ *Blood. 2007; provisionally accepted* ------

Introduction

Under steady-state conditions, cells that undergo apoptosis and necrosis can be safely and silently eliminated by professional phagocytes, i.e. immature dendritic cells (DCs) and macrophages (Mφ) [1-3]. Apoptotic cells are a rich source of autoantigens, which are involved in the induction of self-tolerance and autoimmunity [4]. Compelling evidence has emerged that abnormal clearance of apoptotic cells is associated with development of the autoimmune disease systemic lupus erythematosus (SLE) [5,6].

Soluble factors from the innate immune system such as complement or pentraxins can opsonize apoptotic cells, thereby promoting their removal by phagocytes [6-8]. In humans, homozygous deficiency of any of the early components of the classical pathway of complement activation (C1q, C1r, C1s, C4, and C2) predisposes to the development of SLE [9], suggesting that complement is involved in removal of dying cells and the immune regulation associated with this process. Complement-mediated clearance of apoptotic cells has been well documented both *in vitro* [10] and *in vivo* [11]. Nevertheless, the role of the complement system in the handling of dying cells has been mostly linked to the classical pathway (via C1q) and lectin pathway (via MBL and ficolin) [10-14]. It was suggested that the main product of complement activation, iC3b, facilitates the removal of dead material and mediates peripheral tolerance [10,15,16].

The alternative pathway of complement is thought to be activated following hydrolysis of C3, generation of C3b and formation of a positive feedback loop to mount a rapid local response [17]. The alternative pathway was initially recognized to amplify complement activation triggered by classical and lectin pathways. Properdin, discovered in 1954 [18], is the only known naturally occurring positive regulator of complement activation [19]. It was shown that properdin binds to C3b and increases the stability of the alternative pathway convertases at least 10-fold on target surfaces and immune complexes [20]. Moreover, it has been suggested that properdin amplifies complement activation on a target surface through an organized assembly process of C3 convertase C3bBbP, accounting for a direct complement activation capacity of properdin [21]. Properdin has long been recognized as one of the key players in the regulation of the complement system [18], nevertheless, the interaction of properdin with dying cells and the involvement of the alternative pathway in this process have not been investigated.

In present study, we investigated whether properdin, like C1q and MBL, contributes to the processing of dying cells. We found that properdin binds predominantly to late apoptotic and necrotic cells independent of C3b, but not to early apoptotic cells, leading to alternative pathway-mediated complement activation. Our data suggest that properdin is one of the important regulators involved in the handling of dying

cells via: 1.) activation of alternative pathway complement; 2.) modulation of the immune response by antigen presenting cells by dying cells.

Material and methods

Generation of phagocytes

Generation of dendritic cells (DCs), Mφ1 and Mφ2 from human peripheral blood monocytes was performed using the methods described previously [22]. Briefly, Mφ1 and Mφ2 were generated from $CD14^+$ monocytes in RPMI culture medium (RPMI 1640 containing 10% heat-inactivated FCS, 90 U/ml penicillin and 90 μg/ml streptomycin) (all from Gibco/Life technologies, Breda, the Netherlands) in the presence of 5 ng/ml GM-CSF (Leucomax, Novartis Pharma BV, Arnhem, the Netherlands) and 5 ng/ml M-CSF (R&D systems / ITK Diagnostics, Uithoorn, the Netherlands), respectively, for 6 days. DCs were cultured with combination of GM-CSF and 10 ng/ml IL-4 (PeproTech, Rocky Hill, NJ).

Induction of apoptosis and necrosis

Jurkat cells were cultured in RPMI culture medium. Early or late apoptosis was induced in Jurkat cells by exposure to ultra violet (UV)-C light (Philips TUV lamp, predominantly 254 nm) at a dose of 50 J/m^2., followed by 3 or 30 hours culture in RPMI serum-free medium, respectively. Necrosis was induced by treating cells at 56°C for 0.5 h or 5 cycles of freeze-thaw from -80°C to 36°C. Both apoptosis and necrosis were confirmed by double staining with fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI, VPS Diagnostics, Hoeven, the Netherlands) according to established methods [23]. Alternatively, human umbilical cord endothelial cells (HUVEC), U937 cell (monocytic cell lines), HK-2 cell (Renal tubular epithelial cells) and Epstein-Barr virus-transformed B lymphoblastoid cell lines (EBV-LCLs) were used for the induction of necrosis.

In some experiments, splenocytes were obtained from C3 knockout ($C3^{-/-}$) [24] or C57BL/6 wild type (WT) mice (Harlan). Splenocytes was rendered necrotic by incubating them at 56°C, as described above.

Phagocytosis assay

Phagocytosis of early apoptotic, late apoptotic and necrotic cells was assessed by using a protocol described previously [22]. Briefly, prior to the induction of apoptosis or necrosis, Jurkat cells were fluorescently labeled with carboxyfluorescein diacetate succinamidyl ester (CFSE, Molecular Probes, Leiden, the Netherlands). Labeled early, late apoptotic or necrotic cells (1×10^5) were investigated either alone or following opsonization with normal human serum (NHS) or purified properdin. Dying cells were co-cultured with DCs, Mφ1 or Mφ2 in 1:1 ratio at 37°C for 0.5 h in 100 μl RPMI culture medium in round-bottom glass tubes. As a control, co-culture was performed at 4°C to detect the binding of dying cells to phagocytes. Mφ2 were stained with a PE-conjugated mAb against CD11b and uptake was analyzed by a two-color flow cytometry. The percentage of CD11b-positive cells that stained positive for

CFSE was used as a measure for the percentage of DCs, Mφ1 or Mφ2 that ingested and/or bound apoptotic cells.

Isolation of properdin, C1q and MBL

Properdin was isolated from pooled human serum from volunteer donors. Serum was first precipitated by dialysis against 5 mM EDTA (pH 6.0). The precipitate was dissolved in Veronal-buffered saline (2×VBS, 1.8 mM Na-5,5-diethylbarbital, 0.2 mM 5,5-diethylbarbituric acid, 145 mM NaCl), and then dialyzed against 0.01 M NaAc containing 2 mM EDTA (pH 6.0) and applied to a Sulphopropyl C50 column. Properdin was eluted with a linear salt gradient. Properdin-containing fractions, as determined by ELISA, were pooled, concentrated, and subsequently applied to a Sephacryl S-300 gel filtration column (Pharmacia Biotech, Uppsala, Sweden). Fractions containing properdin were dialyzed against PBS, 2 mM EDTA and further purified using human IgG coupled to a Biogel A5 (Bio-Rad, Hercules, CA) to remove contaminating C1q. Purity of the properdin preparation was determined by analysis on 10% SDS-PAGE gel. C1q and MBL were purified from pooled human plasma obtained from healthy donors as described previously [25,26].

Properdin binding assay

Binding of properdin to viable, early apoptotic, late apoptotic or necrotic cells was investigated by incubating cells with up to 40 µg/ml human purified properdin at 37°C for 1 h in serum-free RPMI culture medium. Then cells were extensively washed and incubated with a rabbit-anti-human properdin polyclonal Ab, and detected with phycoerythrin (PE)-conjugated goat $F(ab)_2$ anti-rabbit Ig (Southern Biotechnology Associates, Birmingham, US). The cells were analyzed by flow cytometry. Data from 10^4 events were acquired.

Binding of C1q (30 µg/ml) and MBL (10 µg/ml) were performed in the same way as properdin binding and detected with a monoclonal antibody (mAb) directed against C1q (mAb 2204) or MBL (clone 3E7), respectively. Binding was visualized with phycoerythrin (PE)-conjugated goat $F(ab)_2$ anti-mouse Ig (DAKO, Glossstrup, Denmark).

In some experiments, cells were pre-incubated with C1q or MBL, followed by incubation of properdin and *vice versa*.

To detect the binding of properdin to DNA, double stranded DNA (dsDNA) from calf thymus (Sigma-Aldrich), single stranded DNA (ssDNA) or human albumin (Sigma-Aldrich) were coated in PBS on microtiter plates overnight, and then blocked with 2%BSA before adding purified properdin. After washing, bound properdin was detected with Dig-labelled rabbit-anti-human properdin. Bound antibody was developed with anti-Dig-HRP (Roche Diagnostics GmbH, Mannheim, Germany), and measured for absorbance at OD 451 nm.

Serum

C4-depleted serum (C4ds) was used as a complement source that is lacking both classical and lectin pathway activity, and was prepared by affinity absorption using goat anti-human C4 IgG coupled to CNBr-activated Sepharose 4 fast flow (Amersham Bioscience Europe GmBH, Roosendaal, the Netherlands). C4ds was free of C4 antigen and hemolytic activity of the classical pathway could be resorted by purified C4.

Properdin-depleted serum (Pds) was obtained by immune absorption using Biogel-coupled monoclonal Ab against human properdin (a gift of Statens Serum Institut, Copenhagen, Denmark). Pds showed normal classical and lectin pathway activities in hemolytic assays.

Complement activation by dying cells

Activation of complement by dying cells was assessed as follows: early, late apoptotic or necrotic cells were pre-incubated with or without properdin at 37°C for 1 h, washed extensively and then exposed to different dilutions of Pds, C4ds or NHS for 0.5 h at 37°C. Deposition of C3, C4 and C5b-9 on the cell surfaces were detected by flow cytometry using mAbs against C3 (RFK22, [27]), C4 (anti-C4-4 [28]) and C5b-9 (AE11, kindly provided by Dr. T. E. Mollnes, Nordland Central Hospital, Bodø, Norway), respectively.

Detection of cytokine production by DCs, Mφ1 and Mφ2.

DCs, Mφ1 and Mφ2 (1×10^5) were incubated for 4 hours at 37°C with necrotic cells that were pre-opsonized with or without properdin, followed by activation with 200 ng/ml LPS for another 20 h in 48-well-plate and supernatants were harvested. The amounts of TNF-α and IL-10 in the supernatants were quantified by ELISA, as described previously [22].

Statistical analysis.

Statistical analysis was performed by one sample *t* test using GraphPad Prism (GraphPad software, San Diego, CA). Differences were considered statistically significant when p values were less than 0.05.

