The role of the nuclear receptor Nr4a1 as mediator of the anti-inflammatory effects of apoptotic cells

Die Rolle des nukleären Rezeptors Nr4a1 als Mediator der anti-inflammatorischen Effekte apoptotischer Zellen

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Table of Contents

Abstract

The Nuclear receptor subfamily 4 group A (Nr4as) are members of the superfamily of nuclear receptors. These orphan nuclear receptors (NRs) are known to play a key role during glucose metabolism, cellular differentiation and the immune response and are expressed in many cell types such as adipocytes, myocytes as well as neuronal and immune cells. Nr4a1, also known as nerve growth factor IB (NGFIB) or Nur77, has been implicated in the control of the inflammatory response, as negative feedback regulator of NF-κB signalling and as key regulator during the differentiation of Ly6C-low resident monocytes.

MΦs are considered responsible for engulfment and non-immunogenic removal of apoptotic cells (ACs). Notably, ACs exert anti-inflammatory effects on MΦs but the underlying mechanisms are still poorly understood. Here, we describe a novel role of Nr4a1 as key mediator of the anti-inflammatory effects of apoptotic cells in tissue-resident MΦs. We observed that ACs strongly and rapidly induced the expression of Nr4a1 in resident MΦs. This effect was dependent on the recognition of phosphatidylserine present on the surface of ACs and required p38- p44/p42-dependent signalling events within the MΦ. The resident MΦs showed an altered cytokine profile as well as increased NF-κB activity. Importantly, the lack of Nr4a1 partially abrogated the anti-inflammatory effects of ACs in resident MΦs. Nr4a1$^{-/-}$ mice showed an aberrant pro-inflammatory response to ACs *in vivo* as well as a break of self-tolerance in the murine model of pristane-induced lupus. Taking together, these data show a so far unrecognized role for Nr4a1, as a major mediator of the anti-inflammatory effects of apoptotic cells and as key factor in the maintenance of self-tolerance to AC-derived autoantigens.

Zusammenfassung

Mitglieder der Nr4a Untergruppe an nukleären Rezeptoren stellen wichtige Regulatoren des Glukose Metabolismus und des angeborenen und adaptiven Immunsystems dar und werden in unterschiedlichen Zelltypen wie z.B. in Fett- und Muskelzellen, Neuronen und Zellen des Immunsystems exprimiert. Nr4a1 (auch bekannt als nerve growth factor IB (NGFIB) oder Nur77) dürfte eine wichtige Rolle in der Kontrolle der Entzündungsantwort und des NF-κB Signalwegs einnehmen und kontrolliert zudem die Differenzierung einer Monozyten Subgruppe (Ly6C-low resident monocytes).

Makrophagen (MΦ) sind zentral an der Phagozytose und nicht-immunogenen Entsorgung apoptotischer Zellen (AZ) beteiligt. Zwar übt die Phagozytose von AZ übt einen hemmenden Einfluss auf die inflammatorische Antwort des beteiligten Makrophagen aus, jedoch sind die zugrundeliegenden molekularen Mechanismen nicht vollständig verstanden. Im Rahmen der vorgelegten Arbeit konnten wir eine bisher unbekannte Rolle von Nr4a1 als Schlüsselmediator dieser anti-inflammatorischen Effekte von AZ in residenten Gewebsmakrophagen beschreiben. Die Phagoytose von AZ induzierte eine schnelle und ausgeprägte Expression von NR4a1 im aufnehmenden MΦ. Sowohl die Erkennung von an der Oberfläche der AZ exponiertem Phosphatidylserin, als auch p38- p44/p42-abhängige Signalwege im MΦ waren hierbei für die Expresion von Nr4a1 vonnöten. $Nr4a1^{-/-}$ MΦ zeichneten sich durch ein verändertes Zytokinprofil, sowie eine überschießende Aktivierung des NF-κB Signalweges aus. Zudem waren die anti-inflammatorischen Effekte in $Nr4a1^{-/-}$ MΦ deutlich abgeschwächt. Ebenso zeigten $Nr4a1^{-/-}$ Mäuse nach Injektion von AZ eine überschießende Zytokinantwort und wiesen einen Bruch der immunologischen Toleranz im Mausmodell des Pristan-induzierten Lupus auf. Diese Daten belegen eine bisher unbekannte Rolle von Nr4a1 während der nicht-inflammatorischen Entsorgung von AZ und deuten auf eine wichtige Rolle dieses Transkriptionsfaktors in der Aufrechterhaltung der immmunologischen Toleranz hin.

1. Introduction

1.1 Macrophages

In 1883, the russian/french biologist Elie Metchnikoff (1845-1916) discovered a population of cells having “capacity to stretch out prolongations” and “capable of consuming foreign bodies”[1]. Considering these characteristics, he named them phagocytes (from the Greek “phago” for devour and “cytes” for cells) and in this way introduced a new concept: inside a body are specialized cells, the phagocytes, taking charge of the defence against invading organism: the starting point of innate immunity (fig.1).

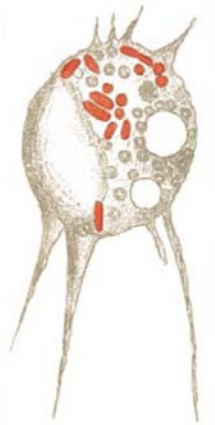

Figure 1: Drawing of bacterial phagocytosis by Elie Metchnikoff [1]

Since this first observation, 131 years ago, our understanding about the origin, the development and the function of macrophages has enormously expanded. However, many key aspects of their biology remain unclear.

1.1.1 Origin and development

Until recently, the origin and development of macrophages were described as clear and well defined processes. Thus, pluripotent bone-marrow haematopoietic stem cells (HSC) are considered as the source of all cellular elements in the blood. Among others, they can differentiate into two common precursors: the common lymphoid progenitor, which gives rise to the lymphoid lineage (B and T lymphocytes and Natural Killer (NK) cells) and the macrophage and dendritic cell progenitor (MDP). The MDP can differentiate into two other groups of precursors, then released in the blood: the common dendritic cell precursors (CDP) which will provide the common dendritic cells (cDC) and the plasmacytoid dendritic cells

(pDC) and monocytes, respectively. In mice, $CD11b^+$ $CD115^+$ monocytes are classified into two distinct subsets, based on their cell-surface marker expression. The $Ly6C^{low}$ $CCR2^{low}$ $CX3CR1^+$ $CD62L^-$ resident monocytes have patrolling activities in the vascular system and can populate the tissue after injury or infection [2]. $Ly6C^+$ $CCR2^+$ $CX3CR1^{low}$ $CD62L^+$ monocytes form another subgroup that is referred to as inflammatory monocytes, due to their capacities to migrate into the inflamed tissues [3] during the early stage of inflammation. The dogma on the Mononuclear Phagocyte System (MPS), largely established in 1968 [4], suggest that under Macrophages Colony-Stimulating Factor (M-CSF) stimulation, monocytes are able to enter the tissues and differentiate into macrophages [5, 6]. From a haematopoietic origin, these cells should be continuously reconstituted by self-renewal and proliferation from the bone-marrow-derived monocytes [3]. However, a lot of observations such as the presence of macrophages at embryonic stage (a time point before the appearance of the bone-marrow and the HSC) [7], their maintenance independently from bone marrow-derived monocytes in steady state [8], or the fact that microglia (a macrophage population from the brain) maintain themselves through adulthood without input of the HSC [9], and the high degree of heterogeneity (in term of function and phenotype) between the different monocyte and macrophage subsets, led the scientific community to call this dogma into question. These findings lead to the more recent hypothesis that inflammatory and tissue-resident macrophages derive from at least two distinct origins (fig.2).

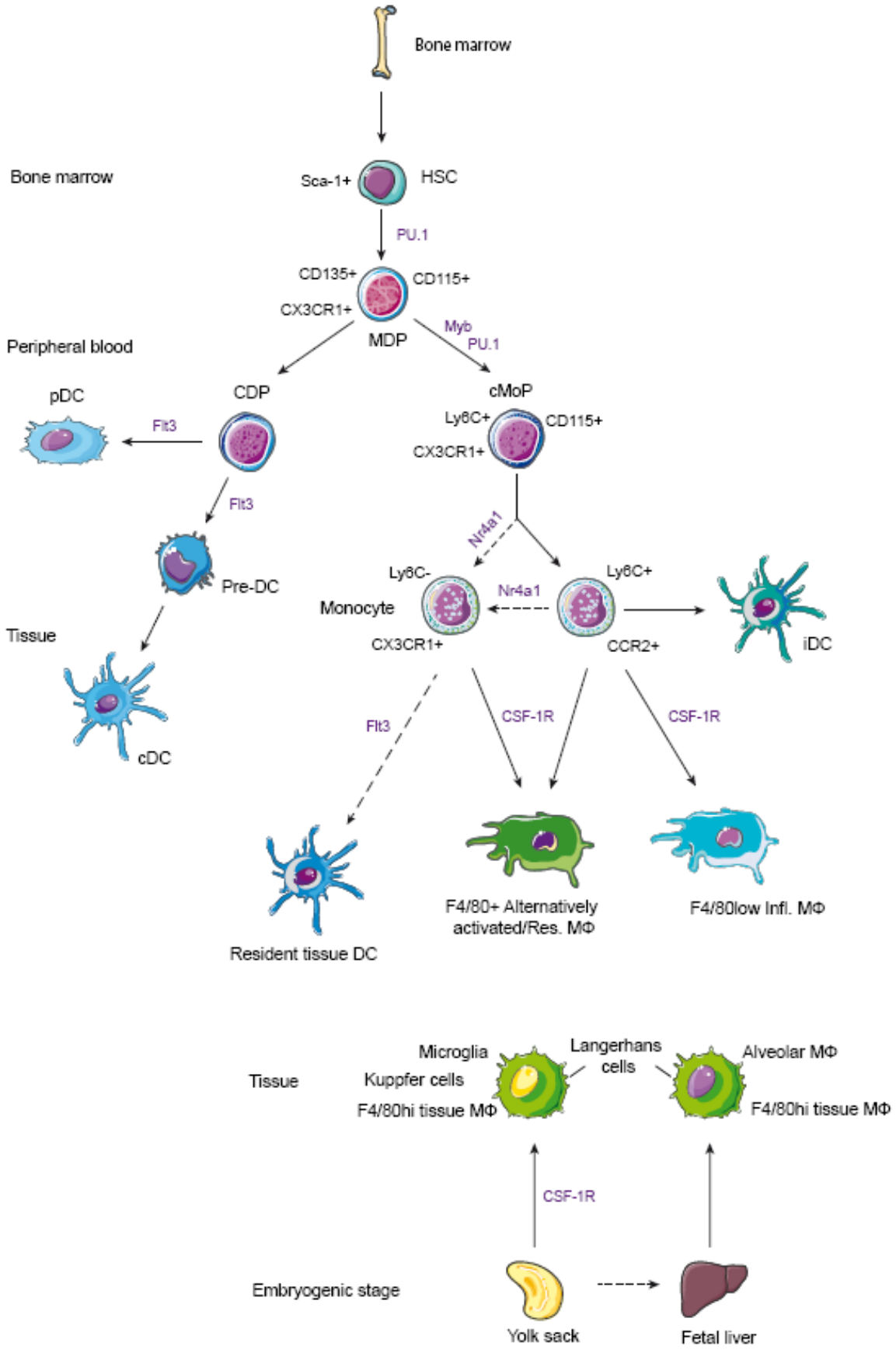

Figure 2: Origin and development of the monocytic lineage. HSC: haematopoietic stem cell; MDP: macrophage and dendritic cell progenitor; CDP: common DC precursor; pDC: plasmacytoid dendritic cell; Pre-

DC: pre-dendritic cell; DC: dendritic cell; MΦ: macrophage; Infl. MΦ: inflammatory macrophage; cDC: conventional dendritic cell; Res. MΦ: Resident macrophage; DC: dendritic cell; iDC: inflammatory dendritic cell.

Moreover, the origin of the Ly6C^{low} CCR2^{-} CX3CR1^{+} resident monocytes remains unclear. Indeed, some evidences suggest that the MDP first develop into Ly6C^{+} CCR2^{+} CX3CR1^{low} monocytes, which either develop into inflammatory macrophages or shut down their Ly6C expression and become Ly6C^{low} monocytes [10]. However, the identification of the nuclear receptor Nr4a1 as a vital factor for the differentiation and survival of Ly6C^{-} monocytes, but not the Ly6C^{+} monocytes [11], suggests the opposite.

1.1.1.1 Resident macrophages

1.1.1.1.1 Origin and self-renewal of tissue-resident macrophages

The origin of tissue-resident MΦs has recently been called into question due to data showing that a subset of macrophages develops into E8 embryonic mouse before the appearance of bone marrow at E10.5. Originated from the yolk sac, these MΦs are clearly independent of bone-marrow-derived monocytes and MΦs [7]. Accordingly, *Schulz et al.* [12] showed that, at the embryonic stage, tissue-resident macrophages follow an independent way of development. These cells are characterized by F4/80^{bright} CD11b^{low} markers. By using *Myb*-deficient mice, which lack the development of hematopoietic stem cells and CD11b^{high} monocytes, the authors showed that F4/80^{bright} MΦs do not need the HSC system or CD11b^{high} monocytes, to develop. Originally found in the YS, the F4/80^{bright} MΦs population give rise to different tissue-resident MΦs such as Kupffer cells in the liver, microglia in the brain and also partially to epidermal Langerhans cells, which are persisting through adult life in mice [9, 13]. Furthermore, other tissue-resident MΦs, such as alveolar MΦs and most Langerhans cells, were shown to originate from fetal liver monocytes (fig.2) [14, 15]. In 2013, Merad´s and Frenette´s group joined their forces and collected evidence that support this concept by showing that tissue-resident macrophages maintain themselves by local proliferation throughout the adult life and are therefore independent from circulating bone marrow-derived monocytes [8, 12]. The exact mechanisms allowing the tissue-resident MΦs to renew themselves in steady state are still unknown. However, their strong tendency to proliferate *in vivo* under inflammatory condition, such as during zymosan- or thioglycollate-induced peritonitis (and especially during the resolution of these forms of inflammation), seems to be

coordinated by local cytokine production, such as IL-4 and M-CSF [16, 17]. Taking together, these recent data clearly show that the different MΦ subsets have distinct origins, which can be tissue-specific. The F4/80^{bright} tissue-resident MΦs originally develop from either the YS or the fetal liver at embryonic stage. However, a possible contribution of bone-marrow-derived MΦs to the tissue-resident MΦ pool, under inflammatory conditions or tissue injury remains incompletely understood [18].

1.1.1.1.2 Phenotype and function of tissue-resident macrophages

The different subtypes of macrophages share common characteristics allowing us to identify and characterize them. Macrophages are in general positive for F4/80, CD68, CD64, and CD11b and can be identify by flow cytometry analysis on their large size and strong granularity (forward light scatter FCS^{hi} and side light scatter SSC^{hi}). Some tissue specific MΦs make an exception on their extracellular markers, such as the alveolar MΦs, CD11b^{low} and CD11c^{+} or the marginal zone MΦs notably positive for MARCO and SIGNR1.

Tissue-resident macrophages, also called resident macrophages, commonly express CD115 and CD11b on their surface. From a pre-natal origin, they derive either from the YS and have a bright F4/80 expression or from the fetal liver and have a lower F4/80 expression. Resident macrophages are characterized by a strong arginase expression. Arginase is an enzyme competing with inducible Nitric Oxide Synthase (iNOS) for the use of a common substrat (L-arginine) and is a major characteristic of the anti- or non-inflammatory profile of these macrophages [19]. Type2 cytokine stimulation, such as IL-4, might increase their expression of chitinase-like 3 (Chi3l3/Ym1) and resistin-like molecule α (RELMα/Retnla/Fizz1) [20]. Their role can depend on their location and be tissue specific. Microglia, as a form of resident macrophages for example, are involved in the immune surveillance and also promote neuronal survival, whereas resident peritoneal MΦs are known to clear apoptotic cells and adipose tissue MΦs are implicated in the control of insulin sensitivity and thermogenesis. In general, resident MΦs produce pro-inflammatory cytokines, but in a much lower amount than inflammatory macrophages, favouring the production of anti-inflammatory cytokine, such as IL-10 and TGF-β1 [21].

1.1.1.2 Inflammatory macrophages

1.1.1.2.1 Origin and renewal of inflammatory macrophages

Absent from the tissue of healthy mice, inflammatory MΦs, characterized by CD11b^{hi} F4/80^{low} expression, are short-lived cells that are found in inflamed tissue rapidly after the induction of inflammation. They are derived from the "classical" inflammatory Ly6C^{hi} monocytes and their recruitment is mediated by adhesion molecules and chemokine receptors such as CCR2 [3, 22-24]. Their proliferation capacity in tissue is not well defined yet, but some hints suggest that they are able to proliferate under classical inflammatory conditions such as during thioglycollate-induced peritonitis. However, also the presence of type 2 cytokines, such as IL-4, has been shown to induce their proliferation [17, 25].

1.1.1.2.2 Phenotype and function of inflammatory macrophages

Thought to be derived from the bone-marrow Ly6C^{hi} monocytes, the inflammatory macrophages also express CD115 (the M-CSF-receptor) and Ly6C and additionally possess higher expression of activation markers such as MHCII and CD86. They are known to be recruited to sites of inflammation, where they react rapidly and strongly to pro-inflammatory stimulation, such as Toll-like receptor (TLR) ligands or interferon gamma (IFNγ) that are in turn characteristic for pathogen invasion, by increasing their production of iNOS and its product Nitric Oxide (NO) and thereby actively participate in the defence against pathogens. Under stimulation, inflammatory macrophages also produce high amounts of pro-inflammatory cytokines, such as Tumor Necrosis Factor-α (TNF-α), IL-12 and the cytokine IL-6, which will initiate the inflammatory response and the clearance of pathogens. Inflammatory macrophages have a short period of life, with a maximum of a few days, and are rapidly eliminated [26].

1.1.2 The M1/M2 classification

Another way to define macrophages is to use the M1/M2 classification. Often approximate, this classification allows a better and simplified understanding and denomination of the monocyte-derived macrophages. The "M1", or classically activated, macrophages develop under pro-inflammatory stimulation such as LPS and IFNγ and produce high amount of iNOS, TNF-α and IL-12; they are comparable to inflammatory macrophages. The "M2", or

alternatively activated, macrophages can be divided into three subgroups called M2a, M2b and M2c, which are based on their way of activation and their gene expression profiles.
IL-4/IL-13 stimulation promotes differentiation to M2a, immune complexes in combination with IL-1β or LPS induces M2b differentiation and IL-10/TGF-β or glucocorticoids promote M2c macrophages [27]. Alternatively activated MΦs are characterized by an increased expression of Chi3l3 and Retnla and by CD206 on the surface of the cells [20]. The M2 MΦs have been shown to promote parasite clearance [28], tissue remodelling [29] and immunoregulation.