Results

Complement-mediated phagocytosis of late apoptotic and necrotic cells

Relatively pure populations of viable (90-98%), early apoptotic (40-70%), late apoptotic (90-100%) and necrotic cells (100%) were obtained (Fig. 1A). In agreement with our earlier findings, M-CSF-driven anti-inflammatory Mφ2 preferentially recognized and ingested early apoptotic cells, as compared to the ingestions of late apoptotic and necrotic cells [22] (Fig. 1B). However, opsonization of early apoptotic cells with normal human serum (NHS) did not enhance their uptake by Mφ2 (Fig. 1B, C), while opsonization of late apoptotic and necrotic cells with

NHS significantly increased their uptake by Mφ2 ($p<0.01$) (Fig. 1B, C). We next questioned whether the observed enhanced phagocytosis is associated with complement deposition on the dying cells. Indeed, NHS-exposed late apoptotic and necrotic cells, but not early apoptotic cells, displayed strong deposition of C3 by flow cytometry (Fig. 1D). Therefore we next assessed the pathways involved in the activation of complement on these cells.

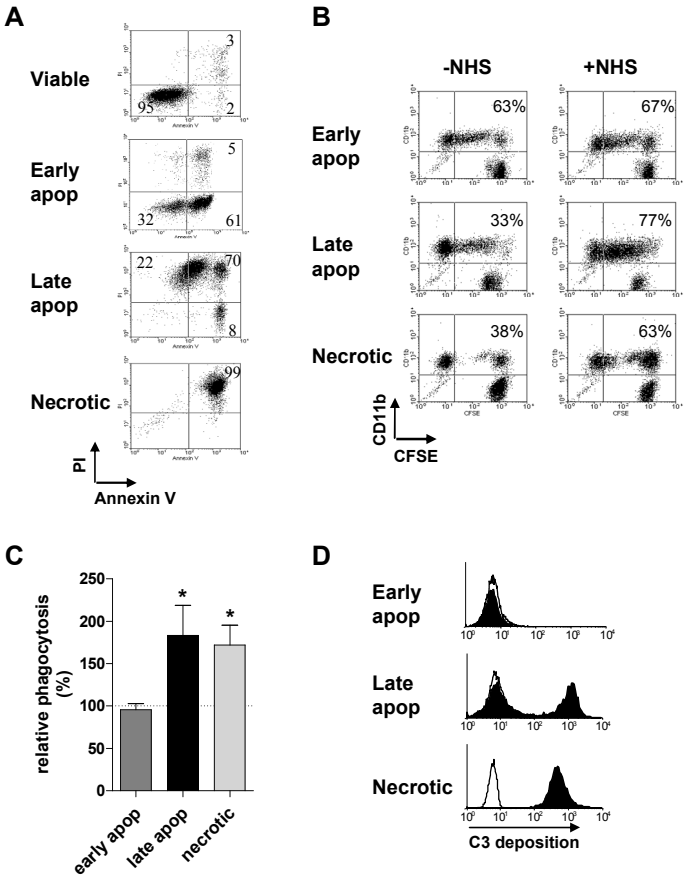

Figure 1. Complement-mediated phagocytosis of dying cells. (A) Early or late apoptosis was induced in Jurkat cells by exposure to ultra violet (UV)-C light at a dose of 50 J/m^2, followed by 3 or 30 hours culture in RPMI serum-free medium, respectively. Necrosis was induced by treating cells at 56°C for 0.5 h. Cells were stained with annexin V and PI by flow cytometry. (B) CFSE-labeled early apoptotic, late apoptotic or necrotic cells (1×10^5) were first opsonized with or without normal human serum (NHS), then co-cultured with Mφ2 in 1:1 ratio at 37°C for 0.5 h. Mφ2 were stained with a PE-conjugated mAb against CD11b and uptake was analyzed by a two-color flow cytometry. CD11b$^+$CFSE$^+$ cells were used as a measure for the percentage of Mφ2 that ingested apoptotic cells. (C) Relative phagocytosis was calculated as uptake of NHS-opsonized dying cells versus non-opsonized cells. Data are mean ±SEM of 3 independent experiments. *, $p<0.01$, one sample paired *t* test. (D) C3 deposition (filled histogram) after NHS opsonization on early, late apoptotic and necrotic cells was detected by flow cytometry. Open histograms are the matched isotype controls.

Properdin binds to late apoptotic and necrotic cells.

It has been shown that several serum factors bind to apoptotic cells (see review [8]). C1q, MBL and properdin are complement factors which activate complement via the respective 3 pathways. We here investigated whether properdin, the only naturally occurring positive complement regulator, can bind directly to dying cells that are at different stages of cell death. Similar to C1q, and MBL, properdin showed a predominant interaction with late apoptotic cells and necrotic cells, and no significant binding to early apoptotic or viable cells (Fig 2A, B). Properdin was shown to bind to both late apoptotic and necrotic cells in a dose-dependent manner (Fig. 2C). To rule out the possibility that the observed binding of properdin is cell type or method specific, different cell lines and methods for induction of necrosis were used. Properdin was shown to bind strongly to necrotic HUVEC, U937, HK-2 and EBV-LCL cells, and also to Jurkat cells that were rendered necrotic by 5 cycles of freeze-thawing (data not shown), suggesting that binding of properdin to necrotic cells is a universal phenomenon and irrespective of specific cell types.

It is accepted for a long time that properdin binds to a pre-existing cluster of surface-bound C3b [29], thereby stabilizing the C3b-dependent C3 convertase C3bBb [20]. However, the data presented above propose a new model for the binding of properdin to dying cells independent of C3b. To confirm our hypothesis, we investigated whether properdin could bind to necrotic splenocytes derived from C3 knockout ($C3^{-/-}$) mice. Properdin was shown to bind strongly to necrotic splenocytes of $C3^{-/-}$mice and the extent of binding was similar to the binding of properdin to necrotic cells from WT mice (Fig. 2D). Importantly, properdin did not bind to viable splenocytes derived from either $C3^{-/-}$ or WT mice (Fig. 2D).

Properdin is a focus point for amplification of alternative pathway complement on dying cells

Since properdin is central in alternative pathway activation, we investigated whether binding of properdin to these dying cells might act as a focus point for local amplification of the complement system. For this purpose, we analyzed complement activation on necrotic cells using properdin-depleted serum (Pds). Cells pre-incubated with purified properdin alone, as expected, do not show C3 and C5b-9 deposition. (Fig. 3A). Also, in Pds, no properdin binding to necrotic cells was seen (not shown), however a reduced C3 deposition was observed, which is accompanied with a lack of deposition of the membrane attack complex C5b-9 (Fig. 3A). Necrotic cells that had been pre-exposed to properdin, washed extensively, and subsequently incubated with Pds, displayed significantly increased C3 and C5b-9 deposition, suggesting that properdin is essential for local amplification of the complement cascade on necrotic cells.

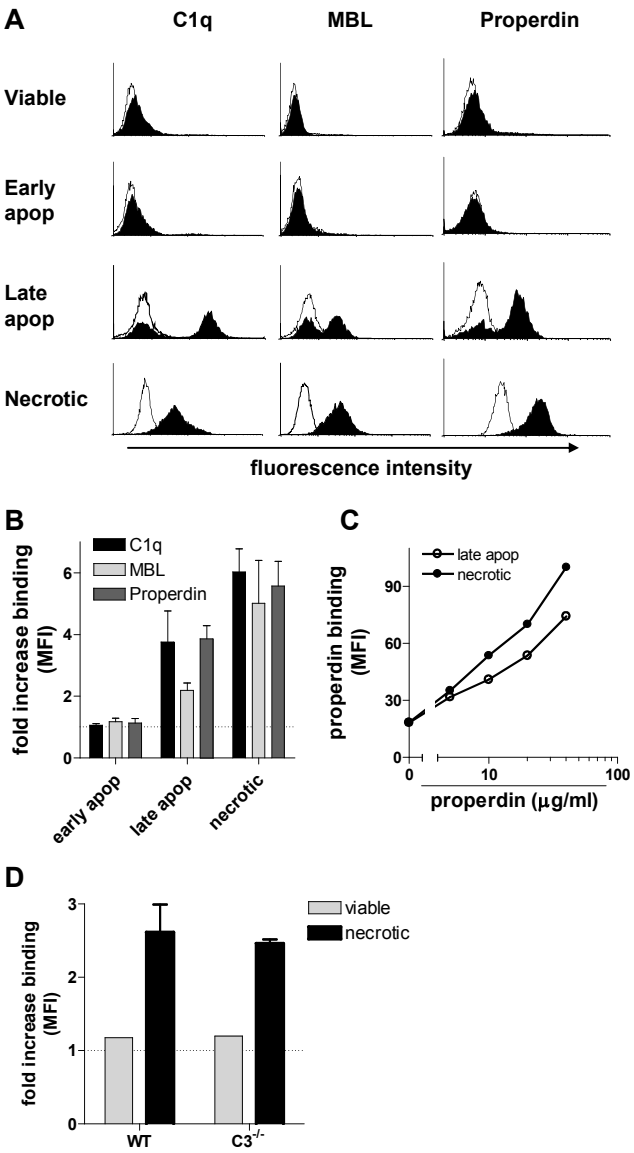

Figure 2. Properdin binds to late apoptotic and necrotic cells. (A) Viable, early apoptotic, late apoptotic and necrotic cells were collected and incubated with C1q, MBL or properdin. Specific binding (filled histogram) of C1q, MBL and properdin were detected by mAb against C1q (clone 2204), MBL (clone 3E7) and rabbit-anti-human properdin. Open histograms are the matched isotype controls. (B) Fold increase of binding was calculated as the mean fluorescence intensity (MFI) of C1q, MBL, or properdin divided by the MFI of matched isotype controls. Data shown are mean ±SEM of at least 4 independent experiments. (C) Late apoptotic and necrotic cells were incubated with increasing concentration of properdin (up to 40 μg/ml), and detected for properdin binding. Data shown are MFI. (D) Splenocytes from $C3^{-/-}$ or WT mice were first rendered necrotic by heating or kept viable, and measured for properdin binding. Fold increase of MFI was shown. Data shown are mean ±SEM of 4 independent experiments.