1.1.3 Macrophages polarization and plasticity

Even if the origin and differentiation of macrophages become clearer, a lot of questions concerning the plasticity of monocytes and macrophages remain unsolved.
The alternative activation and differentiation of monocyte-derived MΦs have been extensively shown under pro- and anti-inflammatory stimulation, demonstrating that TLR-ligands promote the differentiation of inflammatory MΦs and IL-4 the differentiation of alternatively activated MΦs [30]. These data suggest that the phenotype of the BMDM can be strongly influence by their environment, during and after their differentiation.

To better define the MΦs subsets, their tasks and origins, new approaches are developed at the moment. These studies combine single-cell RNA-sequencing, epigenomic analysis and high standardized micro-array analysis [31] (see annexe 1).

1.2 Clearance of ACs

1.2.1 Cell death: a not so sad story

It is estimated that in a healthy adult body, about 1 million cells die every second in order to allow tissue homeostasis [32]. Depending on its origin and its fate, a cell will have its own characteristic lifespan and its own type of death. Every cell type is designed to perform a specific task and thereby possess a distinct lifespan. Some of the best examples are certainly neutrophils which rapidly die (< 24h to 5 days) [33, 34] while red blood cells live about four months and neurons are though not to be replaced during the entire lifespan [35].
To understand and compile all the knowledge accumulated on cell death pathways, origins and consequences, a committee has been created in 2005: the Nomenclature Committee on

Cell Death (NCCD). Every few years, the NCCD meet in order to define more precisely the different cell death and to give an overview of the new concepts. The actual challenge is to define cell death not only via the morphological aspect of the cell but to combine this definition with molecular and biochemical characteristics. In the following paragraphs the most common and well defined forms of cell death will be summarize, as they are relevant for the present work.

1.2.1.1 Apoptosis

The term apoptosis, to define a "cell deletion", has been first used by *Kerr et al* in 1972 [36] and has been directly associated with the process of phagocytosis of the remaining apoptotic bodies. Electron microscopy indicates that apoptosis, also called cell suicide, is characterized by intense changes of the cellular morphology such as the rounding-up of the cell, chromatin and nucleus condensation (pyknosis), nuclear fragmentation (karyorrhexis) and plasma membrane blebbing. Notably, cells undergoing apoptosis maintain their integrity [37]. One major way to define and detect apoptotic cells (ACs) is the detection of the phospholipid phosphatidylserine (PtdSer or PS) that is exposed on their surface. The main feature of apoptosis is the rapid and efficient degradation of intracellular proteins by activation of broad-spectrum cystein-aspartic proteases called caspases. This process allows the cells undergoing apoptosis to avoid the release of intracellular and immunostimulatory components and additionally minimizes damage/disruption of neighbouring cells. On a molecular level, apoptosis can be sub-classified in two groups:

The extrinsic apoptosis that is induced by extracellular stimuli, also called lethal ligands, such as the tumor necrosis factor family: FAS/CD95 ligand (FASL/CD95L), tumor necrosis factor-α (TNF-α) or TNF-related apoptosis inducing ligand (TRAIL). By binding to their respective receptors, also called death receptors, (FAS/CD95, TNF-α receptor 1 TNFR1 and TRAIL receptor TRAILR 1 and 2), the lethal ligands induce the recruitment of several adaptor proteins such as FAS-associated death domain (FADD), which will promote the activation of caspases, final and irreversible step in the apoptosis process [38]. It is also thought that FASL and TNF-α can activate c-Jun N-terminal kinases (JNK) which may inhibit Bcl-2, a pro-survival factor (fig.3).

The intrinsic apoptosis, or mitochondria-induced pathway, is initiated by the cell itself and is either due to a programmed event, cell stress or damage. The activation of BH3-only proteins promotes the creation of BAX-BAX oligomers within the mitochondrial outer membrane, which lead to a major event called the mitochondrial outer membrane permeabilization (MOMP). Proteins contained in the intermembrane space, such as cytochrom *c*, are released in the cytosol and promote the assembly of a major protein complex called apoptosome. The apoptosome is composed of cytochrom *c*, apoptotic protease-activating factor-1 (APAF1) and dATP. This complex recruits and activates caspase-9. This last step induces an activation cascade of other caspases such as caspases-3, 7 and 6. This point of no return leads to the total destruction of the cell (fig.3) [39].

The mechanism of apoptosis is considered to be a programmed event that is used by the organism to eliminate a surplus of cells and to constantly control tissue homeostasis.

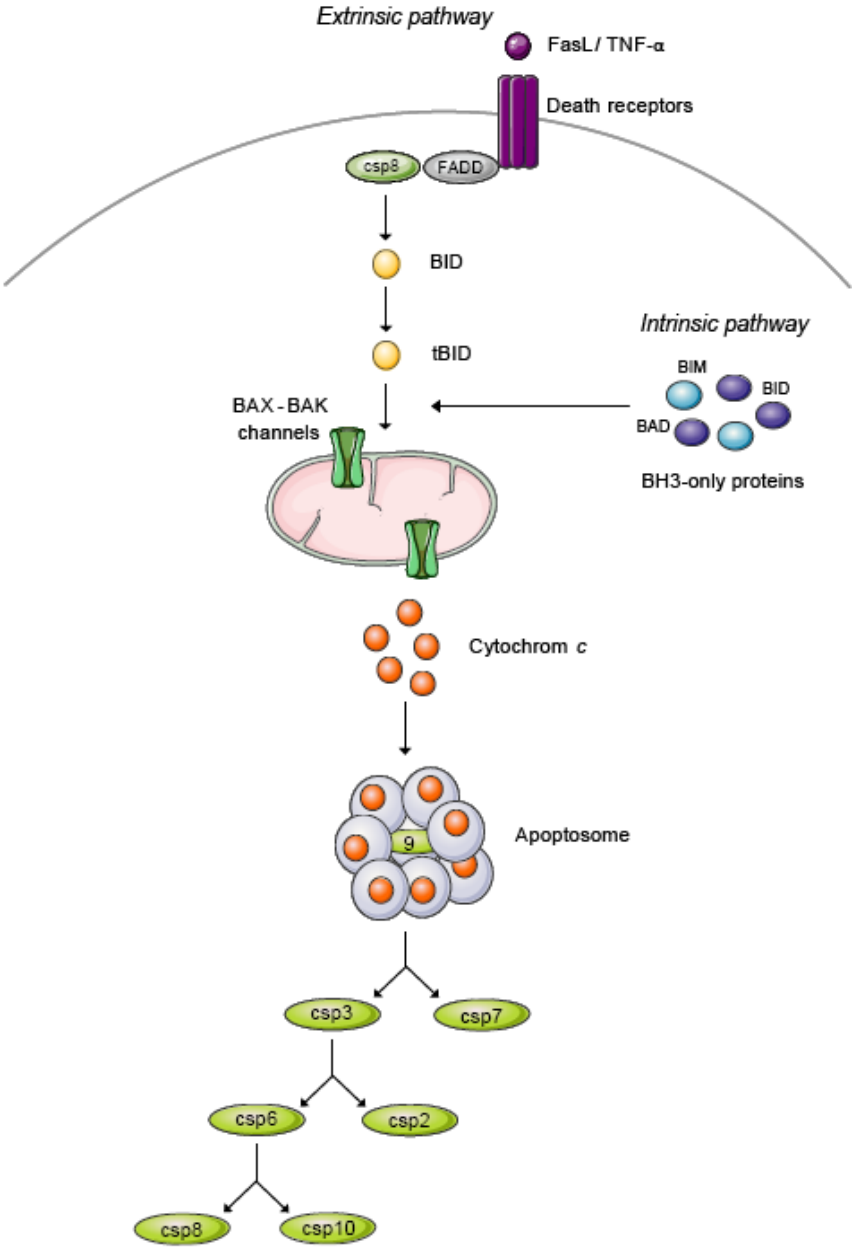

Figure 3: Mechanism of apoptosis. Extrinsic and intrinsic pathways can activate caspases (csp) and cytochrom *c* release which lead to cell death.

1.2.1.2 Necrosis

Intense stress, such as ultraviolet radiation, highly concentrated drugs, DNA damage, pathogens and mechanical stress can lead to a premature and rapid death called necrosis. Per definition, necrosis is a type of cell death lacking the characteristics of apoptosis and autophagy (self-degradation and recycling) and was, until recently, considered to occur uncontrolled and accidental. Necrosis is characterized by a gain in cell volume that is followed by sudden rupture of the cell membrane and release of internal components in the

extracellular space. Thus, necrosis is mostly considered to be harmful and dangerous because of the release of intracellular proteins, DNA and RNA, which are recognized by Antigen Presenting Cells (APC) that eventually initiate an autoimmune response. Likewise, necrotic cell death also releases reactive oxygen species (ROS) and cytokines that can alert the innate immune system and induce local inflammation.

More recently, scientists started to realize that a part of the necrotic events are not uncontrolled, but rather follow a specific program. Indeed, the course of necrosis always follows a certain scheme including the generation of ROS, ATP depletion, deregulation of calcium homeostasis, lysosomal rupture and plasma membrane rupture [40]. Moreover, necrotic cells have been found in some tissue-specific processes also under steady state conditions. For example, the small and large intestine both need apoptosis and necrosis to renew. Similarly, the negative selection of T cells seems to be partially necrosis-dependant [41, 42]. These findings highlight necrosis not only as a dangerous and non-predictable phenomenon, but show a relevant physiologic function of this process, which might serve as a way to alarm the immune system under specific conditions.

1.2.1.3 Secondary necrosis

When a cell undergoing apoptosis is not rapidly removed by its surrounding neighbours, the apoptotic process will go on until it reaches an ultimate step: the destruction of the plasma membrane. This late event is called secondary necrosis and possesses dangers comparable to necrosis. Here, loosing membrane integrity leads to a massive release of the intracellular components into the extracellular space, which might, in turn, activate the immune system [43].

Thus, cell death, either apoptotic or necrotic, allows the maintenance of physiological homeostasis and functionality. However, to avoid uncontrolled immune activation, apoptotic cells have to be quickly and silently removed by professional phagocytes.

1.2.2 The many steps of apoptotic cells clearance

The clearance of apoptotic cells is a crucial process, indispensable for the body to eliminate dying cells without alarming the immune system. If this process is inefficient, the disposal of corpses is impaired and the apoptotic cell enters secondary necrosis, which leads to the release

of intracellular components and macromolecules, such as reactive oxygen species (ROS) alerting and activating neighbouring cells. The silent removal of ACs is mainly performed by professional phagocytes (macrophages and dendritic cells) and involves two major steps. In the first step, the phagocytes are attracted to the AC and recognize it. In the second step, the phagocytes engulf and digest the AC.

1.2.2.1 Recognition: the "find-me" process

When a cell becomes apoptotic, it releases multiple molecules, creating a chemoattractant gradient, which attracts specialized phagocytes [44]. Among these molecules is fractalkine (CX3CL1), a membrane-associated protein, released by apopotic B lymphocytes *via* caspase and Bcl-2-dependent mechanisms [45]. However, fractalkine expression is restricted to few cells, suggesting that it is not the only important attractant. Another example for a chemoattractant that is released by ACs is lysophosphatidylcholine (LPC), a lipid released by ACs in a caspase-3-dependant manner and that binds to the G-protein-coupled receptor G2A [46]. Also sphingosine-1-phosphate (S1P), generated by sphingosine kinase 1 (SphK1) and secreted by ACs, binds to a G-protein-coupled receptor (S1P-R1) and [47] attracts phagocytes. The nucleotides Adenosine-5´-triphosphate (ATP) and Uridine-5'-triphosphate (UTP) are also part of the "find-me" program: they are released during the early stage of apoptosis and attract monocytes and macrophages through their recognition by $P2Y_2$ receptor [48]. However, ATP and UTP are known to be quickly degraded by extracellular nucleotidases, indicating that they most probably do not act on a long-range (fig.4).

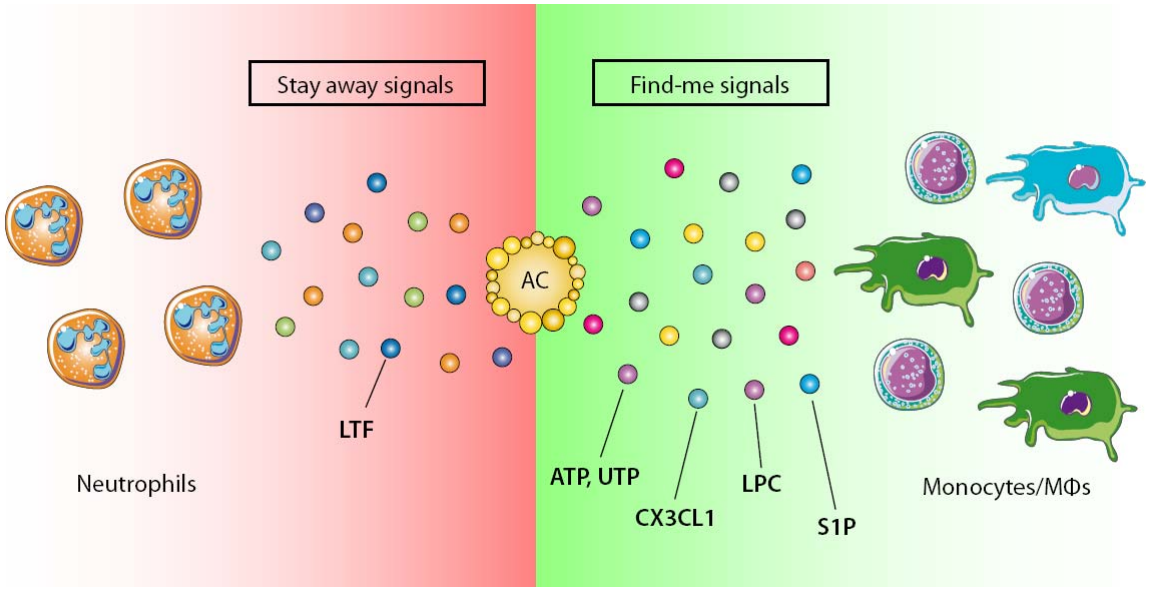

Figure 4: Attraction and hold off signals provided by ACs. ACs secrete simultaneously molecules attracting monocytes and MΦs to help find-them and initiate the process of phagocytosis, and molecules repelling neutrophils to thwart any pro-inflammatory recognition. LTF: Lactoferrin; ATP: Adenosine-5′-triphosphate; UTP: Uridine-5'-triphosphate; CX3CL1: Chemokine (CX3-C motif) ligand 1 or fraktaline; LPC: lysophosphatidylcholine; S1P: sphingosine-1-phosphate.

1.2.2.2 Phagocytosis: the "eat-me" process

During the apoptotic process, cells expose specific molecules at their surface, allowing the environment to recognize them as dying cells. A lot of these "eat-me" signals have been identified [44, 49]. Most "eat-me" signals exposed on the surface of ACs are recognized by a receptor at the surface of the phagocyte, which will recognize the "eat-me" signal and initiate the phagocytosis of the AC. One of the best studied examples is the exposure of the phospholipid phosphatidylserine (PtdSer) by ACs [50]. PtdSer is recognized by surface proteins such as Tim1-4 [51, 52] and BAI1 [53]. However, some receptors cannot directly recognize PtdSer, but need a secreted protein to link it. Among these bridging molecules are Milk-Fat Globule Epidermal Growth factor 8 (MFG-E8) [54], Growth Arrest-Specific 6 (Gas6) and protein S [55]. They recognize the PtdSer and bridge it to several surface receptors: $\alpha_v\beta_3$-integrin and TAM (Axl/MerTK/Tyro3) receptors [56]. It is still unclear, which signals are directly and essentially involved in the process of phagocytosis, or if one of them is a master signal sufficient to drive the process in its entirety, but the current opinion is that the exposure of multiple "eat-me" signals enhances engulfment efficiency [44]. However, the blockade or deletion of specific receptors, such as Mer or SR-A, impairs the clearance of ACs [57, 58] indicating the importance of each of these receptors (fig.5).

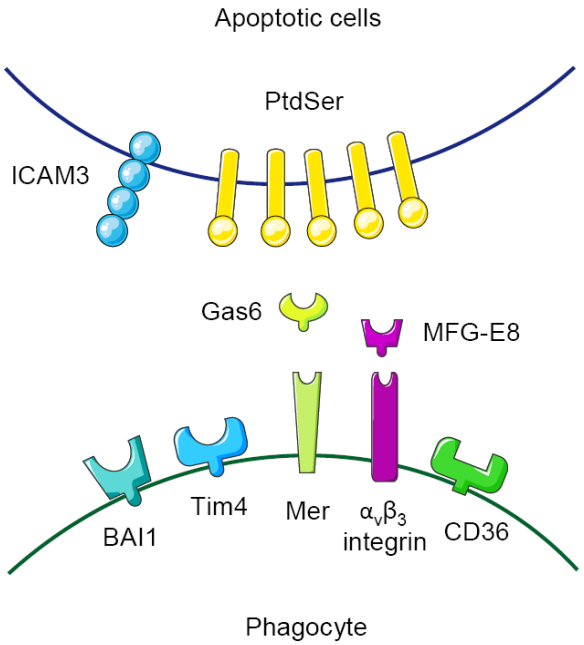

Figure 5: Recognition of apoptotic cells by phagocytes. ACs are recognized through several receptors which can vary between the type of phagocytes or the tissue localisation. PtdSer: phosphatidylserine; MFG-E8: Milk-Fat Globule Epidermal Growth factor 8; Gas6: Growth Arrest-Specific 6.

1.2.2.3 "Stay away" signals

As ACs can attract phagocytes and promote their engulfment, it might also be possible that they produce signals inhibiting their recognition from pro-inflammatory cells. Indeed, it has been shown that Lactoferrin (LTF) is released by ACs and inhibits neutrophils migration (fig.4) [59]. Recently, 12/15-lipoxygenase has been shown to actively participate in the selective clearance of ACs by resident MΦs by blocking the uptake of ACs by inflammatory MΦs [60].

1.2.3 Silent clearance for tolerance

1.2.3.1 "Tolerate-me" signals

Dying NCs or ACs represent a source of ubiquitous auto-antigens such as nucleic acids or histones. To avoid a break in self-tolerance, ACs have hence to be removed without alerting the immune system. ACs themselves can influence the immune response by promoting the production of anti-inflammatory cytokines (IL-10, TGF-β1) and by inhibiting the production of pro-inflammatory cytokines (TNF-α, IL-12) from activated cells, such as LPS-activated

macrophages [61, 62]. Thus, ACs exert anti-inflammatory effects on immune cells but the underlying mechanisms are not fully understood yet. It is known that PtdSer is actively involved in this process but involved receptors are not clearly defined. Recognition of ACs by MΦs seems to strongly alter the intracellular signaling pathways of the MΦ, leading to a reprogramming of the MΦ [63], affecting intracellular gene expression and cytokine production.

1.2.3.2 The stakes of a non-immunogenic removal of apoptotic cells

A defective clearance of ACs will result in the accumulation of ACs in the tissue as well as the occurrence of secondary necrosis and the release of potential pro-immunogenic autoantigens such as nucleic acids, which might additionally activate immune cells via recognition through TLRs. The recognition of ACs by specific receptors seems to be an indispensable key step for the safe removal of ACs. Indeed, *in vivo* studies of knock-out mice reveal that lack of different receptors for ACs triggers the spontaneous development of auto-antibodies and eventually autoimmune diseases such as systemic lupus erythematodes. Notably, the deletion of CD14 leads to the accumulation of ACs in the tissues, but the mice do not develop autoimmunity [64] whereas mice lacking MFG-E8, MerTK or C1qa develop signs of autoimmunity [65-67].
These observations suggest that the immunomodulatory properties of ACs are mediated via specific receptors on phagocytes and that the occurrence of autoantibodies and subsequent organ pathologies such as glomerulonephritis are not necessarily due to direct effects of the accumulation of ACs in the organism but rather due to an aberrant activation of immune cells after accumulation of ACs that results in a break of self-tolerance to AC-derived autoantigens [68].

Even if macrophages are defined as professional phagocytes, their heterogeneity, different origin, tissue localization and individual receptor pattern suggest that they might possess different phagocytic capacities and fulfil different tasks [60, 69]. Moreover, the different subsets of MΦs might react differently to ACs. However, a conscientious comparison of the anti-inflammatory effects of the ACs on the MΦs subset has not been explored, yet.

1.2.3.3 Recognition of self-antigens: development of autoimmunity

Mouse and human studies clearly indicate that a defect in the silent removal of ACs leads to the development of autoimmune diseases [68].

Autoimmunity, first defined as "horror autotoxicus" by the Nobel Laureate Paul Ehrlich in 1901, is a complex phenomenon, defined as the failure of an organism to recognize its own constituent as "self", leading to a "break of self-tolerance" and an immune reaction against itself. In general, autoimmunity is caused by multiple factors such as genetic factors, pathogen exposure, sex and environmental factors (smoke, diet). It is characterized by a deregulation of the immune system resulting in development of antibodies against self-antigens (auto-antibodies), spontaneous inflammation and organ damage as observed in typical autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE) [70].

SLE is a systemic multifactorial autoimmune disease, which can affect any organ/tissue in the body, but most often involves joints, skin and kidneys. The clinical course of the disease remains unpredictable, but the main hallmark of SLE is the production of anti-nuclear autoantibodies, leading to immune-complex deposits in diverse organs, local inflammation and finally, organ damages [71]. The exact cause of SLE and underlying mechanism are still unknown but current evidence suggests that both defective apoptosis and impaired clearance of apoptotic cells are implicated in the development of SLE. Thus, mice deficient in pro-apoptotic factors such as FAS, FASL or Bim, a member of the BH3-only protein, show an accelerated development of autoimmunity [72] as well as severe glomerulonephritis [73]. Moreover, mice lacking factors implicated in the degradation and clearance of apoptotic cells such as DNaseI, MFG-E8 [74] and Mer [58] also produce autoantibodies and develop glomerulonephritis-like clinical signs [71, 75]. Even if the contribution of these molecules in human SLE development is not easy to address, it is highly likely that the (defective) clearance of apoptotic cells is a major factor during this process [76]. Indeed, abnormal accumulation of ACs in SLE patients has been described [77]. Due to its unknown cause and unpredictable development, treatment strategies for SLE patients are difficult to establish. To date, SLE cannot be cured but the symptoms can be diminished by the use of corticosteroids and other immunosuppressive drugs in order to decrease inflammation and suppress autoimmunity.

1.3 Nuclear receptors

1.3.1 Superfamily of nuclear receptors

Nuclear receptors are a superfamily of ligand-activated transcription factors (48 in humans and 49 in mice) [78] having common features. They are widely expressed, present in every cell type and regulate the transcription of specific target genes.

Nuclear receptors are proteins localized in the cytoplasm or in the nucleus of cells. Upon activation by a specific ligand, nuclear receptors bind to a specific responsive element at promoters of target genes as monomers, homo- or hetero-dimers to activate or repress its transcription. Nuclear receptors can be activated by diverse components such as steroid hormones (estrogen, progesterone), various lipid-soluble molecules (retinoic acid, oxysterols, thyroid hormone), fatty-acid and vitamin D_3 [79]. All nuclear receptors share a conserved structure. The N-terminal region, a transactivation domain (A/B domain, also called Activation Function 1 AF1), is not well defined yet and is highly variable in length and sequence. It can be post-translationally modified, can interact with cofactors and is located at the centre of the protein. The DNA-Binding Domain, also called DBD or C domain, is responsible for the binding of the nuclear receptor to its hormone response elements (HREs) on the DNA. The DBD is composed of two zinc fingers and two α-helices. Following the DBD, a small D domain assuring its nuclear localization and a large E domain containing a Ligand-Binding Domain (LBD) and an Activation Function 2 (AF2) are typically found in nuclear receptors. The LBD domain is responsible for both binding of coactivator/corepressor proteins modulating the activity of the nuclear receptor, and facilitates its dimerization with other nuclear receptors. The AF2 domain confers the ability of the LBD to activate the transcription of target genes. The C-terminal region, or F domain is highly variable and has a still unknown function (fig.6) [79] [80, 81].

Figure 6: Common structure of nuclear receptors. AF1: Activation Function 1; DBD: DNA-Binding Domain; LBD: Ligand-Binding Domain; AF2: Activation Function 2; F: F domain.

1.3.1.1 Composition of the nuclear receptor family

Based on their ligands and their DNA-binding properties, nuclear receptors can be classified into three major subgroups. The first group (i) includes steroid hormone receptors such as glucocorticoid (GR), estrogen (ER) and progesterone (PR) receptors. Their ligands are derived from an endogenous endocrine source and bind to their receptor with a high affinity. The second group (ii) is composed of adopted orphan nuclear receptor that act as heterodimers in association with the retinoid X receptor (RXR). They are controlled by so-called "dietary lipids" which bind to the receptors with a lower affinity and are thought to play a major role as lipid sensors and regulators of metabolism [82]. This group includes the Peroxisome Proliferator-Activated Receptors (PPARs) and the Liver X Receptors (LXRs). The third (iii) group includes nuclear receptors without an unknown ligand and is therefore referred to as orphan receptors. Their activation remains incompletely understood [83].

1.3.1.2 Function and action of nuclear receptors

The mechanisms of transcriptional regulation differ between the different subgroups of nuclear receptors. Generally, the process follows several steps: upon binding by its specific ligand, nuclear receptors in the cytoplasm can either homo- or heterodimerize or directly enter the nucleus as a monomer, where they bind to its specific hormone response elements and promotes the transcription of target genes. Other nuclear receptors constitutively bind to the DNA in the promoter region of target genes, where they are associated with co-repressors and block transcription. Upon ligand-binding, the co-repressors are exchanged by co-activators and the transcriptional process is promoted. Finally, some nuclear receptors can act *via* transrepression mechanism: they do not directly bind the DNA, but are associated with co-repressors complexes such as NCoR or SMRT thereby repressing genes that lack a classical nuclear receptor binding motif [84].

1.3.2 The Nr4a family of nuclear receptors

The Nr4a (Nuclear receptor subfamily 4) subfamily of orphan nuclear receptors includes three members: Nr4a1 (also known as Nur77 or NGFI-B), Nr4a2 (Nurr1) and Nr4a3 (NOR1). They have been first identified as early response genes, due to their rapid increase in expression upon stimulation [85-87]. Their structure is common to all nuclear receptors: AF-1, DBD, LBD, AF-2 and C-terminal region. However, so far, no ligand for this nuclear receptor family

has been identified. This can be explained by the 3D structure of the LBD which contains no proper ligand-binding cavity. X-ray crystallography reveals that, unlike other nuclear receptors, the cavity of Nr4a members is occupied by bulky hydrophobic residues, most probably preventing the physiologic binding of any molecules [88]. Nr4a members share a high sequence homology (91 – 95% of homology in the DBD and about 60% in the LBD and C-terminal domain but only about 27% in the N-terminal region) allowing them to bind the same response elements. As monomer, they can bind to the NBRE (NGFI-B Response Element) sequence A/TAAGGTCA. As homo- or heterodimer, they bind to a NurRE (Nur77 response element) composed of two inverted NBRE sequences AAAT(G/A)(C/T)CA that are spaced by 6 nucleotides. Nr4a1 and Nr4a2, but not Nr4a3, can heterodimerize with RXR and bind to a so called DR5 motif consisting of two direct repeats of the consensus nuclear receptor binding motif spaced by five nucleotides [89]. The detailed mode of activation of these nuclear receptors is not known, but they are subject to post-translational modifications. In response to various kinases such as ribosomal S6 kinase (RSK), [90] mitogen-activated protein kinase (MAPK) and Jun N-terminal kinases (JNK), [91] Nr4a members can be phosphorylated at different serine residues [92]. Phosphorylation of NR4a 1-3 is thought to have an inhibitory effect on their activity via two mechanisms: inhibition of the transactivation activity and provision of a signal for nuclear export [91]. For example, Nr4a1 can be phosphorylated on Ser340, Ser350 and Ser354, all residues within the DBD, which changes its conformation and decreases its DNA binding affinity [93-95]. Moreover, Nr4as possesses also sumoylation consensus sites and Nr4a2 sumoylation was shown to modulate its transcriptional activity in a site-specific manner [96].

Nr4a expression is inducible by various stmuli, such as by fatty acid, growth factors and different cytokines [89] and these nuclear receptors were shown to be involved in many biological processes such as apoptosis, brain development, glucose metabolism and cellular differentiation.

1.3.2.1 Biological roles of Nr4as

Nr4a1 controls the development and survival of $Ly6C^{low}$ monocytes in the bone marrow [11]. Moreover, Nr4a2 not only regulates the differentiation of $CD4^+$ T cells by induction of Foxp3 [97] but is also important for the survival of dopaminergic neurons [98].

Nr4a members exert metabolic functions. The expression of Nr4a1 is induced by β-adrenergic receptor agonists in skeletal muscle cells and brown adipocytes [99]. The β-adrenergic signaling is implicated in a lot of metabolic functions such as glucose transport, lipolysis and thermogenesis and targets a lot of tissues such as skeletal muscle, liver, white-adipose tissue (WAT) and brown-adipose tissue (BAT). These findings suggest a role for Nr4as in the metabolic control of these tissues. This idea is supported by the fact that Nr4a1$^{-/-}$ mice show signs of a metabolic dysregulation after receiving a high-fat diet when compared to wild-type (WT) mice. This includes increased weight gain, insulin resistance and slower blood glucose clearance [100]. Moreover, expression of Nr4as has been found in WAT and BAT [101] and may inhibit adipogenesis [102] and promote lipolysis in skeletal muscle [103].

A role of Nr4as in inflammation has also been suggested. Indeed, in macrophages Nr4as are rapidly and highly upregulated following pro-inflammatory stimulation by LPS, TNF-α and poly I:C. Moreover, Nr4as are also induced by oxidized lipids and Nr4a1 expression has been found in atherosclerotic lesions, suggesting that Nr4as play a role in the development of atherosclerosis [104]. However, the exact role of these nuclear receptors during atherogenesis remained elusive as different studies showed inconsistent results. Some studies indicated a beneficial role of Nr4a1 in macrophages polarization and development of the atherosclerotic plaques [105], whereas other studies did not support a role of Nr4a1 in this disease models [106]. Finally, Nr4a2 has a major role in the regulation of the inflammatory activation of microglia and astrocytes, where it blocks the production of pro-inflammatory molecules with the help of the corepressor CoREST and thereby promotes neuron survival [84].

Due to a potential role of Nr4a1 in the control of apoptosis, its role in cancer has been intensively investigated and high expression levels were found in colon, pancreatic, lung and breast tumors in comparison with healthy tissue. Nr4a2 and Nr4a3 are also highly expressed in breast tumors. Although Nr4a1/Nr4a3 double knockout mice develop acute myeloid leukaemia, studies on cancer cell lines indicated rather a pro-oncogenic role of Nr4a1. Moreover, prognosis of patient survival based on Nr4a1 mRNA expression gave opposite results in between two cohorts of patients. Therefore the contribution of Nr4a1 and Nr4a2 to cancer development seems to be dependent on the type of cancer [107].
Even the fact that Nr4as play a major role in different biological events, their induction and activation are still not well defined. Recently, a substance isolated from an endophytic fungus, the compound cytosporone B (Csn-B), was found to interact specifically with the LBD of

Nr4a1. Despite its pro-apoptotic effect on tumor cells, Csn-B seems to be well tolerated in mice, which allows its use in research. 6-Mercaptopurine has been identified as agonist for Nr4a2 [108, 109] and Nr4a3 [109] and prostaglandin A2 as agonist for Nr4a3 [110], but their effects *in vivo* are not clear yet.

Interestingly, despite the major role of these nuclear receptors, $Nr4a1^{-/-}$ mice have no obvious phenotype, suggesting a functional redundancy between the three members of this family of nuclear recepors. In contrast, mice deficient for Nr4a2 die shortly after birth, due to a defective respiratory function. Furthermore these mice lack dopaminergic neurons [111] [112]. $Nr4a3^{-/-}$ mice survive but have inner ear defects and impaired bi-directional circling behaviour [113]. Nr4a1/Nr4a3 double knockout mice have a smaller size and die rapidly after birth, due to acute myeloid leukaemia [114].

Nr4as are also expressed in humans such as in inflamed synovial tissue, colorectal cancer cells, psoriatic lesion and multiple sclerosis, suggesting a role of these nucelar receptors in the pathogenesis or regulation of inflammatory diseases in humans [115].

1.3.3 The role of nuclear receptors in the clearance of apoptotic cells

The importance of NRs in the clearance of apoptotic cells has recently been highlighted. ACs have been shown to induce activity of LXRs [116] and PPARβ [117] and this process appears to be indispensable for the clearance of AC by thioglycolate-elicited peritoneal and bone-marrow-derived macrophages, respectively. Moreover, LXRα/β double knockout and PPARβ deficient mice show a defective clearance of ACs and spontaneously develop autoimmune disease, characterized by production of autoantibodies and development of glomerulonephritis. These data support the idea that the development of autoimmune disease is related to a defective clearance of apoptotic cells by MΦs.

It is now clear that nuclear receptors play a major role not only in the control of inflammation, but also in the clearance of apoptotic cells and in the maintenance of self-tolerance. The phenotype and function of MΦs are partially controlled by nuclear receptors, such as PPARs [27].

The role of the different MΦ subsets in the silent removal of ACs has not been clearly defined yet. To better define it, we focused our studies on the differential role of resident and inflammatory peritoneal MΦs during this process. To better understand underlying mechanisms, we analysed the gene expression of MΦs after their contact with ACs and found Nr4a1 to be a major mediator of the anti-inflammatory effect of the AC.

2. Materiel and methods

2.1 Materiel

2.1.1 Media

R10 medium

Roswell Park Memorial Institute medium (RPMI 1640, Invitrogen) was supplemented with 10 % heat-inactivated fetal calf serum (FCS, PAA Laboratories, Pasching, Austria), 1%L-Glutamine and 1 % penicillin-streptomycin (10 000 U/ml, Gibco).

Bone-marrow-derived macrophages medium

Minimum Essential Media (MEM) Alpha Medium GlutaMAX^{TM} was supplemented with 10% heat-inactivated fetal calf serum (Biochrom), 1%L-Glutamine, 1 % penicillin-streptomycin (10 000 U/ml, Gibco) and 30ng/ml M-CSF (R&D System).

Bone-marrow-derived dendritic cells medium (R10+ medium)

Roswell Park Memorial Institute medium (RPMI1640, Invitrogen) was supplemented with 10 % heat-inactivated fetal calf serum (PAA Laboratories, A15-043), 1%L-Glutamine and 1 % penicillin-streptomycin (10 000 U/ml), 50µM β-mercaptoethanol and 10% of GM-CSF containing-conditioned medium [118].

Ag8653 myeloma cells medium

The GM-CSF-producing cells were cultivated in Roswell Park Memorial Institute medium (RPMI1640) supplemented with 10 % heat-inactivated fetal calf serum Laboratories, 1%L-Glutamine and 1 % penicillin-streptomycin (10 000 U/ml) and 50µM β-mercaptoethanol.

2.1.2 Cell culture

β-mercaptoethanol	GIBCO® by life technologies (#31350010)
FCS	GIBCO® by life technologies (#10499-044)
OC-FCS	Biochrom (#S0415/0879L)

FCS for dendritic cells	PAA Laboratories
Penicillin-Streptomycin	GIBCO® by life technologies (#15140-122)
α-MEM	GIBCO® by life technologies (#32571-028)
DMEM	GIBCO® by life technologies (#41965-039)
RPMI 1640	GIBCO® by life technologies (#11875-093)
L-Glutamine	GIBCO® by life technologies (#25030-081)
PBS	GIBCO® by life technologies (#14200-067)
Plates	Greiner bio-one CELLSTAR®

2.1.3 Buffers

FACS buffer

4% FCS in PBS

ELISA washing-buffer

0,05% Tween 20 in PBS

TE buffer

10 mM TRIS-HCl

1 mM EDTA

in H_2O

pH 8

Tail lysis buffer

100 mM Tris-HCL pH 8,5

5 mM EDTA pH 7,5-8
0,2% SDS
200 mM NaCl
in H_2O

Tris-buffered saline (TBS)
50 mM Tris
150 mM NaCl
pH 7,6

RBC lysis buffer
4g NH_4Cl
2,35g HEPES
100 µl 0,5M EDTA pH8
Add 500 ml H_2O

2.1.4 Chemicals

ELISA substrate I and II	eBioscience (#BMS402/3)
ELISA stop solution	eBioscience (#BMS409)
ECL Western Blotting Substrate	Thermo Scientific (#32106)
Tissue-Tek O.C.T.	SAKURA (#4583)
TWEEN 20	Sigma-Aldrich (#P7949)
Pristane	Sigma-Aldrich (#P2870)
Brewer thioglycollate	Sigma-Aldrich (#B2551)

Laemmli Biorad	Biorad (#161-0737)
Proteinase K	Applichem (#A3830)
LPS from E. coli O111:B4	Sigma-Aldrich (#L2630)
Milk powder	Roth (#T145.2)
BSA	Sigma-Aldrich (#A7030)
Dexamethason	Sigma-Aldrich (#D4902)
peqGOLD TriFast	Peqlab (#30-2020)
Mounting medium	Dako (#S3025)
DNAse I	Thermo Scientific Fermentas (#EN0521)
dsDNA from Calf Thymus	Sigma-Aldrich (#D1501)
Poly-L-lysine	Sigma-Aldrich (#P1274)
Histone from calf thymus	Roche (#10223565001)
Ringer´s solution	B. Braun Ecotainer

2.1.5 Cytokine

Recombinant mouse M-CSF	R&D systems (#146-ML)

2.1.6 Antibodies

Polyclonal rabbit anti-CoREST	Millipore (#07-455)
Rabbit anti-IgG	Santa Cruz (#sc-2027)
Polyclonal goat ant-rabbit HRP	Dako (#2016-03)
PE Rat anti-mouse F4/80	Biolegend (#123110)
Pacific Blue Rat anti-mouse F4/80	Biolegend (#123124)
PE Rat anti-mouse TIM4	Biolegend (#129906)
APC Rat anti-mouse CD68	Biolegend (#137008)
PE Rat anti-mouse CD115	Biolegend (#135506)
TruStain fcX^{TM} (anti-mouse CD16/32)	Biolegend (#101320)
Goat anti-mouse IgG HRP	Southern Biotech (#1030-05)