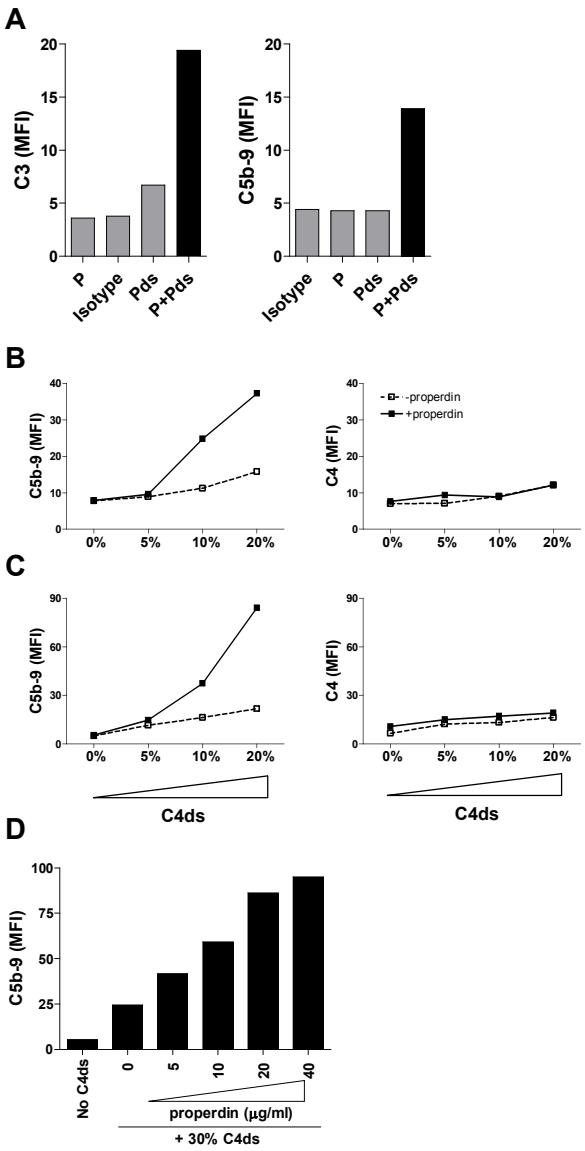

Figure 3. Properdin activates complement via alternative pathway

(A) Necrotic cells were pre-incubated with or without properdin, and then washed extensively before adding 30% properdin-depleted serum (Pds). Data shown are C3 and C5b-9 deposition on the cells. (B) Late apoptotic and (C) necrotic cells were pre-incubated with or without properdin, and then washed extensively before adding increasing concentration of C4-depleted serum (C4ds). C5b-9 and C4 deposition on the cells were measured. (D) Necrotic cells were pre-incubated with increasing concentration of properdin, and then washed extensively before adding 30% C4ds. Cells were measured for C5b-9 formation. Data are representative of 2 independent experiments.

To confirm that cell-bound properdin activates complement via the alternative pathway, we used C4-depleted serum (C4ds) as a source of complement, since C4 is a crucial factor for both the classical and lectin pathways. Late apoptotic and necrotic cells were pre-incubated with properdin and washed extensively before the addition of increasing concentrations of C4ds. Exposure of both late apoptotic cells (Fig. 3B) and necrotic cells (Fig. 3C) that had been pre-incubated with

properdin to C4ds significantly induced the deposition of C5b-9 in a dose-dependent manner, as compared with those without properdin, suggesting that properdin is the limiting factor that mediates complement activation via alternative pathway. As a control, we measured deposition of C4 on both late apoptotic and necrotic cells that had been exposed to C4ds. There was no C4 deposition neither on late apoptotic (Fig. 3B) nor on necrotic cells (Fig. 3C) after opsonization of C4ds, confirming that C4 had been effectively depleted in our C4ds preparation. When a fixed amount (30%) of C4ds was used, the increase of C5b-9 on the cell surface was dose dependently affected by the amount of properdin (Fig. 3D).

Properdin does not compete with binding of C1q and MBL to necrotic cells

We showed previously that C1q and MBL share binding ligands on apoptotic cells [30]. Since properdin was shown to bind to late apoptotic and necrotic cells in a similar pattern as C1q and MBL (Fig.1A), we hypothesized that properdin may bind to a similar structure on dying cells. Necrotic cells was pre-incubated with properdin and followed by increasing concentrations of C1q. A dose-dependent binding of C1q was observed, but properdin did not inhibit the binding of C1q to the cells (Fig. 4A). In a reverse way, pre-incubation of necrotic cells with C1q did not decrease properdin binding either (Fig. 4B). Similarly, pre-incubation of necrotic cells with properdin did not interfere with MBL binding and *vice versa* (Fig. 4C). Therefore, our data suggest that properdin binds to a yet unknown ligand which is different from the one to which C1q and MBL bind.

Properdin binds to DNA

One of the autoantigens exposed on apoptotic cells and necrotic cells is DNA [4] and it has been shown to be released from these cells [31]. Several serum components have been suggested to interact with DNA, including C1q [32], MBL [33], serum amyloid-P component (SAP) [34], and C4b-binding protein (C4BP) [35]. Based on the finding that properdin specifically binds to late apoptotic and necrotic cells, we hypothesized that properdin might bind to DNA exposed on the surface of dying cells. Pre-incubation of properdin with calf thymus dsDNA dose-dependently inhibited binding of properdin to necrotic cells (Fig. 4D), suggesting a strong interaction between DNA and properdin. To further confirm whether properdin binds to DNA, various concentrations of dsDNA and ssDNA were coated on microtiter plates prior to addition of properdin. Human albumin was used as a negative control. The binding was detected by a Dig-labelled rabbit-anti-human-properdin Ab. Properdin showed a strong binding to both dsDNA and ssDNA at concentrations of 1 μg/ml and higher (Fig. 4E).

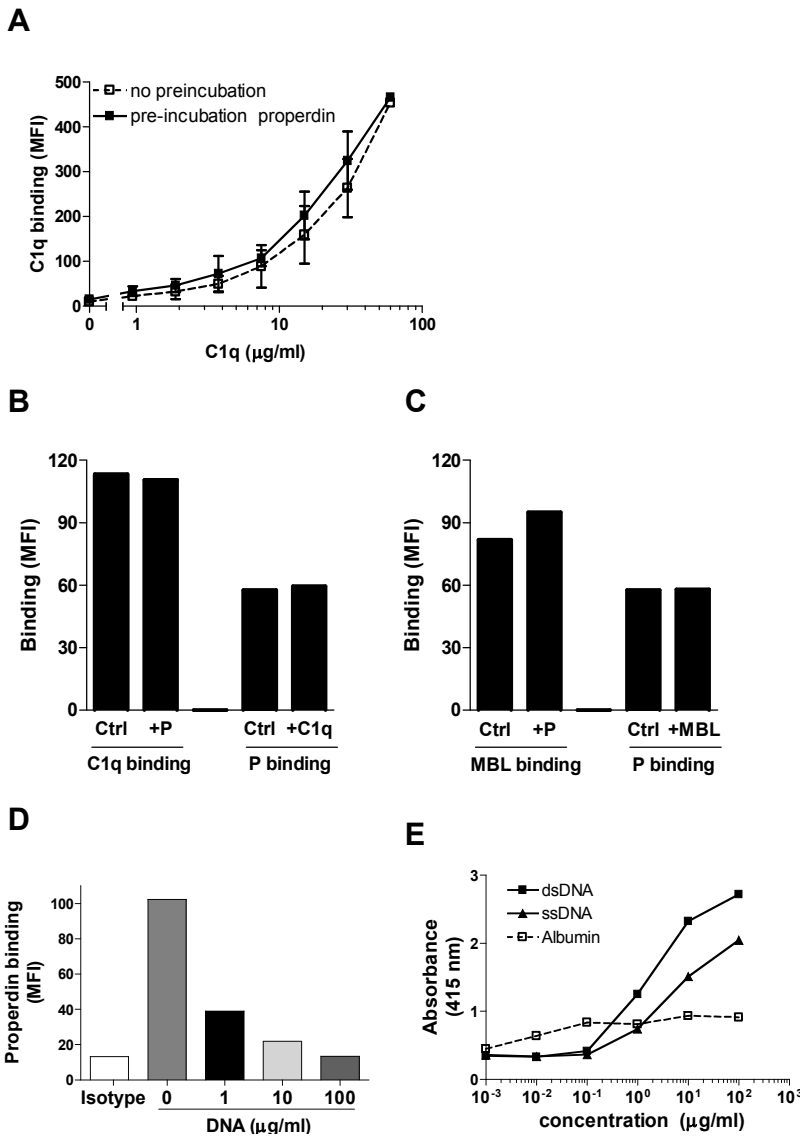

Figure 4. Properdin does not compete for binding with C1q and MBL, but binds to DNA. (A) Necrotic cells were pre-incubated with properdin (40 μg/ml) and followed by incubation with increasing concentrations of C1q (up to 60 μg/ml). C1q binding was measured. Data shown are mean ±SEM of 2 independent experiments. (B) Necrotic cells were pre-incubated with properdin (40 μg/ml) or C1q (30 μg/ml), then followed by incubation with C1q (10 μg/ml), or properdin (20 μg/ml), respectively. C1q and properdin binding were measured by flow cytometry. (C) Competition between properdin and MBL (10μg/ml) was investigated similar as described in (B). (D) Properdin was pre-incubated with increasing concentrations of calf thymus double strand DNA (dsDNA), and then incubated with necrotic cells. Data shown are properdin binding to the cells (MFI). (E) Different concentration of dsDNA and single strand DNA (ssDNA) or human albumin were coated on microtiter plates overnight, and then blocked with 2%BSA before adding properdin. After washing, plates were incubated with Dig-labelled rabbit-anti-human properdin. Signal was developed by anti-Dig-HRP, and measured for absorbance at OD 451 nm.

Purified properdin does not enhance phagocytosis of necrotic cells, but limits their pro-inflammatory potential.