2.1.7 Kit

ChIP-IT™ Express kit	Active Motif (#53008)
ELISA kit (TNF-α, IL12-40, IL-6, IL-10, CXCL1)	R&D System

2.1.8 Primers

Gene name	Sequence
beta-actin	**Forward** 5′-TGTCCACCTTCCAGCAGATGT-3′ **Reverse** 5′-AGCTCAGTAACAGTCCGCCTAGA-3′
Atf1	**Forward** 5′-GCCTTACAGTTGGCCAGTCC-3′ **Reverse** 5′-CGTGGTACGGATCTGGTAGG-3′
Atf4	**Forward** 5′-CTTCTGGCGGTACCTAGTGG-3′ **Reverse** 5′- CCAGACAATCTGCCTTCTCC-3′
Atf6	**Forward** 5′-CCTGCTTCCTTCACCATAGC-3′ **Reverse** 5′-CCATTGTGCTTACTGGAATGG-3′
Cxcl1	**Forward** 5′-AGCACCAGCCAACTCTCACT-3′ **Reverse** 5′-CGTTAACTGCATCTGGCTGA-3′
Egr1	**Forward** 5′-GCACCTGACCACAGAGTCCTT-3′ **Reverse** 5′-AGAAGCGGCCAGTATAGGTG-3′
Egr2	**Forward** 5′-AGGCCGTAGACAAAATCCCA-3′ **Reverse** 5′-ATGTTGATCATGCCATCTCCC-3′
Egr3	**Forward** 5′-TGCCTGACAATCTGTACCCC-3′ **Reverse** 5′- AGTTCCGGATTGGGCTTCTC-3′
Il12b	**Forward** 5′-AGGTGCGTTCCTCGTAGAGA-3′ **Reverse** 5′-AAAGCCAACCAAGCAGAAGA-3′
Junb	**Forward** 5′-ATGTGCACGAAAATGGAACA-3′ **Reverse** 5′-CCTGACCCGAAAAGTAGCTG-3′

Jund	**Forward** 5′-TCTTTTTGTTTGGTTTTGTTTTGC-3′ **Reverse** 5′-GCGAACCAAGGATTACGGAA-3′
c-Myc	**Forward** 5′-GCCCAGTGAGGATATCTGGA-3′ **Reverse** 5′-ATCGCAGATGAAGCTCTGGT-3′
Nr4a1	**Forward** 5′-CGGACAGACAGCCTAAAAGG-3′ **Reverse** 5′-TAACGTCCAGGGAACCAGAG-3′
Nr4a2	**Forward** 5′-GACCGGCTCTATGGAGATCA-3′ **Reverse** 5′-ACCCCATTGCAAAAGATGAG-3′
Nr4a3	**Forward** 5′-TCAGCCTTTTTGGAGCTGTT-3′ **Reverse** 5′-TAACCCAGTTCGCTCTGTGA-3′
Tim1	**Forward** 5′-TCGAGTGGAGATTCCTGG AT-3′ **Reverse** 5′-GGGTTCTGGTTTGTGAGTCC-3′
Tim2	**Forward** 5′-GGTGATGGTGGTCCCTATTG-3′ **Reverse** 5′-TGGCCTCTGGTTTGTAGGTC-3′
Tim3	**Forward** 5′-ATGACCATGGGACCTACTGC-3′ **Reverse** 5′-GGAGGGTCACCAGTGTCTGT-3′
Tim4	**Forward** 5′-TCAAACACCAATCGAGGTGA-3′ **Reverse** 5′-GCTAGTGTCTGGGGTGGTGT-3′
Mfg-e8	**Forward** 5′-CACTTGGGAAGGCTGGATAA-3′ **Reverse** 5′-GTCCACTGCACACCATCATC-3′
Lox-1	**Forward** 5′-CACTTGGGAAGGCTGGATAA-3′ **Reverse** 5′-GTCCACTGCACACCATCATC-3′
Gas6	**Forward** 5′-TGGCTTCCGAGTCTTCTCAC-3′ **Reverse** 5′-TTCAGGCCTGCCATATTTTC-3′
Sr-4	**Forward** 5′-CTGGACAAACTGGTCCACCT-3′ **Reverse** 5′-CTTGCCCCAATATGATCAGG-3′
Cd36	**Forward** 5′-AAGCTCCTTGGCATGGTAGA-3′ **Reverse** 5′-AGAGAGAGCACACACCACCA-3′
Cd14	**Forward** 5′-GCCTTTCTCGGAGCCTATCT-3′ **Reverse** 5′-TGGCTTCGGATCTGAGAAGT-3′

2.2 Methods

2.2.1 Animals

The C57BL/6 mice were purchased from Charles River Laboratories (San Diego, CA, USA) and the NR4a1$^{+/-}$ mice were derived from the Jackson laboratory (Main, USA). Littermates were generated by house-breeding. The mice were housed in the animal facility of the University of Erlangen-Nuremberg and the animal experiments were approved by the government of Mittelfranken (Franconia, Bavaria, Germany).

2.2.2 Genotyping

Genomic DNA was isolated by digestion of a piece of tail of the mice in so called tail lysis buffer overnight by 55°C, and the PCR was performed with the following primers:
primer 1: 5´-CCA CGT CTT CTT CTT CAT CC-3´
primer 2: 5´-TGA GCA GGG ACT GCC ATA GT-3´
primer 3: 5´-CAC GAG ACT AGT GAG ACG TG-3´

and the following program:

94°C 3min		1 cycle
94°C 0:30min	}	
62°C 1min	}	35 cycles
72°C 1min	}	
72°C 2min		1 cycle
4°C		forever

2.2.3 Isolation and generation of cells

Macrophages isolation

Resident peritoneal macrophages were isolated from the peritoneum of naïve 8-10 weeks old mice. Inflammatory macrophages were isolated from peritonitis exudates, 72 hours after intraperitoneal injection of 2,5 ml of 3% Brewer thioglycollate. Both elicited and resident peritoneal macrophages were obtained by peritoneal lavage: 6 ml of ice-cold 4%FCS/PBS were injected in the peritoneal cavity of a sacrificed mouse. After a gently shaking of the

mouse ("sit-ups"), the fluid of the peritoneal cavity was collected in a falcon. When needed, the mice from a same genotype were pooled. After centrifugation, the cells were resuspended in R10 medium, counted and plated at the density of $0{,}5.10^6$ cells/500µl in 24 wells-plate. When indicated, the $Tim4^+$ resident MΦs were removed from the $Tim4^-$ inflammatory MΦs by MACS techniques. Whole cells were stained with Tim4-PE antibody and the resident Mms were excluded by the use of anti-PE beads (Miltenyi).

Generation of bone-marrow-derived dendritic cells

Bone marrows were flushed from the femurs and tibias of 8 to 10 weeks old C57BL/6 WT mice. After lysis of the erythrocytes, the cells were resuspended in R10+ medium and cultured for 8 days at a concentration of 1×10^5 cells per 2 cm well. GM-CSF-containing medium was obtained from Ag8653 myeloma cells transfected with a GM-CSF gene expressing plasmide. After 8 days of culture, 85 to 95 % of the non-adherent cells were $CD11c^+$.

Generation of bone-marrow-derived macrophages

Bone marrows were flushed from the femurs and tibias of 8 to 10 weeks old C57BL/6 WT and $NR4a1^{-/-}$ mice. After lysis of the erythrocytes, all bone marrow cells were let overnight in α-MEM containing 10% FCS and 1% Penicillin/Streptomycin. The next day, the non-adherent cells were collected and resuspended in macrophages differentiation medium made of α-MEM containing 10% FCS, 1% Penicillin/Streptomycin and 30ng/ml of M-CSF and cultured during 7 days.

Generation of apoptotic cells

The thymus from 4 to 6 weeks old C57BL/6 WT mice was isolated and a single cells suspension was generated. After centrifugation, the cells were resuspended in R10 medium at 10.10^6 cells/ml and treated with 1µM of dexamethason for 6h. The degree of apoptosis was checked by the FACS analysis of annexinV/PI staining: 70 to 80% of the cells were early apoptotic. A ratio if 5 apoptotic thymocytes for 1 macrophage was used.

CFSE staining of apoptotic cells

The apoptotic cells were resuspended at 10.10^6 cells/ml and stained in PBS with carboxyfluorescein succinimidyl ester (CFSE, 1:5000) for 5 min at room temperature in the

dark. The reaction was stopped with the addition of 25 ml of PBS. The cells were washed two times more with PBS before to be used.

2.2.4 Phagocytosis assays

In vitro phagocytosis assay

A mixture of WT resident and thioglycollate-elicited peritoneal macrophages was isolated 5 days after i.p. injection of 1ml of 3% Brewer's thioglycollate, plated on coverslips in 24-well plates and incubated with CFSE-labelled apoptotic thymocytes in R10 medium for 30 min at a ratio of 1:5 at 37°C. Unbound cells were washed away with ice-cold PBS and the remaining cells were fixed 15 min with 4% paraformaldehyde (PFA). The macrophages were stained with either CD68 (allophycocyanin, APC) and Tim4 (phycoerythrin, PE) or with F4/80 (phycoerythrin, PE) for 1 h at room temperature in the dark. All antibodies were diluted 1:100 in 5% rabbit serum/PBS. After extensive washing step, the coverslips were mounted on glass slides with mounting medium and let dry overnight at 4°C in the dark. Microscopy was performed with an Eclipse-80i microscope (Nikon) and a monochromatic camera (DS-Qi1MC; Nikon). Pictures are displayed with indicated pseudocolors by NIS elements software BR3.0 (Nikon).

In vivo phagocytosis assay

Five days after i.p. injection of 1ml of 3% Brewer's thioglycollate, 15.10^6 CFSE-labelled apoptotic thymocytes were i.p. injected into WT mice. After 30min, peritoneal lavage was performed as previously described and the collected cells were stained for Tim4 (PE, 1:400), CD115 (APC, 1:400) and F4/80 (Pacific Blue, 1:800). The cells were analysed with a GALLIOS cytofluorometer (Beckmann Coulter) and FlowJo Software. After exclusion of the doublets, Tim4 expression and engulfment capacities of the $CD115^+$ $F4/80^+$ cells (CFSE signal) were analysed.

In vivo apoptotic cells uptake

5.10^7 CFSE-labelled apoptotic thymocytes were i.v. injected into 10 to 12 weeks-old C57BL/6 WT and $Nr4a1^{-/-}$ mice. After 18 h, the mice were sacrificed and the spleens were collected. One part was embedded in OCT Tissue-Tek compound, snap-frozen on dry-ice and conserved at -80°C. The other part was used for RNA analysis.

2.2.5 Phospholipids vesicles preparation

1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC) and 1,2-dimyristoyl-3-sn-phosphoserine (DMPS) were purchased from Avanti Polar Lipids. Phospholipids were dissolved in chloroform and stored at -80°C until their use. For each experiment, indicated lipids were evaporated under a stream of argon, and resuspended in culture medium by vigorous vortexing for at least 30s.

2.2.6 RT-PCR analysis

Total RNA was isolated from cells using peqGOLD TriFast^{TM}. When RNA were isolated from organs, the isolation was followed by a genomic DNA-digestion step, performed with DNAse I. 700ng was used for the first-strand complementary DNA synthesis (Amersham Biosciences), which was then used for SYBR Green–based quantitative real time-PCR (RT-PCR) [119]. Relative quantification was performed by calculating the difference in cross-threshold values (ΔCt) of the gene of interest and a housekeeping gene according to the formula 2-ΔCt. Normalized gene expression values were calculated as the ratio of expression of messenger RNA (mRNA) for the gene of interest to the expression of mRNA for β-actin.

2.2.7 Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants measurement for murine IL-12p40, IL-10, TNF-α, IL-6 and CXCL1 were performed with ELISA-kits (R&D-systems). The protocols from the company were strictly followed.

2.2.8 Auto-antibodies quantification

For the detection of anti-dsDNA antibodies, microtiter plates were coated overnight with 20µg/ml poly-L-Lysine, washed three times with TE buffer and coated again overnight with 20µg/ml calf thymus DNA.

For the detection of anti-histone antibodies, microtiter plates were coated overnight with 50µg/ml histone of calf thymus (dissolved in ethanol) in PBS.

All following washing steps were performed with ELISA washing-buffer and unspecific bindings were blocked in both ELISA with 2%FCS in PBS for 30 min. Sera were added at 1:100 dilution in 2%FCS/PBS for 1h30 at room temperature. Bound IgGs were detected with

a horseradish peroxidase-conjugated goat anti-mouse IgG (IgG-HRP) at 1:4000 dilution in 2%FCS/PBS for 1h30 at room temperature. The detection of the IgG-HRP was performed with ELISA substrate for no longer than 10 min and the reaction was stop by acid (ELISA stop solution).

2.2.9 Western blotting

Cultured cells were lysed by liquid nitrogen and resuspend in Laemmli Buffer (25 µl / 0,5.10^6 cells). Equal amounts of protein extracts were fractionated by sodium dodecyl sulfate–polyacrylamide gel (10%) electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked 1 h with 5% non-fat dry milk in Tris-buffered saline (TBS) and then immunoblotted overnight at 4°C with either anti-phospho-NF-κB -p65 antibody (1:500) or anti-actin antibody (1:1000) diluted in 5% non-fat dry milk/TBS. A polyclonal goat anti-rabbit HRP-conjugated secondary antibody was applied the next day for 1 h at room temperature and the proteins were visualized by enhanced chemiluminescence (ECL) procedure, accordingly to the manufacturer's instruction.

2.2.10 Chromatin immunoprecipitation

Resident peritoneal macrophages were isolated from WT and Nr4a1$^{-/-}$ mice, plated in R10 medium at the density of 300 000 cells/cm^2. The cells were pre-incubated during 2 hours with phospholipids vesicles (10 µg/ml DMPS + 40 µg/ml PAPC) and then stimulated with 100ng/ml LPS during 30min. The cells were harvested and ChiP experiment performed with the ChiP-IT™ Express kit (Active Motif) according to the manufacturer´s intructions. The immunoprecipitation was performed by using a rabbit anti-CoREST antibody (Millipore) and a rabbit anti-IgG (Santa Cruz) as control. The ChiP and input DNA were analysed by quantitative RT-PCR. The fold enrichment was calculated as a ratio of the amplification efficiency of the ChiP sample over that of the IgG:

Fold enrichment = % ChiP / % IgG

with % ChiP = 2^(Input Ct-ChiP Ct)(100)

and % IgG = 2^(Input Ct-IgG Ct)(100).

2.2.11 Flow cytometry (FACS) analyses

The analysis of cell surface molecules was performed with a GALLIOS cytofluorometer (Beckmann Coulter) and analysed with FlowJo software. After isolating and counting of the cells, a step of blocking was performed by resuspending the cells in 20 µl FACS buffer containing FcBlock (1:100). After 10 min incubation at room temperature, 80 µl of staining solution containing the diluted antibodies in FACS buffer at a saturating concentration was added, and the cells were incubated in the dark, at 4°C for 30 min. Afterwards, the cells were washed with 200 µl FACS buffer and finally resuspended in 200 µl FACS buffer.

2.2.12 Immunofluorescence

The spleens were snap-frozen in O.C.T Tissue-Tek compound and kept for at least 2 days at -80°C. Sections of 4 µm were performed and fixed for 2,5 min in a cold acetone bath (-20°C) and stored in -20°C until staining.

The slides were rehydrated with PBS for 5 min and then blocking buffer (10% goat serum in PBS) was added for at least 30 min. The antibodies were diluted in 5% goat serum in PBS and were applied to the sections for 1 h at room temperature in the dark. After 3 washing steps of 5 min with PBS, the slides were covered using mounting medium.

2.2.13 Pristane induced-experimental murine lupus

Autoimmunity was induced in C57Bl/6 and NR4a1$^{-/-}$ mice with a single intraperitoneal injection of 0,5 ml pristane oil at 10 weeks of age. The mice were bled one time before the injection and then every month during 4 months and sacrificed after 4 months. Sera were collected after centrifugation of blood at 5000 rcf for 5 min and frozen at -80°C. Spontaneous autoimmune phenomena were assessed in mice without any stimulus or challenge at 26 weeks of age.

2.2.14 Statistical analysis

All data are shown as means ± SEM. Group mean values were compared by two-tailed Student's t test. Significant differences are indicated as follows: * $p<0.05$; ** $p<0.01$; *** $p<0.001$

3. Results

3.1 Resident and inflammatory MΦs differentially respond to TLR stimulation

Resident and inflammatory MΦs are defined as two distinct MΦ populations having different origins. However, little is known about differences in their function. In general, MΦs are known to be one of the first lines of defence against pathogens and recognize both bacterial and viral particles through Toll-Like Receptors (TLRs). Analysis of the mRNA expression in tissue-resident and inflammatory MΦs isolated from the peritoneal cavity of mice, revealed a differential expression pattern of TLRs (fig.7). Inflammatory MΦs expressed higher levels of TLR1 whereas resident macrophages expressed more TLR2, TLR3 and TLR7.

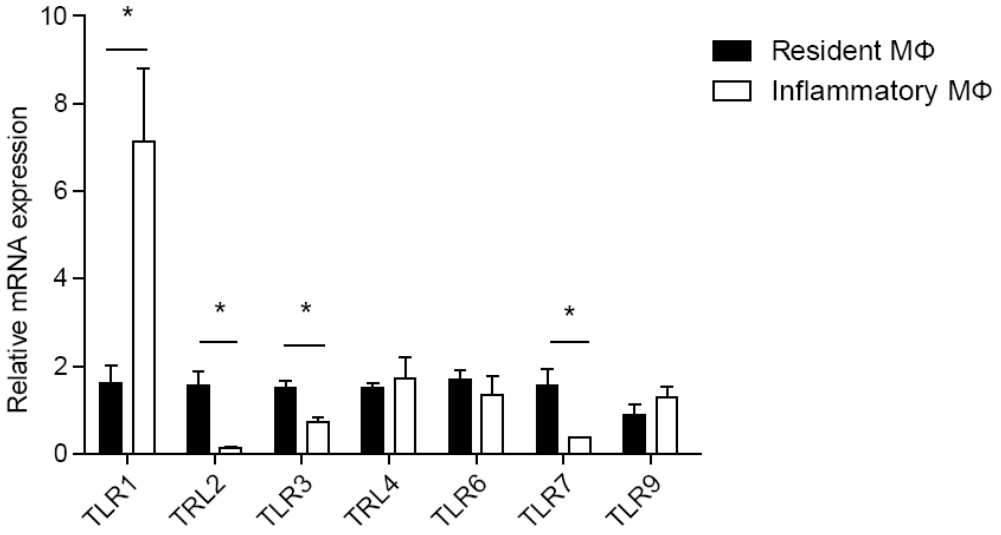

Figure 7: **Resident and inflammatory MΦs differentially express TLRs.** Relative mRNA expression of TLR analysis performed on non-stimulated tissue-resident and inflammatory macrophages (isolated 3 days after 2,5 mL i.p. injection of 3% Brewer's thioglycollate) by real-time PCR (RT-PCR) analysis. Black bars indicate resident MΦs and white bars inflammatory MΦs.

MΦs differentially reacted to TLR stimulation and TLR ligand (TLRL)-stimulated resident and inflammatory macrophages show distinct activation patterns (fig.8). While inflammatory MΦs produced high amount of pro-inflammatory cytokines, such as TNF-α and IL-12p40, tissue-resident MΦs produced more IL-6 and IL-10. The gene expression analysis also revealed an alternative phenotype of resident MΦs (high arginase expression) and a classical activation profile of the inflammatory MΦs (high iNOS expression).

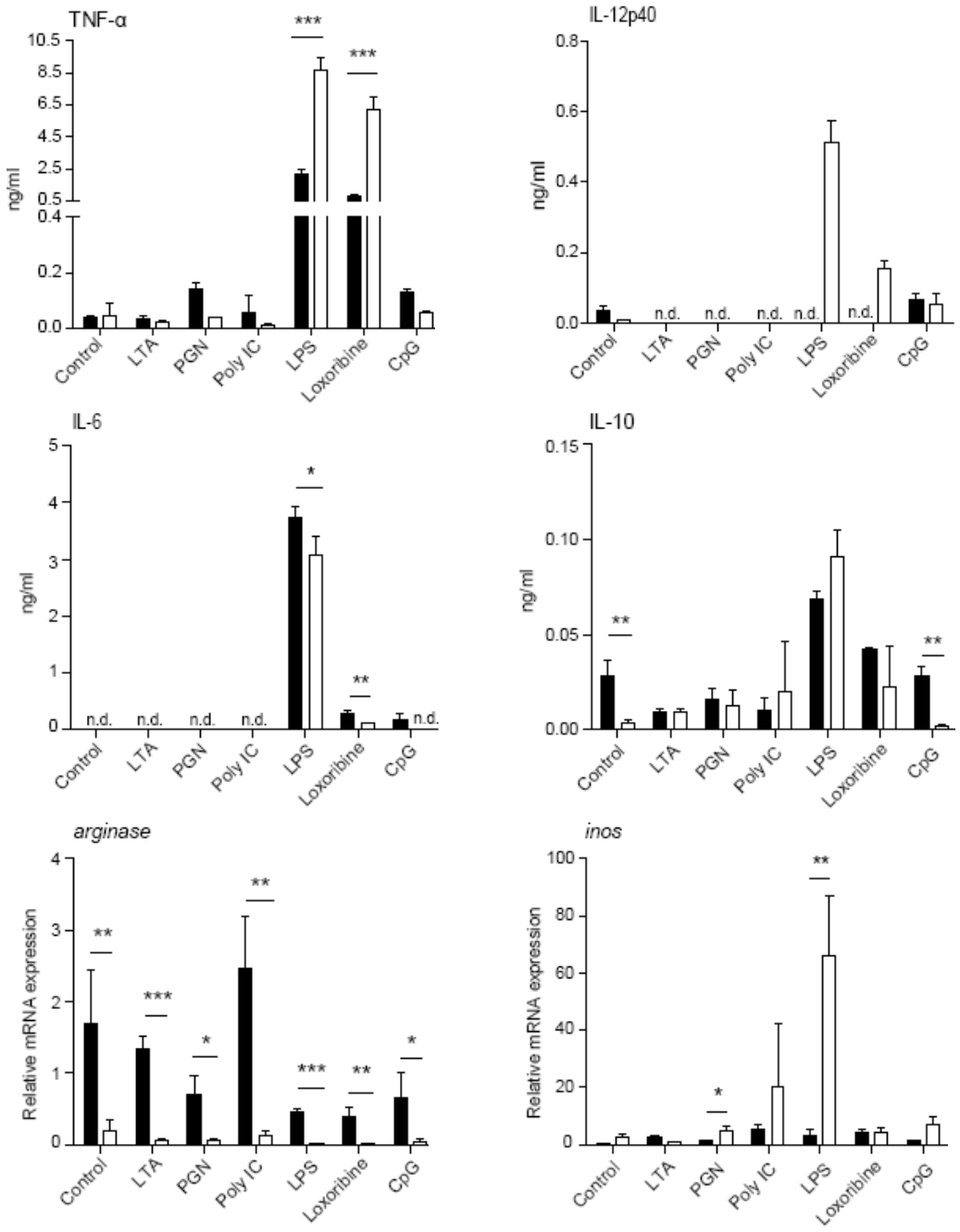

Figure 8: Resident and inflammatory MΦs differentially react to TLR stimulation. Tissue-resident and inflammatory macrophages were isolated from murine peritoneum and stimulated for 6 h with TLRLs. Cytokine production was analysed by ELISA and gene expression was determined by RT-PCR analysis. LTA= Lipoteichoic acid from S. aureus, TLR2L, 1 µg/ml. PGN= peptidoglycan, TLR2L, 4 µg/ml. Poly (I:C)= Polyinosine-polycytidylic acid, TLR3L, 100 ng/ml. LPS= lipopolysaccharide, TLR4L, 100 ng/ml. Loxoribine, TLR7L, 1 mM. CpG, TLR9L, 3,5 µg/ml. Black bars indicate resident MΦs and white bars inflammatory MΦs.

These data clearly showed that resident and inflammatory macrophages do not only differently express TLR, but that they also react differently to pro-inflammatory TLR stimuli.

3.2 Apoptotic cells are preferentially engulfed by resident MΦs

The clearance of apoptotic cells (ACs) by specialized phagocytes is still an incompletely understood process. In particular, it remains to be determined, which subtype of MΦ is responsible for the rapid and non-immunogenic clearance of ACs. Using a model of peritonitis, we sought to compare the function of tissue-resident and monocyte-derived-inflammatory MΦs in terms of their capacity to ingest dying cells. This model easily allows the isolation of these two subtypes of MΦs from the same cavity at the same time. We focused on two major subtypes of macrophages: tissue-resident Tim4^{+} F4/80^{+} macrophages and monocytes-derived-inflammatory Tim4^{-} F4/80^{+} macrophages (fig.9). To analyse the phagocytic capacity of these MΦs, ACs were intraperitoneally (i.p.) injected and their engulfment was analysed by FACS analysis. These experiments indicated that, during peritonitis, apoptotic thymocytes are mainly taking up by tissue-resident Tim4^{+} peritoneal macrophages, whereas inflammatory MΦs hardly participated in the uptake of dying cells (fig.9A). These observations were confirmed by *in vitro* phagocytosis assay (fig.9B). Accordingly, we observed that resident MΦs efficiently and homogenously ingested ACs during steady state, also in the absence of inflammatory MΦs (fig.9C).

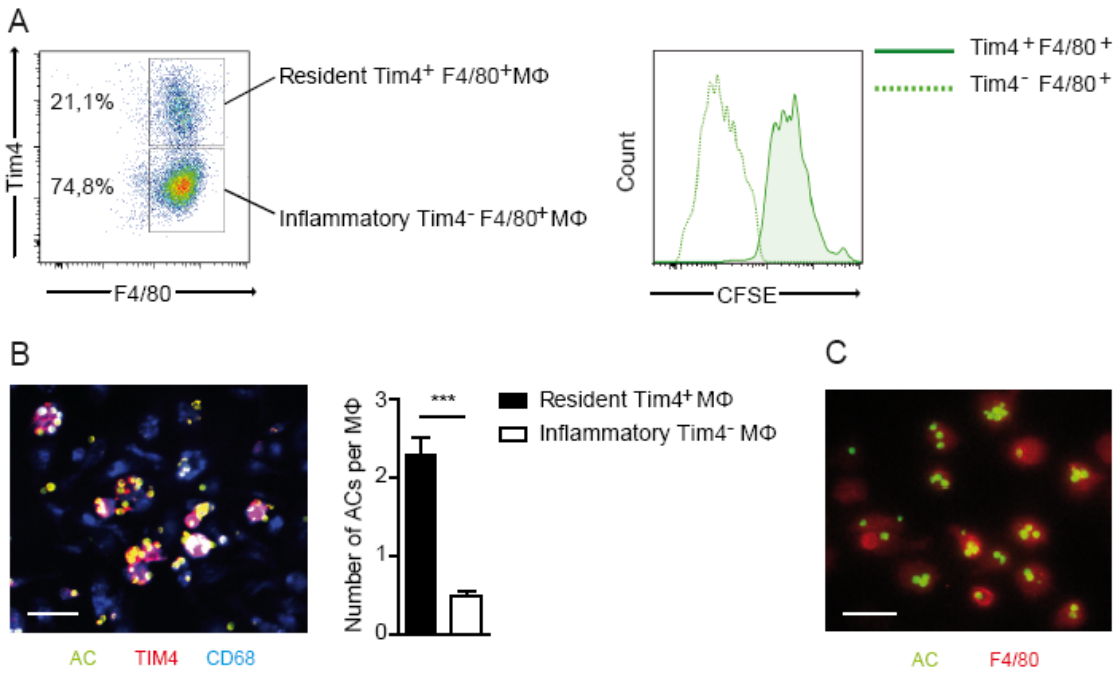

Figure 9: Resident macrophages mainly engulfed apoptotic cells. (**A**) C57BL/6 wild-type (WT) mice were intraperitoneally (i.p) injected with 1 mL of 3% Brewer's thioglycollate. After five days, 15.10^{6} CFSE-labelled C57BL/6 apoptotic thymocytes were i.p. injected. After 30 min, peritoneal lavage was performed and the

isolated cells were analysed by FACS. After exclusion of the doublets, the cells were gated on $CD115^+$ cells and the intensity of CFSE was determined in both $Tim4^+$ and $Tim4^-$ population. (**B**) WT resident and inflammatory macrophages were isolated 5 days after i.p. injection of 1 mL of 3% Brewer's thioglycollate, plated on 24-well plates and co-incubated with CFSE-labelled apoptotic thymocytes (green) for 30 min. The macrophages were stained with CD68 (blue) and Tim4 (red). (**C**) WT peritoneal resident macrophages (F4/80, red) were isolated from healthy mice and co-incubated with CFSE-labelled apoptotic thymocytes (green) for 30 min.

These results indicated that the clearance of ACs is a defined process and restricted to specialized macrophage subpopulations.

3.3 Resident and inflammatory macrophages differentially express AC-binding receptors

One possible explanation for the differential uptake of ACs by MΦs is a difference in the expression profile of AC-binding receptors. Indeed, we could confirm that tissue-resident $Tim4^+$ and inflammatory $Tim4^-$ macrophages showed a distinct mRNA expression profile of various receptors and molecules that have been implicated in the clearance of ACs. Whereas resident MΦs expressed more Tim4, 12/15LO, Axl, Tyro3 and CD14, inflammatory MΦs clearly expressed more Tim3, Mfg-e8, Gas6 and Mertk (fig.10).

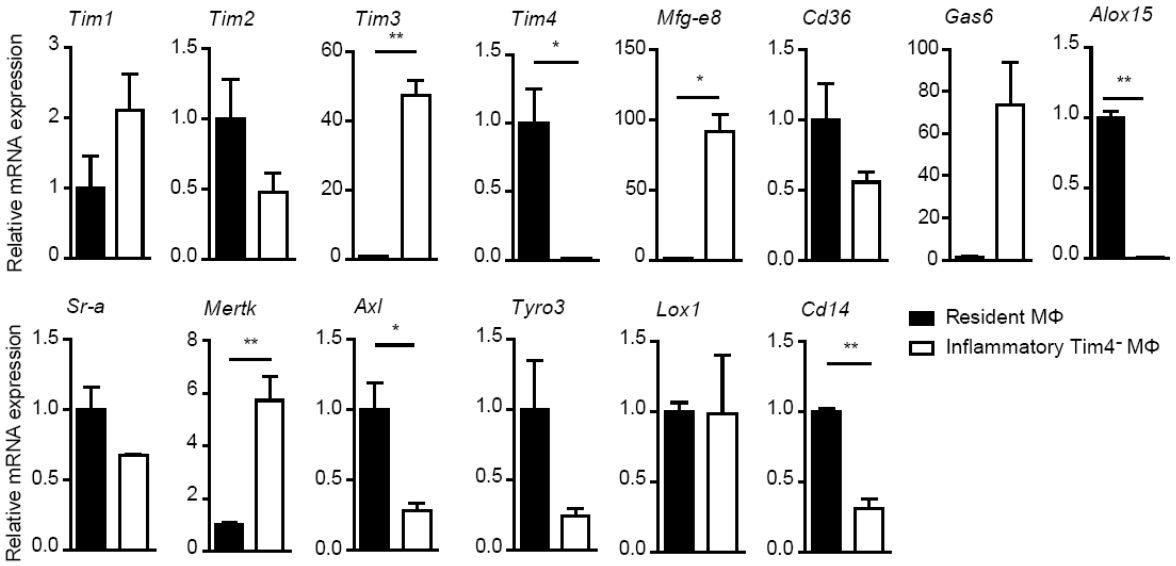

Figure 10: Resident and inflammatory macrophages have a different pattern of receptor expression. Tissue-resident MΦs were isolated from the peritoneum of healthy WT mice. Pure populations of inflammatory $Tim4^-$ MΦs were isolated from the peritoneal cavity of WT mice, 3 days after 2,5 ml i.p. injection of 3% Brewer's thioglycollate. Inflammatory MΦs were separated from resident MΦs by MicroBeads.

3.4 ACs exert anti-inflammatory effects on tissue resident macrophages

Previous studies have already shown that ACs are not only removed in an immunological silent way, but that they also exert anti-inflammatory effect through the inhibition of pro-inflammatory cytokines [62, 63, 120]. We confirmed these observations in our model and studied the effect of ACs on the LPS-induced cytokine expression of MΦs. In accordance with previous data, ACs blocked the expression of pro-inflammatory cytokines such as IL-12p40, TNF-α and IL-6 and boosted the production of anti-inflammatory cytokines such as IL-10 in resident MΦs (fig.10A). These effects were also observed in inflammatory MΦs but to a lower extend (fig.10B) and the cytokine production of bone-marrow-derived dendritic cells (BMDC) was not affected by the presence of ACs (fig.10C). These results indicated that ACs exert the strongest anti-inflammatory effect on resident MΦs as this MΦ subset ingested the highest number of ACs.

It is important to note that ACs did not affect the expression of every cytokine (e.g. CXCL1, TGF-β1), suggesting that ACs do not act through a common and general process that inhibits the production of cytokines, but rather specifically block distinct molecular pathways. However, the anti-inflammatory effects of ACs are still poorly understood and in particular their molecular mode of action to repress the production of pro-inflammatory cytokines is not well defined.

To better address the underlying mechanisms, we performed a set of experiments. First, we added cycloheximide (CHX), a protein synthesis inhibitor, to test whether the synthesis of new proteins was involved in the anti-inflammatory effects of ACs. Incubation with CHX indeed interfered with the AC-induced block of IL-12p40 in resident MΦs (fig.11). These experiments gave a first hint and indicated that the anti-inflammatory effects of ACs are mediated via the production of certain proteins by MΦs, which subsequently interfered with the cytokine production in these cells.

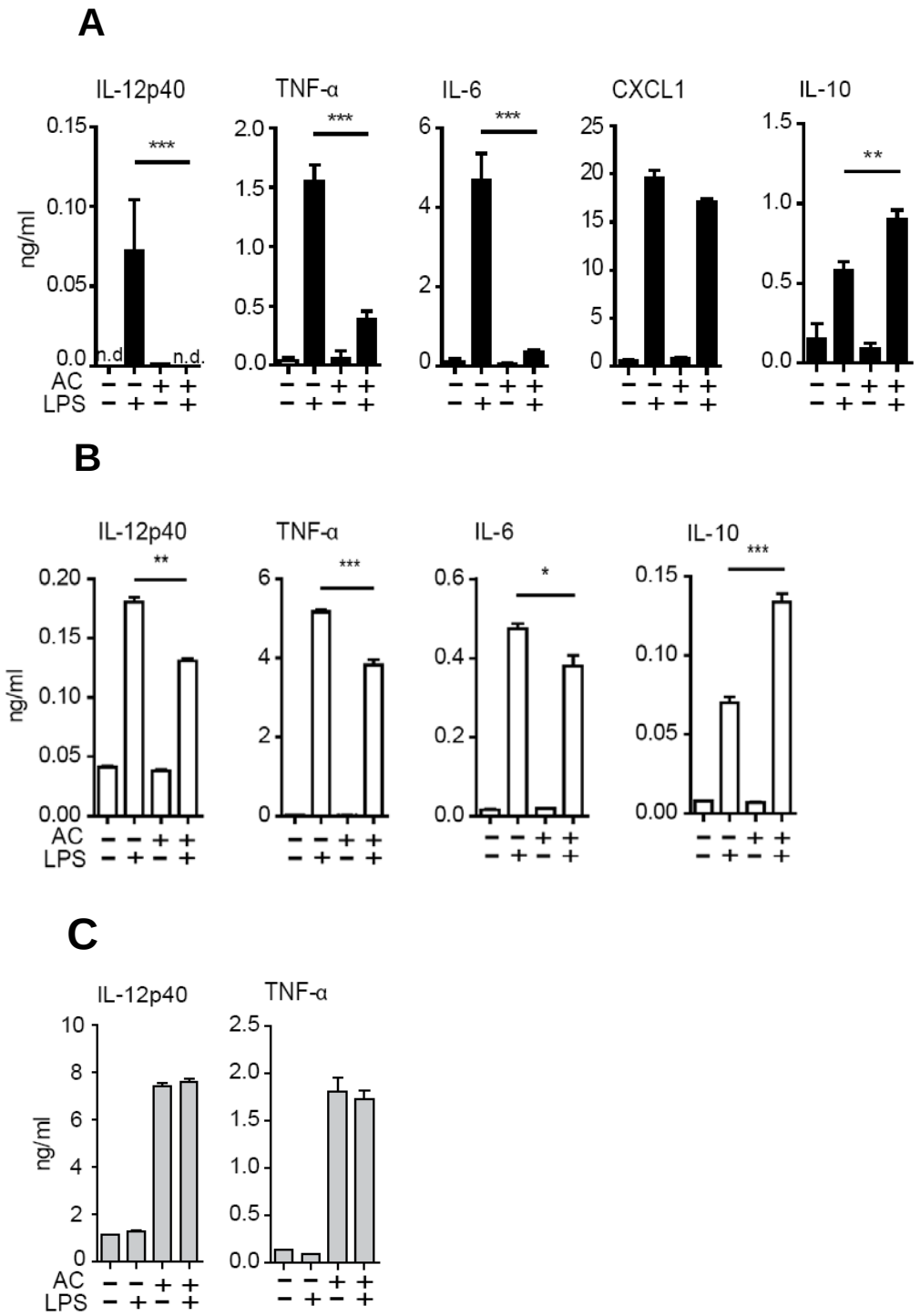

Figure 10: ACs repress pro-inflammatory cytokines production from LPS-stimulated resident MΦs. Resident WT (**A**) and inflammatory (**B**) MΦs and dendritic cells (DC, **C**) were preincubated with a 1:5 ratio of ACs during 1 h and then stimulated overnight with 100 ng/ml LPS. Cytokines production was analysed by ELISA.