It has been shown earlier that both C1q and MBL facilitate phagocytosis of apoptotic cells [12,13] prior to complement activation, accounting for a direct recognition of C1q and MBL by receptors on phagocytes. We here questioned whether properdin can enhance the removal of late apoptotic and necrotic cells by phagocytes. DCs, Mφ1 and Mφ2 generated in parallel from the same donor efficiently phagocytose late apoptotic cells (not shown) and necrotic cells. While opsonisation of necrotic cells with NHS clearly enhanced phagcytosis by Mφ1, opsonisation of necrotic cells with purified properdin did not show any enhancing activity (Fig. 5A). This was found to be true for all three types of phagocytes, namely DCs, Mφ1 and Mφ2 (Fig. 5B), suggesting that there are probably no high affinity properdin receptors on these phagocytes.

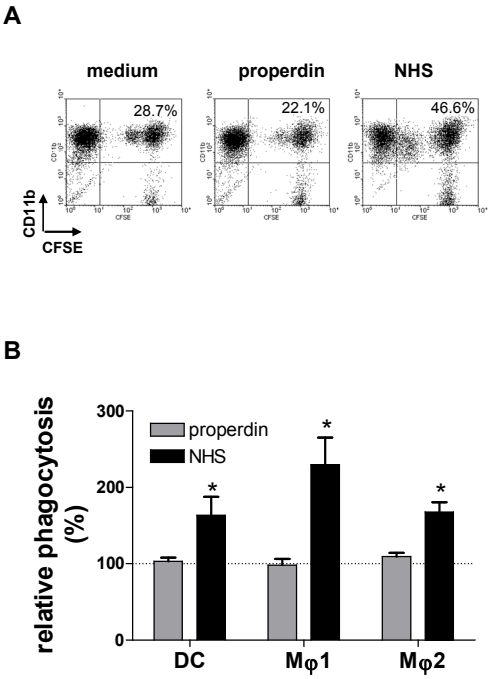

Figure 5. Properdin alone does not enhance phagocytosis

CFSE-labeled necrotic cells (1×10^5) were opsonized with or without properdin or 30% NHS, then co-cultured with DC, Mφ1 and Mφ2 in 1:1 ratio at 37°C for 0.5 h. Phagocytes were stained with a PE-conjugated mAb against CD11b and uptake was analyzed by a two-color flow cytometry. (A) A representative experiment of phagocytosis by Mφ1. (B) Relative phagocytosis was calculated as uptake of properdin- or NHS-opsonized necrotic cells versus non-opsonized cells. Data are mean ±SEM of 4 independent experiments. *, $p<0.01$, one sample *t* test.

Some opsonins from the innate immune system like C1q [13] and C4bP [36] have been suggested to modulate the inflammatory response. We therefore investigated whether properdin might regulate the inflammatory response by phagocytes. Necrotic cells were opsonized with or without properdin and then co-cultured with DCs, Mφ1 and Mφ2 in the presence of LPS. In three independent experiments, properdin significantly inhibited necrotic cells-induced TNF-α production by DCs (mean ±SEM of fold induction: 0.76 ±0.14, $p<0.05$) and Mφ1 (mean ±SEM: 0.59 ±0.29, $p<0.05$) (Fig. 6A). Since the anti-inflammatory Mφ2 did not produce TNF-α

[22], properdin did not show an effect (Fig. 6A). In contrast to the production of TNF-α, properdin opsonization of necrotic cells did not inhibit the anti-inflammatory cytokine IL-10 by both DCs (mean ±SEM: 1.16 ±0.28) and Mφ1 (mean ±SEM: 1.0 ±0.29). Interestingly, a significant increase of IL-10 (mean ±SEM: 2.99 ±1.2, $p<0.05$) by properdin-opsonized necrotic cells on Mφ2 was observed (Fig. 6B). Our data therefore suggest that properdin is a regulatory factor which limits the pro-inflammatory potential of necrotic cells.

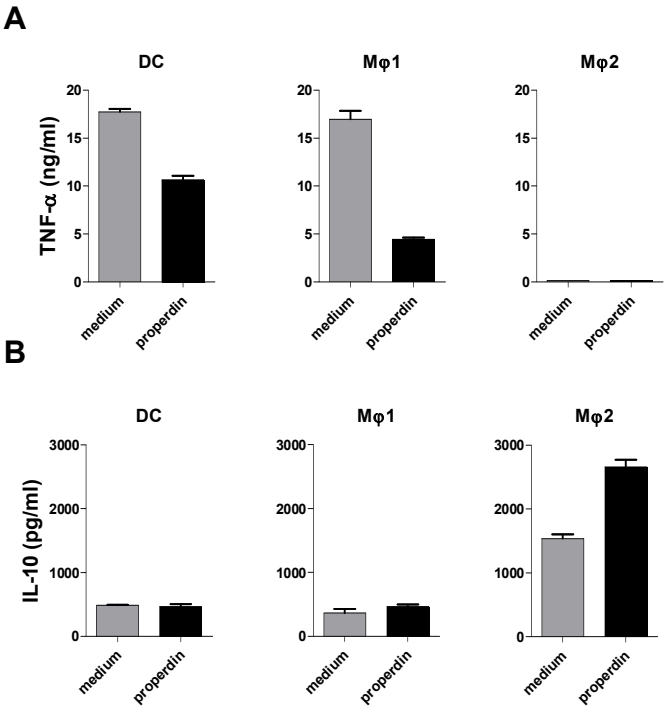

Figure 6. Properdin limits the pro-inflammatory potential of necrotic cells. Necrotic cells were opsonized with or without properdin, then co-cultured with DCs, Mφ1 and Mφ2 for 4 h followed by the addition of LPS (200 ng/ml) for another 20 h. Supernatants were collected for the measurement of TNF-α (A) and IL-10 (B) by DCs, Mφ1, and Mφ2. Data are mean ±SD of duplicate cultures and represent 3 independent experiments.

Discussion

We describe here that properdin, the only known naturally occurring positive regulator of complement, specifically binds to late apoptotic or necrotic cells, but not to early apoptotic cells. Furthermore, properdin binds to DNA and down-modulates the pro-inflammatory response induced by necrotic cells. We provide evidence that binding of properdin to late apoptotic cells and necrotic cells is independent of C3b, and serves as a focus point for the local amplification of the alternative pathway complement activation.

In the past, studies on complement-mediated clearance of dying cells have mainly focused on the classical pathway [10-12]. Properdin is a positive regulator of the alternative pathway, which has been shown to bind to C3b and to stabilize the

labile C3b-dependent C3 convertase C3bBb [19,20]. Two models have been proposed for the role of properdin in alternative pathway activation of complement. The first model suggests that properdin binds to a pre-existing clusters of surface-bound C3b [20]. The other model suggests that properdin first binds to a surface ligand (C3b, C3bB or C3bBb) via one of its subunits and then promotes the assembly of further C3bBb at the ligand-binding sites of its adjoining subunits [21]. Our data showed that properdin binds to late apoptotic and necrotic cells prior to C3 deposition on the cell surface, therefore we exclude the possibility of initial binding of properdin to the cells is via C3b. Evidence that binding of properdin to dying cells is independent of C3b was further supported by experiments showing that properdin binds strongly to necrotic splenocytes derived from $C3^{-/-}$ mice (Fig. 2D). Thus, we suggest that properdin binds to the cell surface of late apoptotic or necrotic cells first in the absence of C3 to ligands like DNA, and serves as a focus point for the local amplification of complement activation as soon as a C3 source is available. Properdin is indispensable for the alternative pathway activation of complement on these dying cells.

We have tried to identify the ligands on late apoptotic and necrotic cells to which properdin binds. Our data suggest that DNA is one of the targets for properdin on dying cells. Indeed, DNA is massively released by apoptotic and necrotic cells [31,37], and has been shown to be one of the major autoantigens exposed on apoptotic cell surfaces [4]. In pathological situations, DNA is considered as one of the immunologically active autoantigens [38], that can stimulate immune cells via Toll-like receptors [39,40]. In autoimmune lupus, DNA is one of the major immunogens to trigger autoantibody production [41]. Our finding that properdin binds strongly to DNA suggests a role for properdin in preventing unwanted immune activation when DNA is exposed on dying cells during a large scale cell death.

Next to DNA, we also showed that properdin does not compete with the binding of C1q and MBL to dying cells, suggesting that properdin binds to other as yet unknown ligands which are different from those for C1q and MBL. It remains a challenging task to identify the exact additional ligands for properdin on dying cells. We have excluded the possibility that C3b is the ligand on dying cells, which initially facilitates binding of properdin. It is also not likely that phosphatidylserine (PS) exposed on the surface of dying cells is the ligand since properdin does not bind to early apoptotic cells although they do express PS. Recent data suggest that properdin binds to sulfatide (sulfated glycosphingolipids) [42]. Whether sulfatide or other phospholipids are one of the key ligands on dying cells for properdin recognition is currently under investigation.

Interestingly, although purified properdin binds strongly to late apoptotic and necrotic cells, it does not lead to an enhanced phagocytosis by DCs or Mφ. This might suggest that there are probably no high affinity properdin receptors on these phagocytes. In agreement with the earlier observations that complement-mediated

phagocytosis is mainly dependent on bound iC3b [10,11], we propose that a role of properdin in the augmentation of phagocytosis is associated with the triggering of alternative pathway activation of complement.

It has been suggested that some serum factors such as C4BP [35] are able to dampen the pro-inflammatory potential induced by necrotic cells. The current paper shows that properdin prevents the pro-inflammatory response of the pro-inflammatory Mφ1 and DCs induced by necrotic cells. Importantly, the anti-inflammatory cytokine IL-10 is not inhibited, or even increased after opsonization of dying cells with properdin. This indicates that although necrotic cells are generally thought to release danger signals and usually uncontrolled in their dying process, the host is able to minimize the danger. Since we did not find any evidence of the presence of a potential properdin receptor on these phagocytes which could directly interact with properdin, it is tempting to hypothesize that down-regulation of pro-inflammatory potential of dying cells by properdin might be via an indirect way where properdin inhibits the pro-inflammatory signals exposed on dying cells. Therefore, we propose that properdin plays a regulatory role in the resolution of inflammation and maintenance of peripheral tolerance towards self antigen.