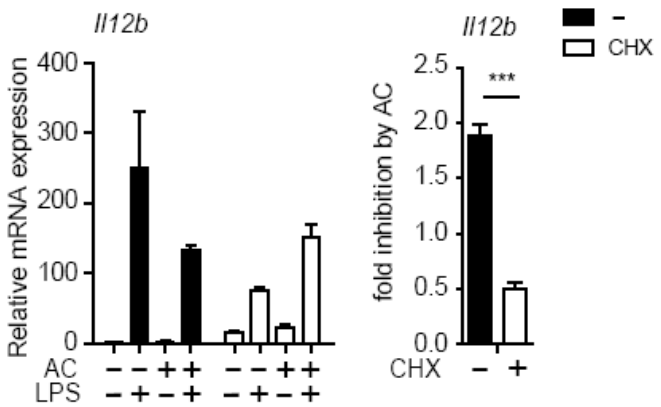

Figure 11: IL12-p40 blockade by ACs is protein synthesis dependant. Resident peritoneal MΦs were pre-incubated for 30 min with 10 mg/ml of cycloheximide (CHX) and then incubated with ACs (ratio 1:5) for 1 h before being stimulated for 4 h with 100 ng/ml of LPS. The relative mRNA expression of IL-12p40 was analyzed by RT-PCR.

3.5 ACs induce Nr4a1 expression

The stimulation of MΦs with ACs leads to strong and rapid molecular changes. Already 1 to 4 h after stimulation, the expression of a wide panel of immediate early response genes was heavily changed. This included genes such as Early growth response protein 1 (Egr-1), JunD and Activating transcription factor 4 (Atf4) (fig.12). Moreover, one gene family was even stronger affected: the nuclear receptor (NR) family Nr4a. All members of this subfamily (Nr4a1-3) – and especially Nr4a1 – were highly and rapidly induced upon co-incubation of MΦs with ACs. In accordance, previous studies showed an important role of Nr4a1 in the regulation of inflammation [121] and immune cell development [11, 122, 123], which suggested a potential role for Nr4a1 in the non-inflammatory removal of ACs by resident macrophages.

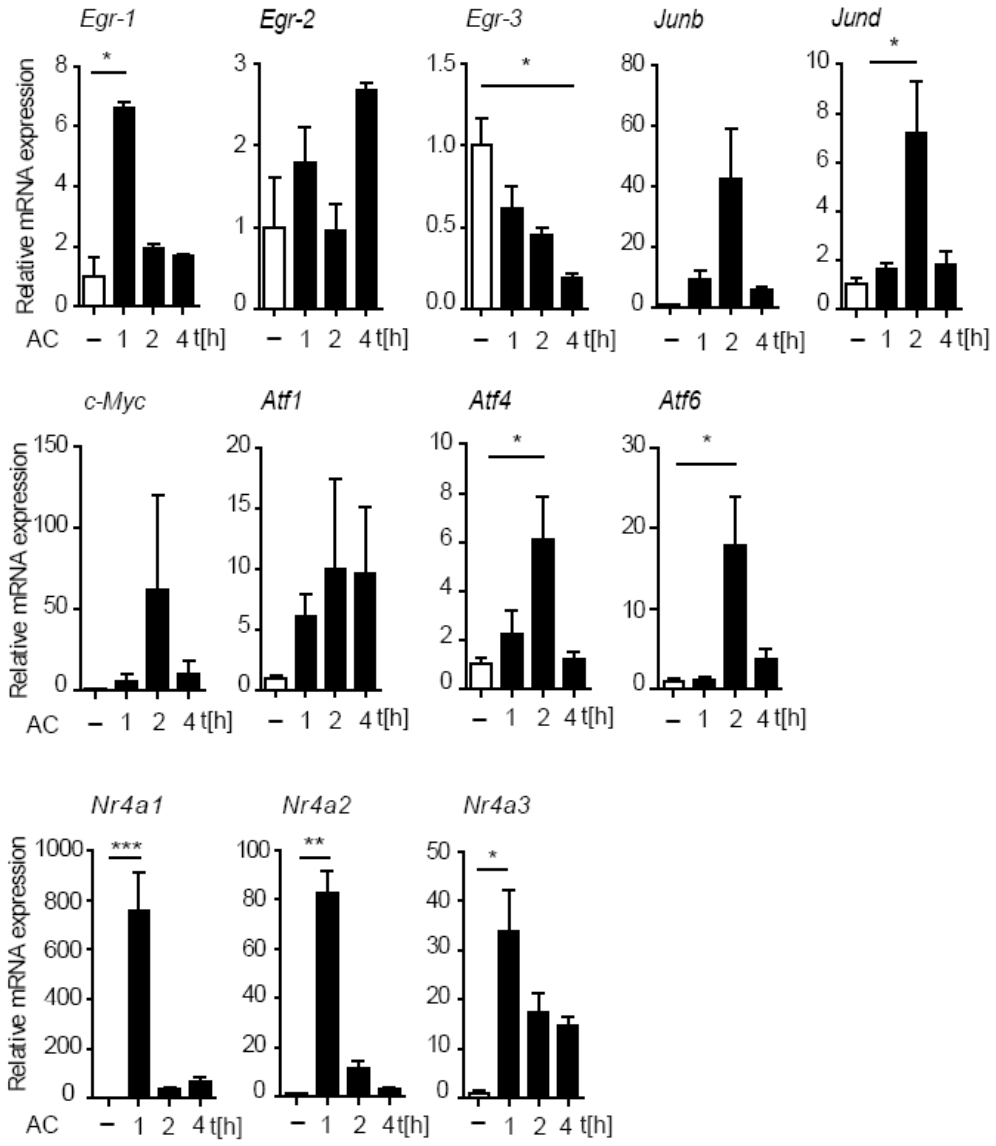

Figure 12: ACs induce Nr4a1 expression. Resident peritoneal macrophages were co-incubated with ACs (ratio 1:5) for the indicated times and the relative mRNA quantification of indicated genes was performed by RT-PCR analysis.

To test whether the induction of Nr4a1 was restricted to resident MΦs, we performed the same experiment with thioglycollate-elicited inflammatory MΦs and BMDCs. When incubated with ACs, these cells only showed a slight and delayed increase in Nr4a1 expression (fig.13A and B), which indicated a cell-type-specific role of Nr4a1 during the clearance of ACs by myeloid cells. Moreover, the incubation of resident MΦs with necrotic cells (NCs) did induce only a very minor increase in Nr4a1 expression (fig.14), suggesting that Nr4a1 was induced by a specific signal coming from ACs and not from NCs.

Alternatively, signals derived from NCs might exert an inhibitory influence on Nr4a1 expression.

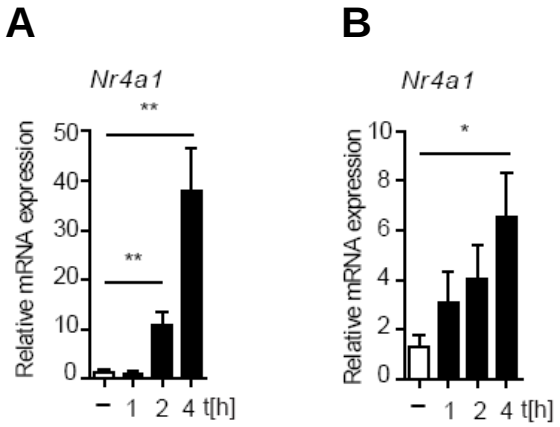

Figure 13: ACs only slightly induce Nr4a1 expression in inflammatory MΦs and dendritic cells. Thioglycollate-induced inflammatory MΦs (**A**) and Bone-Marrow-Derived Dendritic cells (BMDC, **B**) were co-incubated with ACs (ratio 1:5) for the indicated times. Relative mRNA analysis of Nr4a1 was performed by RT-PCR analysis.

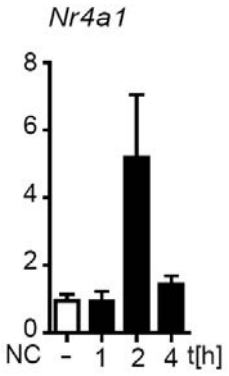

Figure 14: NCs do not induce Nr4a1. WT peritoneal macrophages were co-incubated with necrotic cells (NC, ratio 1:5) for the indicated times and the relative mRNA quantification of Nr4a1 was performed by RT-PCR analysis.

Since Nr4a1 was reported to potently block inflammatory cytokine expression, we hypothesized a major role of this transcription factor during the anti-inflammatory effects of ACs. Therefore we first investigated the mechanisms controlling the AC-induced expression of Nr4a1 in resident MΦs.

3.6 Nr4a1 induction is phosphatidylserine-, p38- and ERK-dependent.

When incubated with latex beads, tissue-resident macrophages did not show an increase in Nr4a1 expression (fig.15A), suggesting that the induction of Nr4a1expression was not due to the phagocytosis process *per se*, but rather specifically induced by the AC.

Nr4a1 induction was a rapid process, happening during the first steps of the uptake of ACs, where the dying cell is recognized by the receptor-mediated binding of phosphatidylserine (PS) molecules present on the surface of the AC. This suggested that NR4a1 is directly induced via receptor-mediated recognition. As shown earlier, resident and thioglycollate-induced inflammatory MΦs expressed a differential receptor profile (fig.10) which could explain the differential expression of Nr4a1 within the phagocyte subsets. To address a potential involvement of PS exposure during the effects exerted by ACs, we performed phagocytosis assays in the presence of Annexin V, which masks PS molecules on the AC surface. Pre-incubation of ACs with Annexin V significantly blocked the AC-induced Nr4a1 expression in resident MΦs, demonstrating a PS-dependent process (fig.15A). Next we incubated resident MΦs with PS-containing liposomes alone and observed again a rapid and significant induction of Nr4a1 expression (fig.15B). Together, these data suggest a key role for PS recognition by resident MΦs during the AC-mediated induction of Nr4a1.

To further define the mechanisms underlying the induction of Nr4a1, we specifically blocked different molecular pathways by pre-incubating resident MΦs with specific pathway inhibitors, before we co-incubated them with ACs. Notably, AC-induced Nr4a1 expression was significantly blocked by p38 and p44/p42 inhibitors, whereas protein kinase A (PKA), protein kinase C (PKC) and NF-κB inhibitors did not interfere with the effects exerted by ACs (fig.15C). These data clearly indicate that ACs induce Nr4a1 expression via PS recognition and through p38 and ERK pathway.

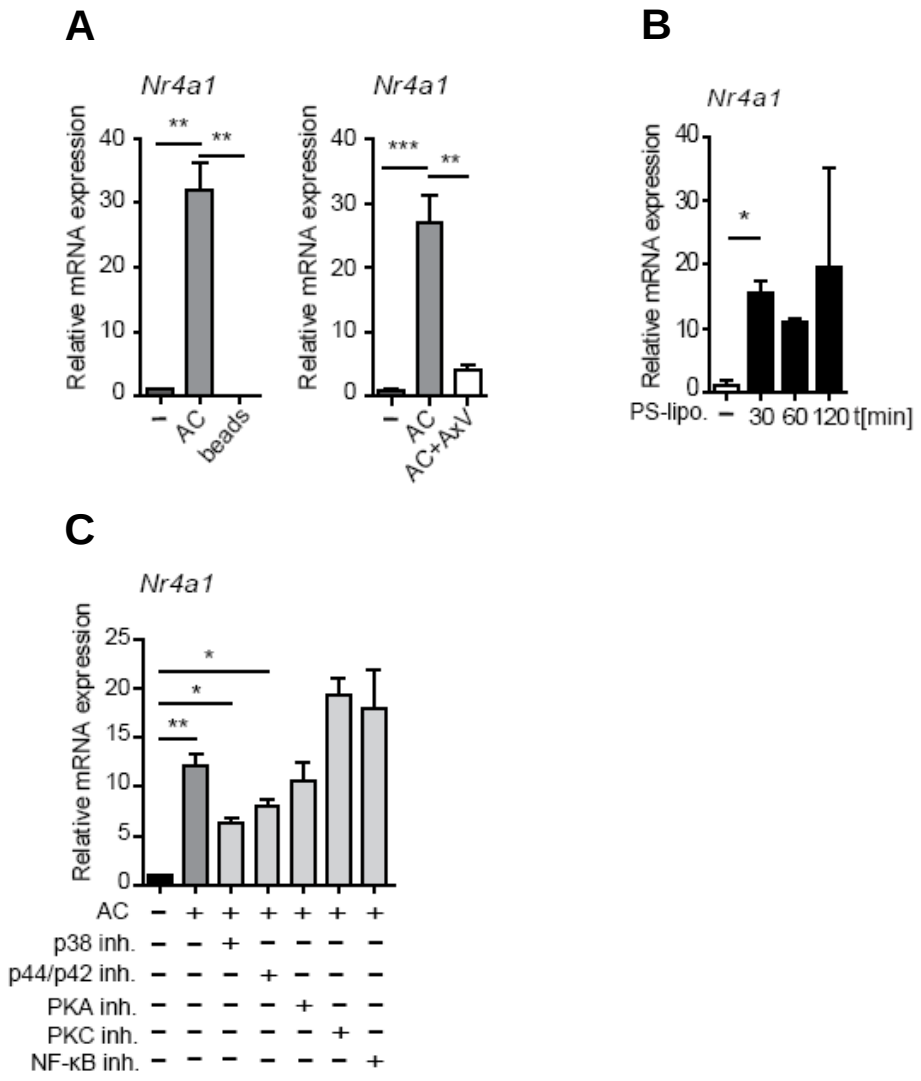

Figure 15: ACs induce Nr4a1 expression via PS recognition and p38, p44/p42 pathway. Resident peritoneal MΦs were incubated ACs (ratio 1:5) or latex beads (2%) (**A**) or ACs coupled with Annexin V (AC+AxV) and Nr4a1 mRNA expression was analysed by RT-PCR. (**B**) Resident peritoneal MΦs were incubated for the indicated times with PS-containing phospholipid liposomes (PS-lipo.) and Nr4a1 mRNA expression was analysed by RT-PCR. (**C**) Resident peritoneal MΦs were pre-incubated for 30 min with the indicated inhibitors before addition of ACs (ratio 1:5) for 1 h. RT-PCR analysis was performed to quantify Nr4a1 expression.

3.7 Nr4a1 mediates the anti-inflammatory effect of apoptotic cells

Nr4a1 was shown to be induced by pro-inflammatory stimuli such as LPS [104] and plays a major role in the regulation of inflammation. Our current data indicated that ACs also strongly induced its expression. Moreover, an interesting synergistic effect on Nr4a1 expression was observable when MΦs were simultaneously stimulated by ACs and LPS (fig.16A) and this effect inversely correlated with IL-12p40 production. Whereas ACs blocked the production of

IL-12p40 after LPS stimulation, they rather boosted Nr4a1 expression, suggesting a potential role for Nr4a1 as mediator of the anti-inflammatory effects of the ACs, and especially during the block of IL-12 production.

To determine the exact role of Nr4a1 in the anti-inflammatory effect of ACs, resident peritoneal MΦs were isolated from both WT ($Nr4a1^{+/+}$) and Nr4a1-deficient ($Nr4a1^{-/-}$) mice. Subsequently, we analysed the anti-inflammatory effects of ACs on both populations of MΦs. As previously shown, pre-incubation with ACs blocked IL-12p40 production in LPS-stimulated macrophages (fig.16B). Notably, these anti-inflammatory effects of ACs were strongly reduced in the absence of Nr4a1. These data indicate that Nr4a1 contributes to the anti-inflammatory effects of ACs.

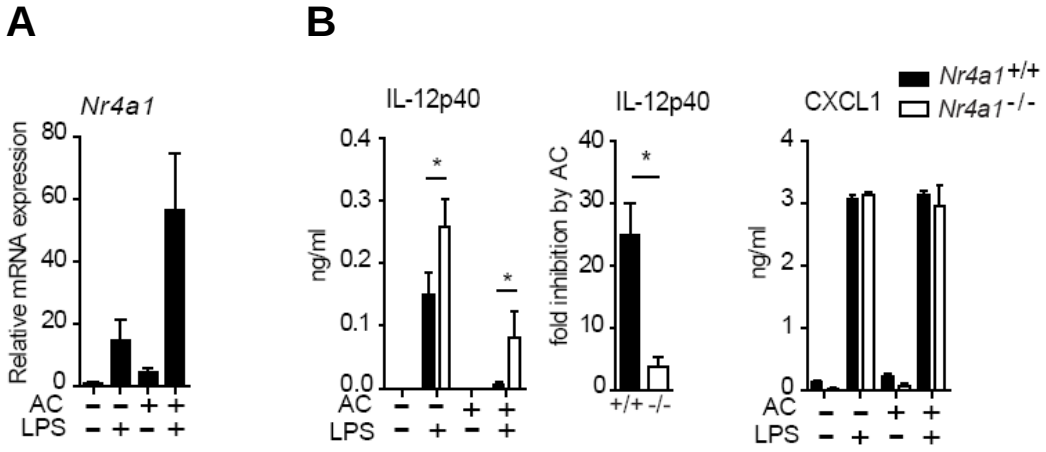

Figure 16: The anti-inflammatory effects of ACs are Nr4a1 mediated. (**A**) Resident peritoneal WT MΦs were pre-incubated 2h with ACs before to be stimulated with 100 ng/ml LPS. Relative mRNA expression of Nr4a1 was quantified by RT-PCR. (**B**) Resident peritoneal MΦs from WT and $Nr4a1^{-/-}$ mice were pre-incubated 1 h with ACs (ratio 1:5) before to be stimulated overnight with 100 ng/ml LPS.

Phosphatidylserine is thought to be one of the major "eat-me" signal on the surface of ACs [54, 124, 125] and our data showed that it contributed to the induction of Nr4a1 expression (fig.15B). Consequently, we investigated the role of phosphatidylserine in the mediation of the anti-inflammatory effects of ACs via Nr4a1 induction. As shown by previous work [126, 127], phosphatidylserine-containing liposomes exerted anti-inflammatory effect on LPS-stimulated MΦs (fig.17) including a block of IL-12p40 production. CXCL1 was again not affected by PS exposure. Moreover, this PS-mediated effect was strongly inhibited in $Nr4a1^{-/-}$ resident peritoneal MΦs, suggesting that the anti-inflammatory effects of PS-liposomes are also Nr4a1-mediated.

Tacking together, these results indicate that ACs specifically modulated the inflammatory response of LPS-stimulated resident peritoneal MΦs through induction of Nr4a1.