Involvement of properdin in the handling of dying cells was initially suggested by Kemper *et al.* reporting that properdin binds to early and late apoptotic cells [43]. Here we demonstrate that properdin binds predominantly to late apoptotic and necrotic cells, but not to early apoptotic cells, which is consistent with the binding of other complement opsonins like C1q, MBL (review in [8]) and Ficolin [14]. Indeed, not only complement factors, but also natural IgM [44] and pentraxin family members such as SAP [45] and PTX3 [46], bind to late apoptotic and necrotic cells, but hardly to early apoptotic cells. Therefore, it seems that most, if not all, of the serum opsonins engage with late apoptotic or necrotic cells. It has been accepted that removal of dying cells during their early stage of cell death ensures a silent process, whereas late apoptotic or necrotic cells might provide danger signals that activate phagocytes and thereby break peripheral tolerance [47-49]. Therefore, soluble opsonins are critical to promote a safe clearance of late apoptotic and necrotic material. Together with our previous findings that early apoptotic cells are preferentially cleared by anti-inflammatory macrophages [22], we suggest that a hierarchy exists in the clearance mechanism of dying cells [9], where uptake of early apoptotic cells by local macrophages with anti-inflammatory properties is an initial step, whereas complement-mediated processes via all three pathways are a rather late event, most likely ensuring a safe clearance when an overload of apoptosis or defects in phagocytic capacity occur.

In conclusion, we provide evidence that properdin binds specifically to late apoptotic and necrotic cells independent of C3b, and acts as a focus point for the local amplification of alternative pathway complement activation. Furthermore, properdin binds to DNA and limits the pro-inflammatory response by necrotic cells.

We propose here that properdin plays a dual role in the handling of dying cells. Firstly, it binds to DNA and probably other structures on late apoptotic and necrotic cells, potentially preventing the immune activation by the “danger” signals. Secondly, it is the limiting factor and focus point for local alternative pathway complement activation on these dying cells, thereby generating the vital opsonin iC3b to ensure a safe clearance.

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8

Summary and General Discussion

1. Summary of the thesis

1.1. Anti-inflammatory Mφ may be the default phagocytes that promptly and silently phagocytose apoptotic cells

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with a yet unclear pathogenesis. To date, different cytokines, soluble mediators, genetic factors and as well as many immune cells such as T cells, B cells, and antigen presenting cells (APCs), are thought to be involved in the initiation and progression of this disease [1-6]. The major targets in SLE are nuclear components (DNA, histones, ribonucleoproteins) [1,2,7], which are mainly derived from dying cells (apoptotic and necrotic cells) that serve as a reservoir for such autoantigens [8,9]. It has been suggested that defective clearance of dying cells may lead to the breakdown of peripheral tolerance and initiation of autoimmune SLE [3,4,10,11]. In this thesis, we have investigated the role of the innate immune system in the processing of dying cells and its immunological consequences.

We first discussed the current understanding of different subsets of DCs and Mφ in the handling of dying cells (**chapter 2**). Both *in vivo* and *in vitro* data suggest that there are different subsets of professional phagocytes that are actively involved in the clearance of dying cells. Presumably, also neighboring non-professional phagocytes might actively participate in these processes. We hypothesized that under steady-state conditions, the fate of dying cells is ultimately determined by local conditions and the composition of the tissue. Therefore a further dissection of distinct functional properties of phagocyte subsets is of particular importance to understand how the clearance of apoptotic cells by phagocytes is regulated and how this may lead to induction or loss of peripheral tolerance.

To address the questions mentioned above, we generated 3 types of phagocytes, namely DCs, Mφ1 and Mφ2 in parallel from the same donor and compared their capacity for phagocytosis of different types of particles (**chapter 3**). We found that M-CSF-driven Mφ2 are potent phagocytes that have the unique capacity to preferentially bind and ingest early apoptotic cells. This macrophage subset has intrinsic anti-inflammatory properties, as characterized by high IL-10 production in the absence of pro-inflammatory cytokines, such as IL-6 and TNF-α. Importantly, whereas the IL-6 and TNF-α production by GM-CSF-driven Mφ1 is inhibited upon uptake of apoptotic cells, the anti-inflammatory status of Mφ2 is retained during phagocytosis. These findings are consistent with earlier data that ingestion of apoptotic cells by Mφ is a non-inflammatory process [12]. We have tried to identify the specific receptors on Mφ2 that are responsible for the unique recognition of early apoptotic cells. CD14 is one of the important molecules which recognize apoptotic cells [13] and it is highly expressed by Mφ2. However, we found that CD14 was only used by Mφ2 for the tethering of apoptotic cells, but not for ingestion. Interestingly, Mφ2 showed more potent macropinocytosis compared to DCs and

Mφ1, and uptake of apoptotic cells was inhibited by a macropinocytosis inhibitor. Our studies suggest that, under steady-state conditions, IL-10-producing Mφ2 are prominently involved in the clearance of early apoptotic cells.

In a normal adult, resident tissue Mφ are derived from circulating bone marrow–derived monocytes, and are largely heterogeneous [14,15]. GM-CSF and M-CSF are two primary growth factors for the differentiation of macrophages from monocytes [16]. Under steady-state conditions, M-CSF is the only primary Mφ growth factor that is detectable in peripheral blood [17]. In contrast, GM-CSF is a pro-inflammatory cytokine, mostly generated during inflammation and hardly detectable in the circulation [18]. Furthermore, knockout mice lacking M-CSF or GM-CSF confirm that M-CSF is a more crucial growth factor than GM-CSF in Mφ differentiation [19-21]. Thus under steady-state conditions, M-CSF could be the default cytokine in driving Mφ differentiation. A relevant question therefore is: do in vitro polarized Mφ1 and Mφ2 exist in vivo?

In **Chapter 4**, a partial answer to the question raised above was obtained. We isolated human peritoneal Mφ (pMφ) freshly from patients on peritoneal dialysis and found that pMφ have phenotypical characteristics, including CD163 surface expression and lack of CD16, as Mφ2 generated *in vitro* upon stimulation of monocytes with M-CSF. Furthermore, we show that like Mφ2, pMφ have the capacity for endocytosis and macropinocytosis, and preferentially bind and ingest early apoptotic cells. These cells produce large amounts of IL-10 upon stimulation with LPS. Moreover, upon LPS stimulation both pMφ and Mφ2 exhibit a down-regulation of CD86, resulting in a reduced capacity of these cells to stimulate the proliferation of allogeneic T cells and an inhibition of Th1 cytokine release by these T cells. Thus, our data provide evidence for the first time that a countpart of *in vitro*-polarized Mφ2 exist in vivo, and that human pMφ resemble anti-inflammatory Mφ2. We propose that pMφ potentially maintain an anti-inflammatory condition in the peritoneal cavity.

Based on the data from **chapter 3** and **chapter 4**, we were intrigued by the opposing functions of polarized Mφ1 and Mφ2. Therefore, we questioned whether Mφ1 and Mφ2 are stable in terms of phenotype and function. In **chapter 5**, we described that Mφ1 exposed to M-CSF for an additional 6-day culture showed an increase in expression of CD163 and CD14, an increased capacity for phagocytosis of early apoptotic cells, and decreased capacity to stimulate T cell proliferation and inhibition of production of pro-inflammatory cytokines. *Vice versa*, Mφ2 exposed to GM-CSF converted these cells into Mφ1-like cells as envisioned by phenotype, cytokine profiles and capacity for phagocytosis and T cell stimulation. The data described above indicate that Mφ1 and Mφ2 can undergo reversible functional changes, namely into cells with anti-inflammatory or pro-inflammatory properties depending on the exposure to GM-CSF or M-CSF, respectively. Our

data support the importance of dynamic changes in cytokine environment in driving Mφ plasticity and provide moral perspectives for local therapeutic targeting.

1.2. Properdin is an important regulator in the processing of dying cells

The innate immune system has a key role in host defense against invading pathogens. Recent studies indicate that molecules of the innate immune system, including complement components and pentraxins, can also target their effect to self tissue such as dying cells. **Chapter 6** reviewed the pivotal role of the innate immune system in the clearance of apoptotic cells. Among the opsonins of apoptotic cells, complement factors, including C1q, and complement-activating members of the pentraxin family play an important role. We proposed that some soluble factors, such as C1q, prevent autoimmunity and maintain self-tolerance by supporting the efficient clearance of apoptotic material, as well as by actively modulating phagocyte function.

Consequently, we identified that another complement component, properdin, binds predominantly to late apoptotic and necrotic cells, but not to early apoptotic cells (**chapter 7**). Although previous studies indicated that properdin binds to pre-existing clusters of C3b on surfaces of cells, our current data suggest that binding of properdin to late apoptotic and necrotic cells can occur independently of C3b. Furthermore, we showed that properdin serves as a focus point for local amplification of alternative pathway complement activation. Importantly, properdin exhibits a strong interaction with DNA that is released and exposed on these cells. It is of interest to note that properdin does not compete with C1q and MBL for binding to late apoptotic or necrotic cells, although C1q and MBL interact with DNA as well [22,23], suggesting that next to DNA, properdin may bind to other as yet unknown ligands. We found no direct effect of purified properdin in the enhancement of phagocytosis of late apoptotic or necrotic cells. However, we did find that opsonisation of necrotic cells by properdin limits the pro-inflammatory potential of necrotic cells by reducing TNF-α release by DCs and Mφ, whereas IL-10 production is retained. Our data indicate that recognition of dying cells by properdin is essential to drive alternative pathway complement activation and that properdin actively promotes an anti-inflammatory response. The studies above provide additional knowledge on complement-mediated phagocytosis of dying cells, involving all three pathways of complement activation [24-26].

It will be a major challenge in the near future to integrate the information obtained in the past years and to distinguish the relative contribution of different components of the innate immune system to the clearance of apoptotic cells. However, also other parts of the immune system might have a direct or an indirect role. Opsonization of apoptotic cells with autoantibodies could potentially facilitate uptake, either via Fc receptor (FcR)-mediated process or via activation of the

complement system. We recently observed that SLE sera contain variable amounts of antibodies (Abs) directed against early apoptotic, late apoptotic /necrotic cells. The contribution of autoantibodies to phagocytosis is still under investigation. Importantly, as already stated in our hypothesis, since expression of FcR is different among phagocytes (**Xu, unpublished**), the effector mechanisms will also strongly depend on the type of phagocyte which encounters the apoptotic cells.

2. General discussion and future directions

2.1. Is SLE a DC-driven disease?

Most experiments described in this thesis have been performed with Mφ and Mφ subsets. However, several non-published experiments have been performed in parallel using monocyte-derived DCs. In this part of the discussion some of these results will be presented and these findings will be discussed in the context of recent developments on the involvement of DCs, or specific DC subsets, in the pathogenesis of SLE.

Many factors are involved in the initiation and progression of SLE, such as cytokines, soluble mediators, genetic factors as well as many immune cells such as T cells, B cells, and cytokines [1-6]. Among the latter, one important cytokine is type I interferon (IFN-α) [5,6], as in agreement with an earlier report that IFN-inducible genes are up-regulated in peripheral blood mononuclear cells (PBMCs) from patients with SLE [27]. Importantly, it was suggested that IFN-α mediates unabated differentiation of DCs, which drive the autoimmune response [28]. DCs acquire antigens from apoptotic cells and (cross-)present these antigens to class I- or class II-restricted T cells [29-31]. It was suggested that late apoptotic cells and necrotic cells, but not early apoptotic cells induce maturation of DCs, and present antigens in the absence of exogenous danger signals [31,32]. However, others suggest that such maturation is due to possible contamination /infection [33]. Therefore it remains controversial whether late apoptotic and necrotic cells one the one hand activate DCs, and that early apoptotic cells on the other hand are immunologically “null”.

To address this controversy, we have tried to study how dying cells at different stages of cell death influence DC biology. We found that DCs loaded with early apoptotic, late apoptotic, or necrotic cells all inhibited pro-inflammatory cytokine release (**Xu, unpublished**). In contrast to the findings of others, we observed that neither late apoptotic nor necrotic cells induce DC maturation. Furthermore, We were unable to demonstrate that DCs loaded with early apoptotic, late apoptotic, or necrotic cells (cross-)present antigen to T cells. This finding is in agreement with a recent study showing that DCs that had captured apoptotic cells only in the presence, but not in the absence of TLR4 triggering by LPS, could present antigens to CD4 T cells [34]. Thus extra danger signals provided by TLR ligands

could make a substantial difference in the initiation of autoimmunity by apoptotic cell-loaded DCs.

Plasmacytoid DCs (pDCs) are a specialized subset of DCs that produce IFN-α upon activation by virus. Although *in vitro* pDCs hardly ingest apoptotic cells [35], they do internalize immune complexes (ICs). It has been shown that apoptotic or necrotic cells combined with IgG from patients with SLE induce the production of IFN-α by purified pDCs [36]. Furthermore, ICs derived from patients with SLE, upon intracellular delivery via CD32, were able to activate pDCs through toll like receptor 9 (TLR9) [37]. This observation was extended in another study showing that small nuclear RNAs within ribonucleoprotein particles activate pDCs through TLR7 [38], suggesting a link between pDCs and autoimmmunity to autoantigens containing DNA or RNA. These studies have provided novel insights in the possible mechanisms of loss of peripheral tolerance to autoantigens in SLE.

Nevertheless, it is too early to conclude that SLE is indeed a DC-driven disease. Firstly, whether late apoptotic or necrotic cells activate DCs is still a matter of debate. Secondly, the pivotal contribution of soluble factors in controlling danger singles requires further investigation. Existing data suggest that factors such as properdin (**Chapter 7**) and C4 binding protein (C4BP) [39] are able to bind DNA exposed or released by necrotic cells, and potentially limit their pro-inflammatory potential. Furthermore, we recently found that complement component C1q is able to modulate DCs into a cell type with tolerogenic properties (**Castellano *et al*., Eur. J. Immunol. in press**). These findings indicate that the host itself is able to minimize the effect of danger signals and control inflammation by the use of inherent innate immune factors.

2.2. Tolerogenic Mφ

Plasticity of pro-inflammatory Mφ1 and anti-inflammatory Mφ2 is a matter of interest for further exploration. We showed in this thesis that both Mφ1 and Mφ2 are not at the end stage of cell differentiation and are adaptable to changes in the microenvironment concerning their immunological functions (**chapter 5**). For example, exposure of pro-inflammatory Mφ1 to M-CSF resulted in down-regulation of their pro-inflammatory cytokine profile such as IL-6 and TNF-α production and up-regulation of the production of the anti-inflammatory cytokines such as IL-10. Similar functional changes were also reflected in their capacity to stimulate T cells.

Next to growth factors, mediators from the innate immune system might impact these polarized cells to mount or tolerize an immune response. For example, during an infection pathogens trigger Toll-like receptors (TLRs) on myeloid cells and activate these cells. It is of interest to investigate how Mφ1 and Mφ2 respond to TLR triggering. Our preliminary data showed that TLR2 engagement e.g. by

peptidoglican (PGN) is able to boost both IL-10 and IL-6 production by Mφ1 (Fig 1) (**Xu, unpublished**). In contrast, anti-inflammatory Mφ2 failed to produce IL-6 by any means of TLR triggering as indicated. Our data imply a role for TLR agonists in modulation of immunological function of myeloid subsets (under investigation).

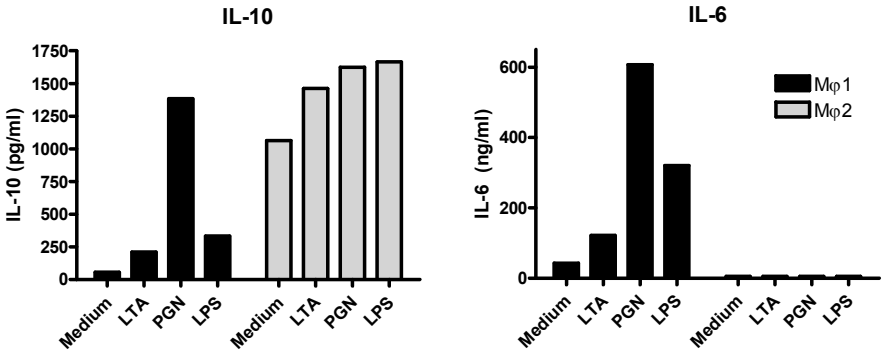

Figure 1. TLR activation by DCs, Mφ1 and Mφ2. Mφ1 and Mφ2 (25,000 cells) were stimulated with TLR agonists Lipoteichoic Acid (LTA) or peptidoglican (PGN) (1 ug/ml), or with the TLR4 agonist LPS (200 ng/ml) for 24 hours in 48-well plates. Supernatants were harvested and measured for IL-10 and IL-6 production by ELISA.

Another way to activate Mφ is via T cell cytokines such as IFN-γ (Th1 cytokine) or IL-4 (Th2 cytokine) [14]. IFN-γ has long been considered the most powerful cytokine produced by Th1 cells to exert antiviral and bactericidal activities against pathogens [40]. However, recent data provide evidence that both DCs [41] and Mφ [42] are able to secrete IFN-γ themselves. Therefore these cells are susceptible to autocrine activation by IFN-γ. Furthermore, IFN-γ actively induces indoleamine 2,3-dioxygenase (IDO) expression on DCs and Mφ. IDO is an tryptophan-degrading enzyme involved in immune suppression and tolerance induction [43,44]. In pilot experiments, we showed that IFN-γ induces IDO expression on both Mφ1 and Mφ2 at RNA level, as measured by RT-PCR (Figure 2A) and real-time PCR (Figure 2B) (**Xu, unpublished**). Furthermore, we found that IFN-γ-stimulated Mφ1 and Mφ2 are impaired in the induction of allogeneic T cell proliferation (data not shown). We are currently actively investigating the mechanisms that are involved in the inhibition of T cell proliferation by IFN-γ.

Together, the plasticity of Mφ subsets opened the door for further characterization of these cells, and we have obtained insight in modulating their functions towards cells with tolerogenic properties. It sheds some light on the potential application of these tolerogenic Mφ in preventing transplant rejection.

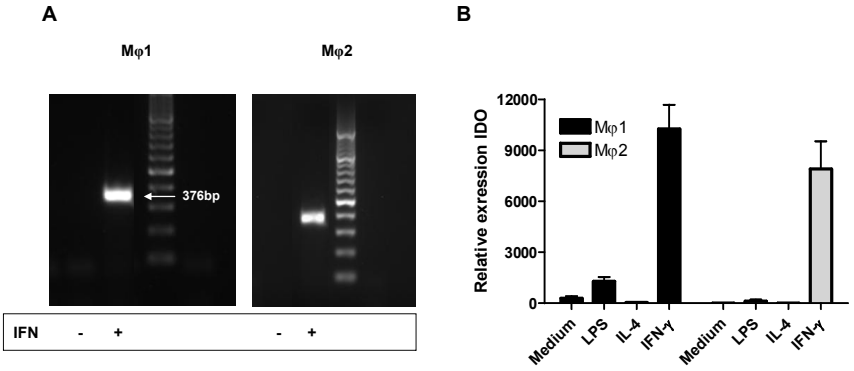

Figure 2. Induction of IDO by IFN-γ on Mφ1 and Mφ2. Mφ1 and Mφ2 were stimulated with IFN-γ for 24 h and RNA was isolated from these cells. IDO expression was measured by RT-PCR (A) or real-time PCR (B).

2.3. Apoptotic cells in the regulation of immune tolerance via regulating Treg and TH17 cells

As discussed throughout the current thesis, apoptotic cells play an essential role in the regulation of immune system in terms of self-tolerance and autoimmunity. How such a balance is regulated is not fully understood. We showed here (**Chapter 3**) that a subset of phagocytes with anti-inflammatory properties (namely Mφ2) have the capacity to preferentially phagocytose early apoptotic cells, resulting in a sustained high production of IL-10. These type of phagocytes exist also *in vivo*, namely in the peritoneal cavity and share characteristics with *in vitro*-polarized Mφ2 (**Chapter 4**). We therefore speculate that *in vivo* clearance of early apoptotic cells is largely confined to a specialized subset of phagocytes with anti-inflammatory properties, leading to the maintenance of self tolerance. If overloaded early apoptotic cells are not cleared promptly, these cells may progress into late apoptosis or secondary necrosis, which consecutively activate newly recruited pro-inflammatory phagocytes. Thus an immunogenic self-antigen presentation might occur which facilitates the development of autoimmune disease.