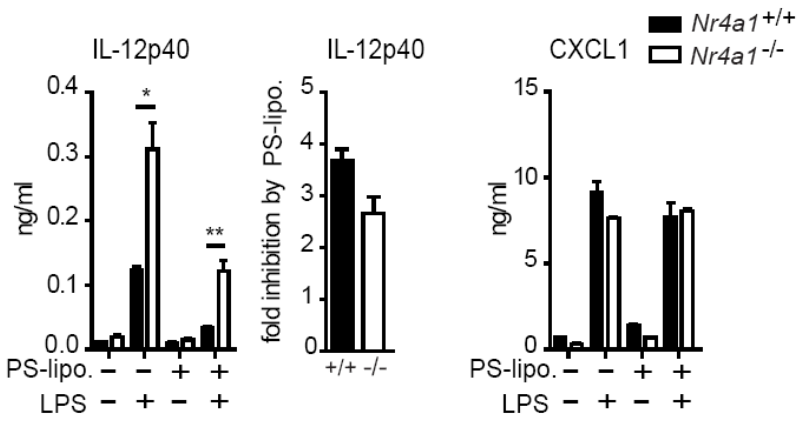

Figure 17: PS-liposomes mediate anti-inflammatory effects through Nr4a1. Resident peritoneal MΦs from WT and Nr4a1$^{-/-}$ mice were pre-incubated 1 h with PS-containing phospholipid liposomes (PS-lipo) and stimulated overnight with 100 ng/ml LPS. The cytokine production was analysed by ELISA.

The NF-κB pathway is known to be a major regulator of inflammatory signaling in MΦs [128] and the production of many pro-inflammatory cytokines, such as IL-12p40, is known to be NF-κB-dependent [129]. ACs have been reported to attenuate the activity of the NF-κB pathway [130], but the underlying mechanisms are still incompletely understood. To evaluate the contribution of Nr4a1 to this process, we pre-incubated resident MΦs with ACs and stimulated them for different time points with LPS. We followed the phosphorylation of the p65 subunit of NF-κB at serine 536 by western blot analysis. p65 phosphorylation serves as a hallmark for NF-κB pathway activation.

The phosphorylation of the p65 subunit of NF-κB that was induced by LPS stimulation in resident MΦs, was strongly blocked by ACs (fig.18A). Notably, this AC-induced inhibition of NF-κB phosphorylation was abrogated in Nr4a1$^{-/-}$ MΦs. This attenuation of NF-κB pathway activity was also observed when MΦs were pre-incubated with PS-containing liposomes (fig.18B). This effect was again abrogated in Nr4a1-deficient MΦs. Together, these results indicate that ACs block p65 phosphorylation and this effect is PS-recognition- and Nr4a1-dependent.

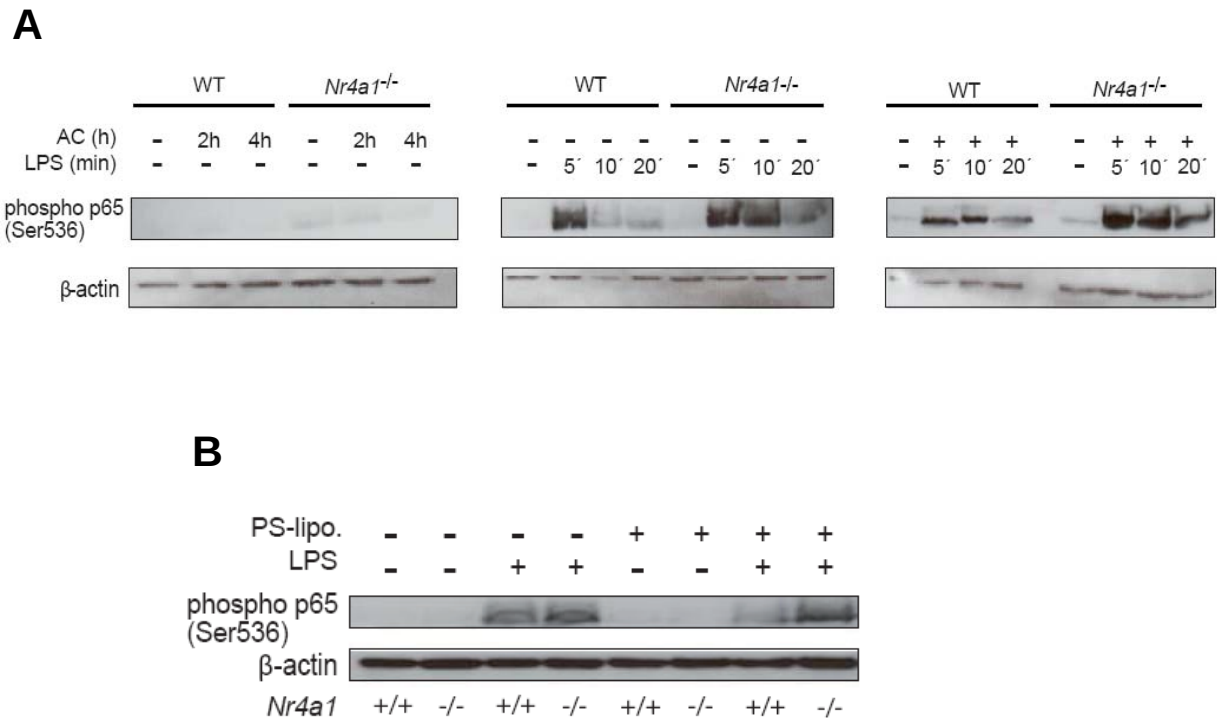

Figure 18: Nr4a1 mediates the block of NF-κB pathway activation by ACs through PS recognition. Resident peritoneal WT and Nr4a1$^{-/-}$ macrophages were pre-incubated either with ACs (ratio 1:5, **A**) for the indicated time or PS-containing liposomes (PS-lipo., **B**) for 2 h and stimulated with 100 ng/ml LPS for the indicated time (**A**) or for 5 min (**B**). Serine 536 phosphorylation was analysed by western blot and detection of β-actin was used as control.

3.8 Nr4a1 participates in the recruitment of corepressor

The reduction of NF-κB activity mediated by ACs/PS-containing liposomes provided an explanation for the reduction of pro-inflammatory cytokines such as IL-12p40. However, the mechanisms leading to cytokine inhibition might be related to a more complex process. Nuclear receptors were shown to recruit corepressor molecules to the promoter of pro-inflammatory target genes and thereby inhibit their transcription. Thus, a decrease in cytokine production can be potentially explained by an increased binding of corepressor molecules to the promoter of the target gene. Here, corepressors were shown to specifically block NF-κB activity and thereby prevent cytokine transcription [79, 131].

We decided to investigate a possible role of Nr4a1 during recruitment of corepressors on NF-κB binding sites at selected promoters. Another member of the Nr4as family, Nr4a2, was recently shown to directly interact with a CoREST-containing-corepressor complex and

thereby inhibit NF-κB activity at the transcriptional level [84]. Thus, we hypothesised that Nr4a1 might also interact with CoREST and thereby block IL-12p40 production.

To test this hypothesis, WT and Nr4a1$^{-/-}$ resident MΦs were pre-incubated with PS-containing liposomes and then stimulated with LPS (fig.19). The binding of the corepressor CoREST on NF-κB binding sites at the IL-12p40 promoter was subsequently analysed by chromatin immunoprecipitation (ChIP). During the steady state, WT and Nr4a1$^{-/-}$ showed the same amount of CoREST binding to the IL-12p40 promoter. However, the recruitment of CoREST was strongly increased in WT MΦs stimulated with PS-containing liposomes and LPS, whereas it was abolished in Nr4a1$^{-/-}$ MΦs. These results suggested that Nr4a1 participated in the recruitment of the corepressor CoREST to the IL-12p40 promoter and thereby contributed to the control of inflammation on a transcriptional level.

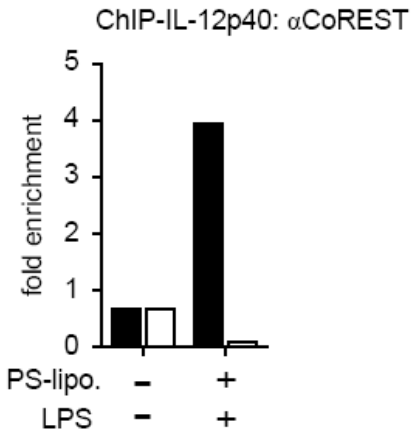

Figure 19: Nr4a1 mediates the recruitment of CoREST induced by PS-containing liposomes. Resident peritoneal WT and Nr4a1$^{-/-}$ MΦs were pre-incuabated 2 h with PS-containing liposomes before a stimulation with 100 ng/ml LPS for 30 min. ChIP experiment was performed with anti-CoREST antibody. Black bars indicate resident WT MΦs and white bars resident Nr4a1$^{-/-}$ MΦs.

3.9 Nr4a1 promotes the non-inflammatory clearance of ACs in vivo and participates in the maintenance of self-tolerance

Our *in vitro* data indicated that Nr4a1 plays a major role in the control of the non-inflammatory clearance of ACs by tissue-resident MΦs. A failure during this process can eventually lead to a deregulation of the immune system and to the development of autoimmunity.

To investigate the role of Nr4a1 in the clearance of ACs *in vivo*, Nr4a1$^{+/+}$ and Nr4A1$^{-/-}$ mice were intravenously (i.v) injected with CFSE-labeled ACs and their spleen were analysed by immunofluorescence microscopy (IF) and mRNA quantification. In accordance with previously published data, we observed an accumulation of i.v. injected, ACs within the splenic marginal zone, where they were bound by marginal zone SIGNR1^{+} Tim4^{+} MΦs (fig.20A) [132]. Notably, injection of ACs strongly induced the expression of Nr4a1 in the spleen of WT mice; whereas the expression of the two other family members, Nr4a2 and Nr4a3, were barely affected (fig.20B). These data supported the concept of a possible role of Nr4a1 in the clearance of ACs *in vivo*. Furthermore, i.v. injection of ACs induced the expression of IL-12p40 in the spleen of Nr4a1-deficient, but not of wild-type mice (fig.20C), whereas the expression of other cytokines such as CXCL1 remained unchanged. These data suggested that the lack of Nr4a1 partially abrogates the non-inflammatory clearance of ACs *in vivo* and render these dying cells pro-inflammatory and potentially pro-immunogenic.

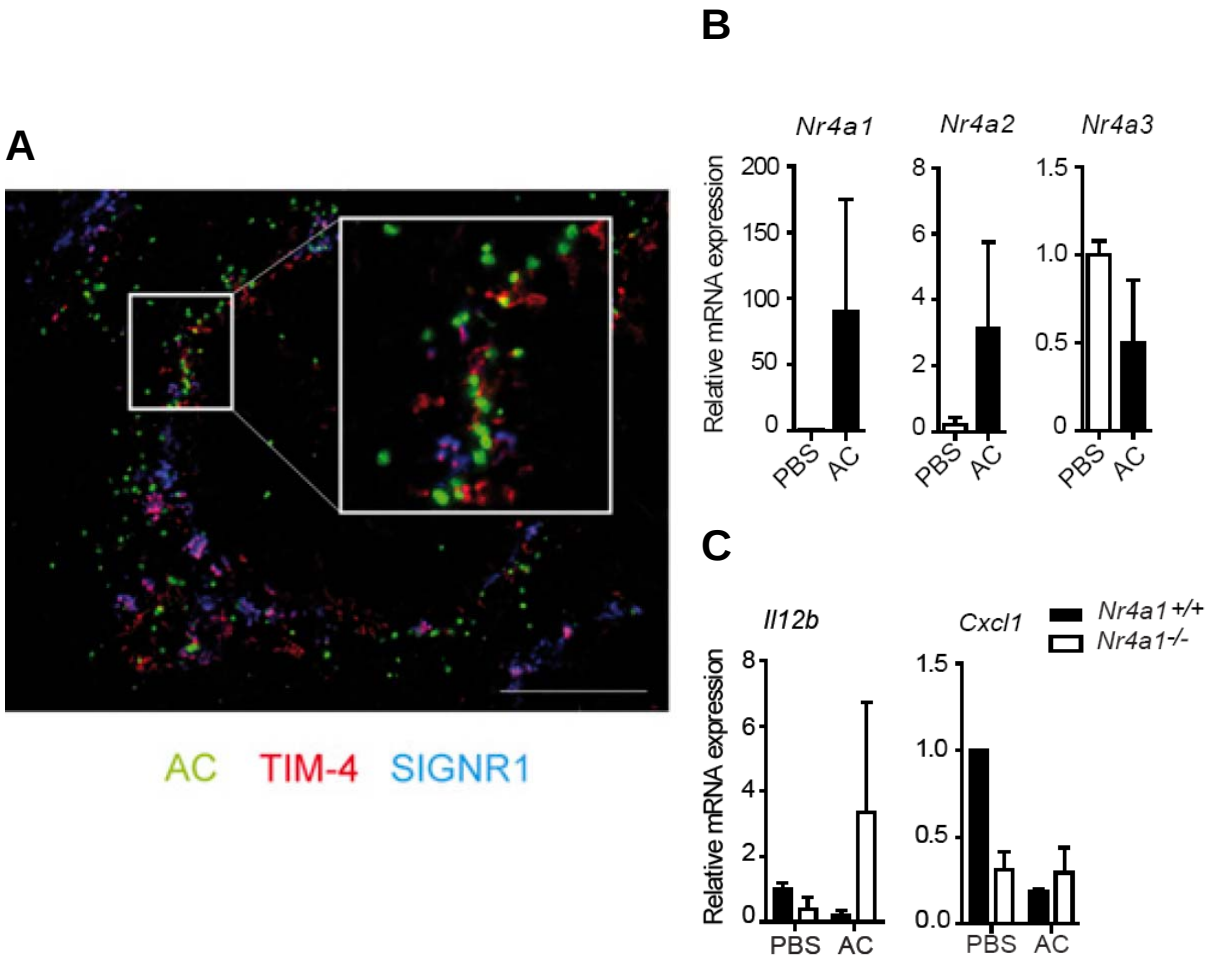

Figure 20: Nr4a1 mediates the anti-inflammatory effects of ACs *in vivo*. C57BL/6 WT mice were intravenously (i.v.) injected with 50.10^{6} CFSE-labeled ACs. After 18h, the spleens were collected and the localization of the ACs was analysed by immunofluorecence staining (**A**) and the relative mRNA expression by

RT-PCR (**B**). (**C**) WT and Nr4a1$^{-/-}$ mice were i.v. injected with 50.10^{6} ACs and relative mRNA expression in the spleen was analysed by RT-PCR.

A defective non-inflammatory clearance of ACs might result in a break of self-tolerance to AC-derived autoantigens and a subsequent production of autoantibodies that are directed against self-antigens. Such autoantibodies are observed during systemic lupus erythematosus (SLE) in humans, where an inefficient clearance of ACs was reported to contribute to the pathogenesis of this autoimmune disease. We therefore decided to address a potential role of Nr4a1 during the onset of autoimmuity and the break of tolerance, and performed the pristane model of experimental murine lupus in Nr4a1$^{+/+}$ and Nr4a1$^{-/-}$ mice. The mice were challenged with a single intraperitoneal injection of pristane oil, which induce massive cell death and chronic sterile inflammation in the peritoneal cavity. Together, these events impose a challenge to the endogenous "clearance machinery" and can trigger a break of tolerance to AC-derived antigens in mice that are prone to the development of lupus-like disease. Such mice develop both autoantibodies and glomerulonephritis [133].

Four months after pristane injection, the analysis of the sera of Nr4a1$^{+/+}$ and Nr4a1$^{-/-}$ mice revealed the production of anti-double strand DNA (dsDNA) and anti-histone autoantibodies in Nr4a1$^{-/-}$ mice, whereas their WT littermates developed significantly lower titers of autoantibodies (fig.21). These data thus confirmed a critical role for Nr4a1 in the maintenance of immunogenic tolerance to AC-derived self-antigens.

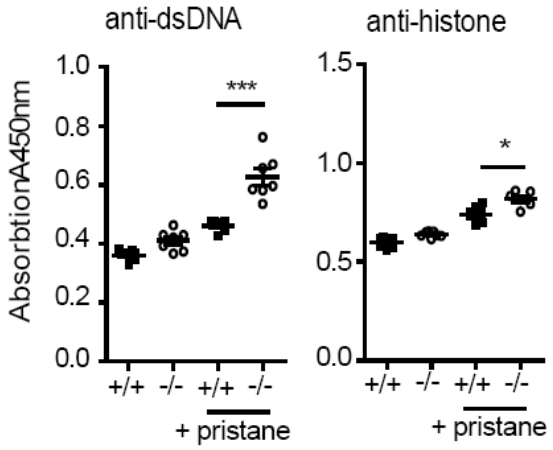

Figure 21: Nr4a1$^{-/-}$ mice develop stronger break of tolerance. Nr4a1$^{+/+}$ and Nr4a1$^{-/-}$ mice were i.p. injected with 500 µl of pristane oil. Four months later, the development of autoantibodies in the sera was evaluated by ELISA.

4. Discussion

The development of autoimmune diseases is still poorly understood. Alterations in the processing of apoptotic cells appear to be one of the major events in the initiation and progression of systemic autoimmune diseases, such as systemic lupus erythematosus (SLE) [76, 134]. However, the mechanisms leading to the recognition of self-molecules as antigens and to a break of tolerance remain unclear. An adult body deals every day with millions of dying cells and their silent removal is one of the key steps to avoid the development of self-antigen recognition by our immune system [75, 135, 136]. The silent clearance of dead cells is characterized by an early recognition of the dying cells by specialized phagocytes, a rapid phagocytosis process and absence of immune response to self-molecules that are derived from the dying cell. An overwhelming accumulation of apoptotic cells, either due to massive apoptosis or to a defect in the phagocytosis process, is though to foster the recognition of self-molecules as self-antigens. However, the presence of self-antigens might have to be accompanied by a pro-inflammatory environment, either provided by infection or by reduced anti-inflammatory effects of the AC, to promote a systemic break of tolerance [71].

The phagocytic capacities and responses of phagocytes to ACs are depending on multiple factors and have to be precisely regulated. These properties can be influenced by the phenotype, origin and tissue localization of MΦs. The differential response of resident and inflammatory MΦs to ACs has not been thoroughly investigated yet. MΦs show differences in their gene expression profiles and differ in surface-receptor expression, all factors that can influence their response to ACs. The phenotype of MΦs and their response to stimulation have been shown to be regulated by the expression of key transcription factors and nuclear receptors, such as LXRs, PPARs and Nr4as [137]. LXRs and PPARβ have been recently identified to play a major role in the clearance of ACs [116, 117]. The members of the nuclear receptor subfamily 4 (Nr4a1 and Nr4a2) play an essential role in monocytes development [11], in the response to inflammatory signals and regulation of inflammation by immune cells [104]. However, the role of the Nr4as in the clearance of ACs has not been established yet.

In this work, we confirmed the differential phagocytic capacities of resident and inflammatory MΦs and identified a so far unrecognized role of Nr4a1 in this process. Indeed, Nr4a1 appeared to be a major mediator of the anti-inflammatory effects of ACs, specifically in resident MΦs. Moreover, Nr4a1 played an important role in the silent removal of ACs *in vivo* and in the maintenance of self-tolerance.

4.1 The differential response of resident and inflammatory macrophages to pro-inflammatory stimuli and apoptotic cells

In the present study, we focused our work on two major MΦ subsets isolated from the peritoneal cavity of mice. Resident MΦs were isolated from naïve mice, whereas inflammatory MΦs were isolated after intraperitoneal injection of thioglycollate. Originally coming from different sources, the embryonic-derived resident MΦs and the monocyte-derived inflammatory MΦs reacted differentially to pro-inflammatory stimuli, such as TLR-ligands. After stimulation, inflammatory MΦs showed a strong pro-inflammatory response, whereas resident MΦs produced much lower amount of inflammatory cytokines. These observations could only partially be explained by the differential TLR expression profile of resident and inflammatory MΦs. Although inflammatory MΦs expressed more TLR1, the expression level of their other TLRs was either equal or lower than in resident MΦs. The differential response to pro-inflammatory stimuli of these two MΦ populations could be potentially explained by a differential molecular signature, promoting either the production of pro-inflammatory cytokines, e.g. via classical NF-κB pathway activation, or the expression of regulatory genes, involving e.g. STAT6 pathway activation [30].

Interestingly, resident and inflammatory MΦs showed great discrepancies in their capacity to remove ACs. Indeed, the phagocytosis of ACs during peritonitis was nearly exclusively performed by resident MΦs. This observation could be explained by variations in the expression of receptors implicated in the phagocytosis of ACs. While resident MΦs expressed receptors such as Tim4, Axl or CD14, inflammatory MΦs strongly expressed a different set of receptors, such as Mertk, Tim3 and Gas6. These results suggest that these receptors have cell-specific roles during the engulfment and uptake of ACs and that each MΦ subset might have a distinct phagocytosis capacity.

The differential phagocytic capacities of resident and inflammatory MΦs could also be related to their origin. Apoptosis is especially important during embryogenesis. During embryogenesis, resident MΦs are the first set of phagocytes that arise, which might explain the need of an especially high phagocytosis capacity. Recent work in our group showed that resident MΦs even have the capacity to inhibit phagocytosis from inflammatory MΦs, which ensures a non-immunogenic clearance of ACs [60].

4.2 Immunomodulatory effects of ACs

One major property of ACs is their capacity to modulate the inflammatory response of immune cells. However, ACs might differentially affect distinct immune cell populations. ACs exert strong anti-inflammatory effects on resident MΦs, whereas these effects were reduced in inflammatory MΦs and bone-marrow-derived dendritic cells (BMDC), at least *in vitro* and in our setting of experiments. However, these effects might vary if the individual phagocytes encounter ACs from other sources (e.g. apoptotic neutrophils versus lymphocyte).
Our results thus showed that the anti-inflammatory effects of ACs were mainly restricted to resident MΦs. It is known that BMDM can replace resident MΦs in case of depletion [18]. However, it is still not clear whether they could fulfil the proper function of resident MΦs and thus silently remove ACs. This hypothesis should be tested in the future.

4.3 Nr4a1 as key mediator of the anti-inflammatory effects of apoptotic cells

Interestingly, ACs rapidly induced the expression of a large panel of early response genes implicated in the regulation and mediation of many intracellular pathways such as Egr-1, JunD and Atf6. Nr4a family members were the strongest genes induced, especially in resident MΦs, meaning that ACs induce Nr4a expression through a mechanism specific to this type of phagocyte. Interestingly, this effect was less pronounced or absent when MΦs were in contact either with necrotic cells (NCs) or latex beads. These results confirm that Nr4a1 induction is dependant on signals received from ACs and is not due to the phagocytic process itself.
Similar effects were observed when resident MΦs were stimulated with phosphatidylserine-containing liposomes. Phosphatidylserine (PS) is a molecule present at the surface of ACs, already during the first step of apoptosis. As a crucial “eat me” signal, it is recognized by many phagocytic receptors located on the surface of MΦs. These data as well as the finding that Nr4a1 expression was blocked by AnnexinV, indicated that Nr4a1 induction is mediated by PS recognition. However, the induction of Nr4a1 by PS-containg liposomes was much lower than by ACs, indicating that Nr4a1 induction might also involve other mechanisms, such as soluble factors released by ACs (e.g. “find-me” signals). To test this hypothesis, experiments using transwell system have to be performed.

Interestingly, we showed that Nr4a1 induction is p38 and p44/p42 pathway dependant. These results indicated that the recognition of PS molecules by resident MΦs might activate these pathways which then promote Nr4a1 transcription.

We could demonstrate a key role of Nr4a1 during the silent removal of ACs by the use of $Nr4a1^{-/-}$ cells. Indeed, the anti-inflammatory effects of ACs observed in wild-type (WT) resident MΦs were strongly reduced in Nr4a1-deficient MΦs. These findings point out a major role of Nr4a1 as mediator of the anti-inflammatory effect of ACs. We also confirmed that these effects were partially PS-mediated, as the anti-inflammatory effects of PS-containing liposomes were also largely abrogated in $Nr4a1^{-/-}$ resident MΦs.
As mediator of the anti-inflammatory effects of ACs, Nr4a1 might strongly influence essential pathways implicated in the production of pro-inflammatory cytokines. It has already been shown that ACs have the capacity to block activation of the NF-κB pathway [130], so we sought to determine a possible role of Nr4a1 in this process. As expected, ACs and PS-containing liposomes blocked the LPS-induced NF-κB activation in WT resident MΦs. These effects were strongly abrogated in $Nr4a1^{-/-}$ MΦs, suggesting that Nr4a1 interferes with the phosphorylation of p65 and thereby blocks the induction of IL-12p40 expression.
To investigate the mechanism of action of Nr4a1 in more detail, chromatin immunoprecipitation experiments were performed. Here we focused on the promoter of IL-12p40. Interestingly, when WT resident MΦs were stimulated with PS-containing liposomes and LPS, the corepressor CoREST strongly bound to IL-12p40 promoter. However, when Nr4a1 was missing, the binding of CoREST to the IL-12p40 promoter was strongly reduced. These data indicate that Nr4a1 facilitates the binding of corepressors to the promoter of pro-inflammatory genes. Our results correlate with already published work on Nr4a2, which is able to recruit CoREST to the promoter of pro-inflammatory genes, such as iNOS [84]. However, the role of other corepressors, such as NCoR or SMRT during the Nr4a1-mediated effects should be investigated in the future.

4.4 Nr4a1 in the maintenance of self-tolerance

Because our data highlight a crucial role of Nr4a1 in the regulation of inflammation and as mediator of the anti-inflammatory effects of ACs *in vitro*, we evaluated its role *in vivo*.
Intravenous injection of ACs into mice resulted in the upregulation of the expression of Nr4a1 in the spleen, suggesting a role for Nr4a1 in the clearance of ACs *in vivo*. When mice were

deficient for Nr4a1, the massive injection of ACs led to the spontaneous induction of pro-inflammatory genes, such as IL-12p40 confirming that Nr4a1 also participated in the immunological-silent removal of ACs *in vivo*.

The mechanisms that underlay the splenic accumulation of ACs after i.v. injection are not fully understood yet. It has been shown that marginal zone MΦs (MZM), and eventually red pulp macrophages, are responsible for the removal of ACs in the spleen [132]. It has to be determined, which cell population in the spleen expresses Nr4a1 and which cells are responsible for the increase in IL-12p40 expression when Nr4a1 is missing. These experiments should be carefully performed in order to determine if Nr4a1 is important in other immune cells during this process. Mice lacking Nr4a1 expression in specific cell population, such as $Nr4a1^{fl/fl}$ $LysM^{cre}$ mice that allow deletion of this nuclear receptor in the monocytic compartment, $Nr4a1^{fl/fl}$ $CD4^{cre}$ mice for the deletion in T cells or $Nr4a1^{fl/fl}$ $CD19^{cre}$ mice for the deletion in B cell populations might be useful tools to answer this question.

Finally, we monitored the long term consequences of an absence of Nr4a1 during the clearance of dying cells. It is known that mice repetitively injected with ACs develop autoantibodies and IgG deposit in kidneys [138], signs of break of tolerance. The lack of Nr4a1 might boost these effects and lead to an early break of tolerance. We choose an alternative model to evaluate the role of Nr4a1 in the maintenance of self-tolerance *in vivo* and challenged WT and $Nr4a1^{-/-}$ mice with a single i.p. injection of pristane oil, which induces overwhelming cell death and inflammation within the peritoneal cavity, finally resulting in a lupus-like disease in mice that show a defective clearance capacity. The massive apoptosis and inflammation induced by the pristane oil within the peritoneal cavity lead to an exacerbated production of autoantibodies in Nr4a1-deficient mice. These results indicated a major role of Nr4a1 in the maintenance of self-tolerance to AC-derived autoantigens. In the presence of ACs, Nr4a1 is induced and mediates the anti-inflammatory effects of ACs. Subsequently, Nr4a1 contributes to the maintenance of tolerance.

As the lack of Nr4a1 disturbs the mechanisms maintaining self-tolerance, potential beneficial effects of its activation by specific Nr4a1 agonists, such as cytosporone B, should be examined in the future. Here, Nr4a1 activation might diminish or even prevent the development of autoimmune diseases.

All these results should be carefully analysed, keeping in mind that Nr4a1 is member of a family of three. Nr4as share high sequence homology and a functionally redundancy [89]. The lack of Nr4a1 might be partially compensated by Nr4a2 or Nr4a3 and thus ameliorate the

phenotype observed. Accordingly, the knock-down of two members of the Nr4a family was shown to be lethal [114], hindering the use of such approaches. However, cell specific deletion using flox/cre system might help to answer these questions.

4.5 Concluding remarks

Apoptosis is a major process, essential for tissue homeostasis and renewal. The rapid and silent clearance of apoptotic cells is critical to minimize the number of dying cells present in the body. Specialized phagocytes, such as resident macrophages, have the ability to recognize ACs and remove them without alerting the immune system. However, these events are not fully understood, yet. The identification of the involved processes will help to better understand mechanisms that contribute to self-tolerance and identify novel and better therapies for the treatment of auto-immune diseases. Our work identifies Nr4a1 as a key player in the regulation of the anti-inflammatory effects exerted by ACs on MΦs and important factor during the maintenance of self-tolerance. These new findings thus highlight nuclear receptors, and in particular Nr4a1, as a new possible targets in the treatment of autoimmune diseases.

Abbreviations

α-MEM	Minimum Essential Medium α
AC	Apoptotic cells
APC	Antigen Presenting Cell
ATP	Adenosine-5´- triphosphate
BM	Bone Marrow
BMDM	Bone Marrow Derived Macrophages
CFSE	Carboxyfluorescein succinimidyl ester
CDP	Common dendritic cell precursor
ChiP	Chromatin Immunoprecipitation
cm	Centimeter
DBD	DNA-binding domaine
cDC	common Dendritic cell
DC	Dendritic cell
DMPS	1,2-dimyristoyl-3-sn-phosphoserine
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FSC	Forward light scatter
Gas6	Growth Arrest-Specific 6
h	Hour
hi	High
HSC	Haematopoietic stem cells
HRE	Hormone response element
HRP	Horseradish peroxidase
iNOS	inducible Nitric oxide synthase
i.p.	Intraperitoneal
i.v.	Intravenously
LBD	Ligand-binding domain
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide

LTA	Lipoteichoic acid
M-CSF	Macrophage colony-stimulating factor
MDP	Macrophage and dendritic cell progenitor
MFG-E8	Milk-Fat Globule Epidermal Growth factor 8
min	Minute
ml	Millilitre
MOMP	Mitochondrial outer membrane permeabilization
MPS	Mononuclear phagocyte system
МΦ	Macrophage
NGFI-B	Nerve Growth factor IB
Nr4a	Nuclear receptor subfamily 4
NurRE	Nur77 response element
PAPC	1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PGN	Peptidoglycan
PFA	Paraformaldehyde
PtdSer/PS	Phosphatidylserine
Poly (I:C)	Polyinosine-polycytidylic acid
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RT-PCR	Real-time Polymerase chain reaction
RT	Room temperature
RXR	Retinoid X receptor
SLE	Systemic lupus erythematosus
SSC	Side light scatter
TLR	Toll-like receptor
UTP	Uridine-5'-triphosphate
YS	yolk sac
µM	micrometre

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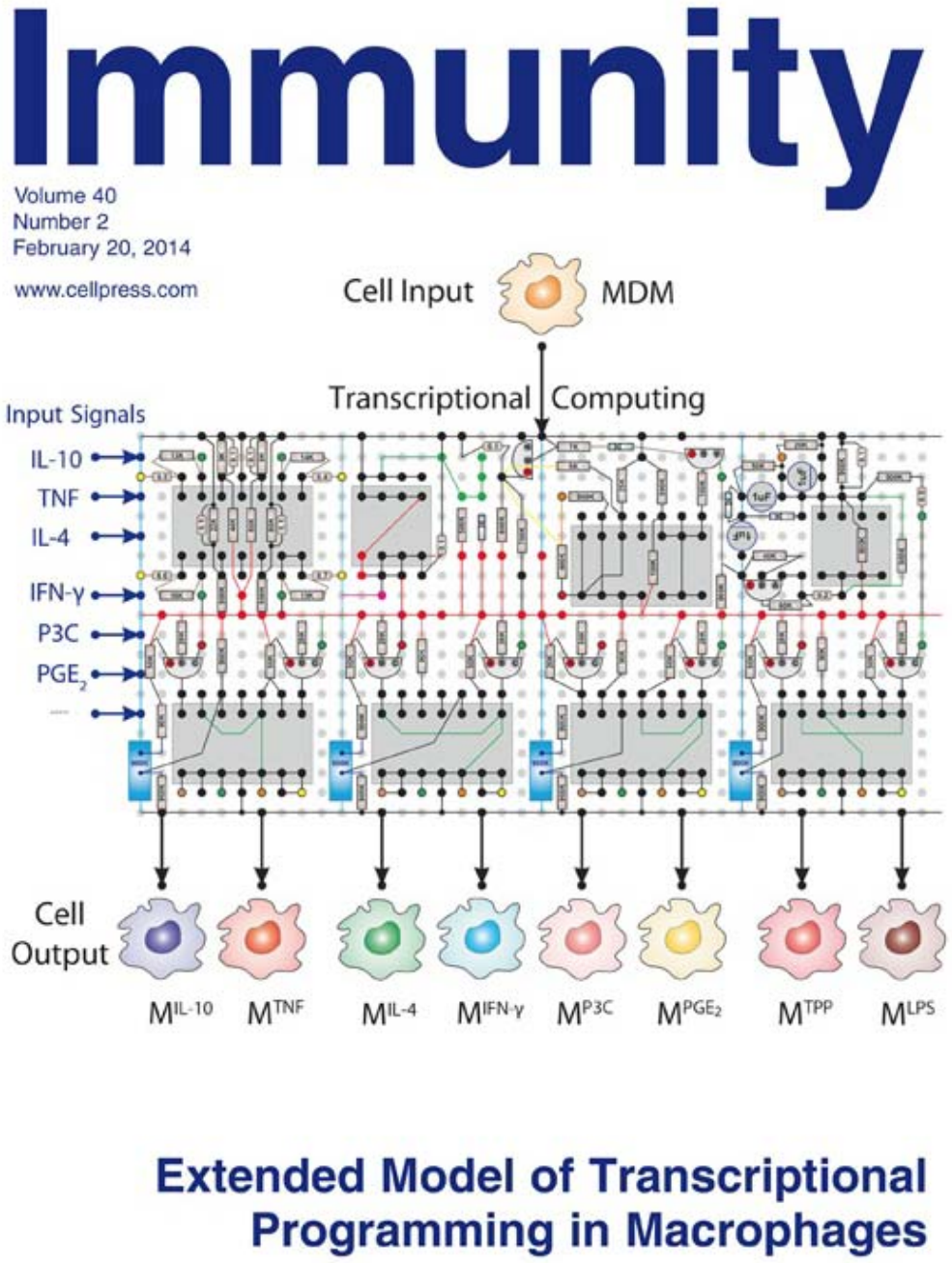
Annexe



Annexe 1: Cover page of the February 2014 edition of Immunity, made by the group of J. Schultze to illustrate the complexity of MΦs activation network.

Curriculum vitae

Personal data

Name: Natacha Ipseiz
Date of birth: 30. 01. 1987
Place of birth: Metz, France
Citizenship: French
Personal address: Gerhart Hauptmannstr.15 / 8L, 91058 Erlangen, Germany

Education

2005-2008: Bachelor of Science at the University Henri Poincaré of Nancy I, Nancy, France
2008-2009: Master 1 of Molecular Biology and Biochemistry at the University Henri Poincaré of Nancy I, Nancy, France
2009-2010: Master 2 of Cellular and Molecular Biology and Medical Application at the University of Montpellier II, Montpellier, France

Scientific expertise

July-August 2008: Student at the laboratory INRA of Dr. Pr. Jacquot "Interaction tree-microorganisms", at the University of Nancy I, Nancy, France. Studying of thioredoxins of poplar tree by using molecular techniques.

January-February 2009: Student at the laboratory of Dr. Frippiat "Immunogenetic", at the University of Nancy I, Nancy, France. Study of the production of IgM in the Pleurodele Waltl after a stay in space.

July-August 2009: Student at the laboratory INRA Supagro of Dr. Cabello "Mitochondrial receptor and thyroid hormone" of Montpellier, France, to learn new techniques: cell culture, *in vivo* experiments and microscopy.

October 2009-June 2010: Master thesis at the laboratory INSERM U844 of Pr. Jorgensen "Mesenchymal stem cells and cartilage reparation" of Montpellier, France. Project: the role of the transcriptional factor GILZ on the properties of mesenchymal stem cells.

Since 10/2010: PhD thesis in immunology at the Department of Internal Medicine 3, Institute for Clinical Immunology, University of Erlangen-Nuremberg, Erlangen, Germany. Supervisors: Prof. Georg Schett and Dr. Gerhard Krönke. Main subject: Nr4a1 in the clearance of apoptotic cells.

Computer skills

MS-Office, Adobe Illustrator, Graph Pad Prism, Flow Jo, Kaluza

Language skills

French	mother language
English	fluent
German	fluent

Others

General first aid 2013, ASB, Erlangen, Germany

Posters

09/2012: NR4A1 mediates anti-inflammatory effects of apoptotic cells.

European Congress of Immunology, Glasgow, Scottland.

04/2013: NR4A1 mediates anti-inflammatory effects of apoptotic cells.

Keystone Symposia meeting on "Nuclear Receptors and Friends: Roles in Energy Homeostasis and Metabolic Dysfunction", Alpbach, Austria.

06/2013: NR4A1 mediates anti-inflammatory effects of apoptotic cells.

Gordon Research Conference on "Apoptotic Cell Recognition & Clearance", Biddeford, USA.

10/2013: NR4A1 mediates anti-inflammatory effects of apoptotic cells.

27th annual conference of the European Macrophage and Dendritic Cell Society, Erlangen, Germany.

Publications

The nuclear receptor Nr4a1 mediates anti-inflammatory effects of apoptotic cells.
Ipseiz N, Uderhardt S, Scholtysek C, Steffen M, Schabbauer G, Bozec A, Schett G, Krönke G. **J Immunol**. 2014 May.

PPARβ/δ governs Wnt signaling and bone turnover.
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