Failure of self-tolerance often leads to the development of autoimmune disease, whereas maintenance of self-tolerance is critically engaged with the regulatory CD4 T cells (Treg) [45]. Treg cells produce immune suppressive cytokines such as IL-10 and /or TGF-β. One of major Treg cells is defined as $CD4^+CD25^+FOXP3^+$ T cells, and can be differentiated by TGF-β [46]. A breakthrough has been made recently by Bettelli *et al.* who showed that IL-6 completely inhibits the generation of $Foxp3^+$Treg cells induced by TGF-β. Instead, IL-6 and TGF-β together induce the

differentiation of newly described pathogenic Th17 cells from naïve T cells [47]. Th17 cells are the cells producing IL-17 and have a crucial role in the induction of autoimmune tissue injury [48]. Thus there is a reciprocal developmental pathway for the generation of pathogenic TH17 cells and protective Treg cells in the immune system depending on the cytokine balance between IL-6 and TGF-β, probably some other unknown cytokines. As many cells produce IL-6 and TGF-β, the main source of these cytokines remains to be investigated [49], and in addition it is of importance to study how the cytokine balance might be ticked.

As discussed earlier, IL-10 and TGF-β are the main cytokines released by phagocytes which have ingested (early) apoptotic cells. Therefore, we hypothesize that a local production of cytokines during clearance of apoptotic cells *in vivo* by anti-inflammatory phagocytes (such as Mφ2 like cells) might server as a primary source of IL-10 and TGF-β to initiate the differentiation of Treg cells for the maintenance of peripheral tolerance. It has been proposed that apoptotic cells induce transplant tolerance via the generation of Treg cells [50,51]. The very first study showed infusion of apoptotic spleen cells induces TGF-β-dependent CD4+CD25+ T cell expansion in a bone marrow transplantation setting [52]. It was further demonstrated in another study that administration of donor apoptotic cells results in indefinite graft survival mediated by generation of Treg [53]. Therefore, use of the inhibitory effects of apoptotic cells on the anti-donor response provides a new approach to treat transplant rejection.

On the other hand, delayed clearance of apoptotic cells (or overload with late apoptotic cells /necrotic cells) might be a trigger to shift the cytokine balance in terms of IL-6, TGF-β and probably other cytokines, and thereby leads to a skewing of differentiation of TH17 cells over Treg cells (see Figure 3). It is worthwhile to explore how local apoptotic cells and phagocytes regulate T cell subset differentiation. It should be noted that most of data mentioned above have been obtained in the murine system. Although it could be anticipated that a similar balance of Th cells exists in the human system, the exact regulation by the cytokine environment is not fully established.

3. Concluding remarks

Throughout this thesis, we have tried to obtain insight in how apoptotic cell clearance is regulated by cells or mediators from the innate immune system. We provide evidence that anti-inflammatory Mφ2 fulfil the demand to efficiently clear early apoptotic cells in a silent manner. This probably provides an explanation on how apoptotic cell clearance contributes to the maintenance of self-tolerance under steady-state conditions. However, it remains largely unclear how the handling of dying cells translates into a break in peripheral tolerance and induction of autoimmunity.

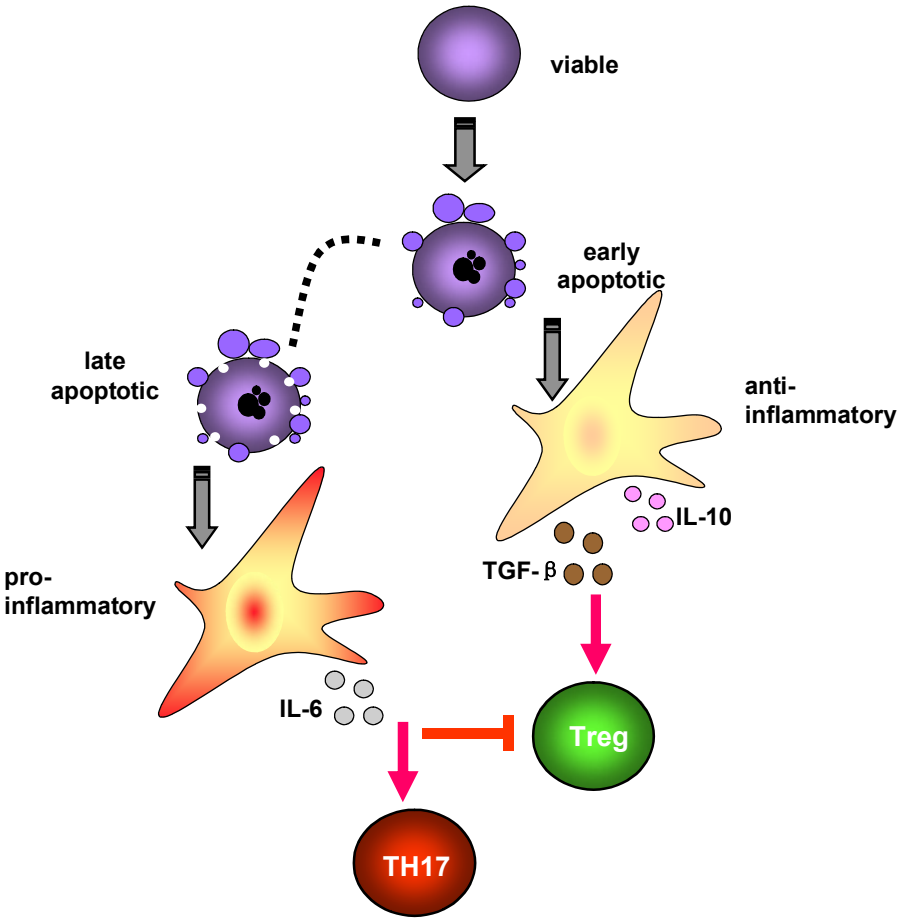

Figure 3. Hypothesis on apoptotic cells in the regulation of immune tolerance via regulating Treg and TH17 cells. We speculate that early apoptotic cells are cleared promptly and efficiently by resident anti-inflammatory phagocytes, leading the release of anti-inflammatory cytokines such as IL-10 and TGF-β. These cytokine serves the primary triggers to the differentiation of Treg cells. When early apoptotic cells are not cleared promptly, they may progress into late apoptosis or necrosis, leading to recruitment of newly elicited pro-inflammatory phagocytes to participate in the clearance. Consequently, pro-inflammatory cytokines such as IL-6 are release, and a cytokine balance is disturbed. As a result, Treg cells are inhibited by IL-6, and pathogenic TH17 cells are promoted by the combination of IL-6 and TGF-β.

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Samenvatting (Dutch summary)

Systemische lupus erythematosis (SLE) is een auto-immuunziekte met reactiviteit tegen verschillende lichaamseigen weefsels en oplosbare moleculen. De oorzaak van deze ziekte is nog niet volledig opgehelderd. De betrokkenheid van zowel T en B cellen als antigen presenterende cellen (APC's) is genoegzaam aangetoond. Ook cytokines en chemokines lijken een rol te spelen, zowel bij de inductie als tijdens de progressie van de ziekte. Daarnaast is duidelijk aangetoond dat de autoantilichamen tegen lichaamseigen componenten zoals tegen DNA, histonen, ribonucleaire eiwitten en tegen complement factoren van belang zijn bij deze ziekte. Een belangrijk aantal van deze antigenen is afkomstig van cellen die een geprogrammeerde celdood (apoptose) hebben ondergaan. Het is bekend dat dagelijks miljarden cellen in het menselijke lichaam in apoptose gaan om de homeostase in stand te houden. De gedachte is dat bij patiënten met SLE er een abnormale presentatie en/of verwerking van apoptotisch materiaal plaatsvindt waardoor er een abnormale immuunreactie optreedt. In dit proefschrift is vooral aandacht besteed aan de rol die het natuurlijk immuunsysteem speelt in de wijze waarop cellen die dood gaan het immuunsysteem beïnvloeden.

In **hoofdstuk 2** wordt de stand van zaken aangaande de verwerking van dode cellen door dendritische cellen (DC's) en macrofagen (Mø's) samengevat. Er zijn duidelijke aanwijzingen dat zowel in vitro als in vivo verschillende subpopulaties van DC's en Mø's bestaan die allen direct betrokken zijn bij de klaring van dode cellen. Verder onderzoek is nodig om na te gaan hoe en in welke mate deze verschillende subpopulaties van DC's en Mø's betrokken zijn bij het proces van klaring en het effect van deze dode cellen op de DC's en Mø's zelf.

Om deze vragen te benaderen hebben we 3 typen van fagocyten, nl. DC's, Mø1 en Mø2 in parallel van dezelfde donoren vergeleken aangaande hun vermogen om verschillende soorten dode cellen te herkennen en te elimineren (**hoofdstuk 3**). Gevonden werd dat M-CSF-gedreven Mø2 zeer potente fagocyten zijn met een uniek vermogen om preferentieel vroeg apoptische cellen te binden en op te nemen. Deze macrofaag subpopulatie heeft intrinsiek anti-inflammatoire eigenschappen met een hoge productie van IL-10, in de afwezigheid van de productie van pro-inflammatoire cytokines zoals IL-6 en TNF-α. Terwijl de productie van pro-inflammatoire cytokines zoals IL-6 en TNF-α door Mø1 geremd wordt door opname van apoptische cellen, wordt de productie van het anti-inflammatoire cytokine IL-10 door Mø2 juist versterkt. In dit proces lijkt de herkenning van apoptische cellen door deze macrofaag subpopulaties gereguleerd te worden door CD14, dat in hoge dichtheid op het oppervlak van Mø2 gevonden wordt. Daarnaast werd gevonden dat Mø2 ook sterke macropinocytose vertoonde verleken met Mø1 en DC's. Daarom denken wij dat onder normale fysiologische

omstandigheden vooral IL-10 producerende Mø2 een prominente rol spelen in de klaring van apoptische cellen.

De vraag die zich voordoet is of er ook vergelijkbare subpopulaties van macrofagen te vinden zijn in vivo. Een eerste aanwijzing dat dat inderdaad zo is, werd gevonden uit onderzoek bij patiënten met een nierziekte die behandeld worden met peritoneaal dialyse. Deze patiënten worden frequent gespoeld met dialyse vloeistof die ingebracht wordt in hun peritoneum. Na de dialyseperiode wordt deze vloeistof geledigd uit het peritoneum. Uit de vloeistof van verschillende patiënten werden macrofagen geïsoleerd en onderzocht op membraan kenmerken en functie. Gevonden werd dat peritoneaal macrofagen duidelijke karakteristieken gemeen hebben met Mø2 (**hoofdstuk 4**). De peritoneaal macrofagen produceren ook grote hoeveelheden IL-10 na stimulatie met LPS. Ook hebben deze cellen een lage expressie van CD86 en hebben ze een geringer vermogen om T cellen te stimuleren. Dus peritoneaal macrofagen lijken in vele opzichten op de subpopulatie van in vitro gegenereerd Mø2. We stellen daarom voor dat peritoneaal macrofagen de equivalent zijn van anti-inflammatoire Mø2, betrokken bij de handhaving van de normale homeostase in het peritoneum.

Wij werden geïntrigeerd door de tegenovergestelde functie van Mø1 en Mø2. Daarom vroegen wij ons af of deze twee subpopulaties van macrofagen een stabiel phenotype hebben. In **hoofdstuk 5** beschrijven wij dat na generatie van Mø1 en Mø2 in vitro en vervolgens stimulatie met de tegenovergestelde stimulus resulteert in een reversibel fenotype en functie van beide typen macrofagen suggererend dat Mø1 en Mø2 geen eindtype fenotypes zijn en dat deze macrofagen onderhevig kunnen zijn aan dynamische veranderingen, mogelijk geïnduceerd door locale omstandigheden in het micromilieu.

Een nieuwe dimensie aan de herkenning van apoptische cellen en regulatie van DC's en macrofagen werd ingegeven door onze vondst dat properdine, een positieve regulator van het complement systeem, bindt aan laat apoptische en necrotische cellen. Interessant is dat de binding van properdine aan apoptische cellen een gelijksoortig patroon van binding vertoont als andere moleculen van het natuurlijk immuunsysteem zoals MBL, C1q, CRP en SAP. De literatuurgegevens over de betrokkenheid van het natuurlijke immuunsysteem bij de klaring van apoptische cellen is uitgebreid samengevat in **hoofdstuk 6**. In **hoofdstuk 7** beschrijven wij dat DNA mogelijk de bepalende factor is in de binding van properdine aan apoptische cellen en necrotische cellen. Belangrijk is de observatie dat properdine binding leidt tot een anti-inflammatoire reactie van macrofagen en DC's op herkenning van apoptisch materiaal. Deze bevindingen samen met onze observaties over de differentiële effecten van subpopulaties van fagocyten suggereren dat de herkenning en de daaropvolgende immuunrespons een delicate balans vormen tussen de verschillende spelers in dit veld. Daarom zal bij mogelijke

therapeutische interventies een tussenweg gevonden moeten worden om bij ziekte de balans op de juiste wijze te corrigeren, zonder dat de herstelde homeostase weer verstoord wordt.

Uit het onderzoek beschreven in dit proefschrift is het ook duidelijk geworden dat de initiële hypothese dat een beperkt aantal spelers in het immunologische veld van belang zijn voor de klaring van dood materiaal te simpel is en dat systematisch onderzoek van belang is om ons inzicht in dit complexe proces verder te verscherpen. De uitdaging voor de komende tijd is om deze en nog te verkrijgen resultaten op een begrijpelijke wijze te integreren.

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Curriculum vitae

Wei Xu was born on Nov. 6th 1976, in Zhangjiagang city, P.R. China. He went to Nanjing University of Traditional Chinese Medicine in 1994 and obtained his Bachelor Degree of Medicine in 1999. He was then appointed as a resident at Zhanjiagang Traditional Hospital. From 2001 to 2003, he was enrolled as one of the first international students of the Master program in Biomedical Sciences at the Faculty of Medicine of the Leiden University. During that period, he conducted two practical training periods at the Dept. of Pathology (Dr. J.J. Baelde and Dr. E. de Heer) and at the Dept. of Hematology (Dr. W.A. Noort and Prof. Dr. W.E. Fibbe), the Leiden University Medical Center (LUMC). After completion of his Master degree, he started his PhD program at the Dept. of Nephrology, LUMC, in July 2003, under the supervisions of Dr. C. van Kooten and Prof. Dr. M.R. Daha. He received the Chinese Government Award for Outstanding Self-Financed Students Abroad (2006) from the Chinese Scholarship Council /Ministry of Education. Starting from December 2007, he will work as a postdoctoral fellow at the Baylor Institute for Immunology Research (Dallas, USA), under the supervision of Prof. Dr. J. Banchereau.

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Color figures

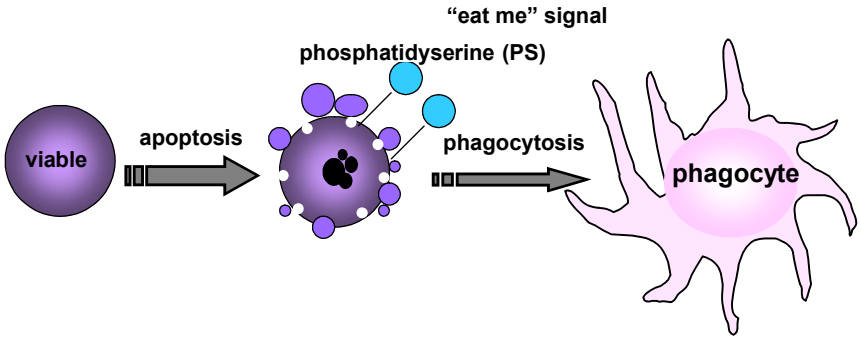


Figure 3, chapter 1, page 12

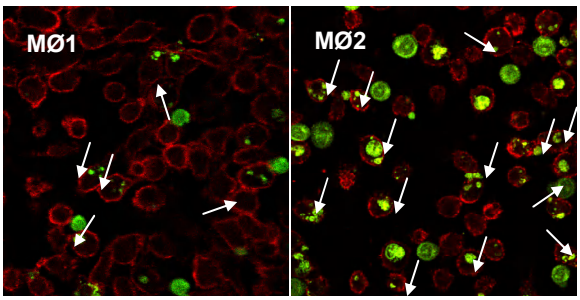


Figure 2C, chapter 3, page 40

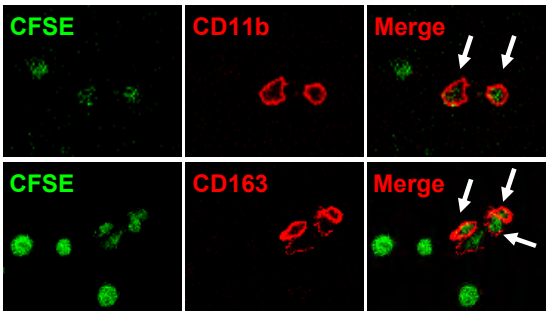
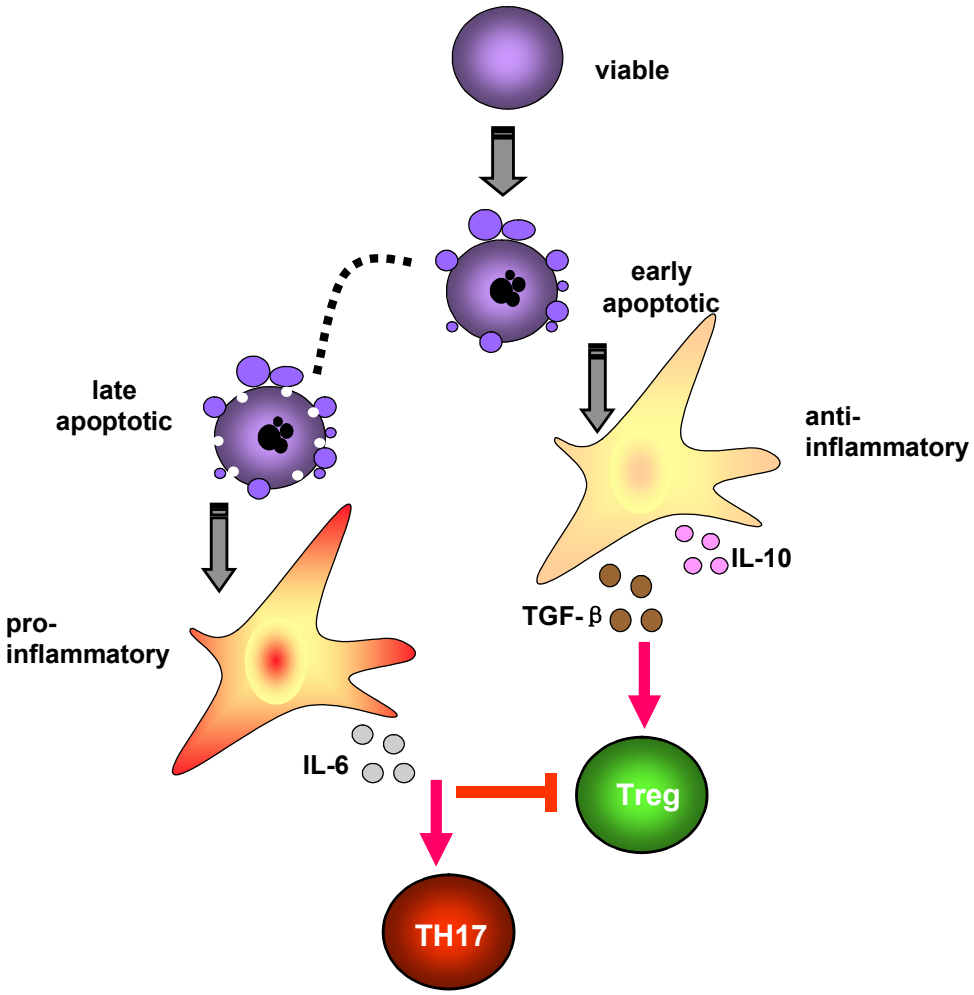


Figure 1B, chapter 4, page 57



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