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The Nuclear Receptor Nr4a1 Mediates Anti-Inflammatory Effects of Apoptotic Cells

Natacha Ipseiz,*,† Stefan Uderhardt,*† Carina Scholtyssek,*† Martin Steffen,*† Gernot Schabbauer,‡ Aline Bozec,*† Georg Schett,* and Gerhard Krönke*§†

Uptake of apoptotic cells (ACs) by macrophages ensures the nonimmunogenic clearance of dying cells, as well as the maintenance of self-tolerance to AC-derived autoantigens. Upon ingestion, ACs exert an inhibitory influence on the inflammatory signaling within the phagocyte. However, the molecular signals that mediate these immune-modulatory properties of ACs are incompletely understood. In this article, we show that the phagocytosis of apoptotic thymocytes was enhanced in tissue-resident macrophages where this process resulted in the induction of NF-κB signaling and repression of inflammatory cytokines, such as IL-12. In parallel, ACs induced a robust expression of a panel of immediate early genes, which included the Nr4a subfamily of nuclear receptors. Notably, deletion of Nr4a1 interfered with the anti-inflammatory effects of ACs in macrophages and restored both NF-κB signaling and IL-12 expression. Accordingly, Nr4a1 mediated the anti-inflammatory properties of ACs in vivo and was required for maintenance of self-tolerance in the murine model of pristane-induced lupus. Thus, our data point toward a key role for Nr4a1 as regulator of the immune response to ACs and of the maintenance of tolerance to “dying self.” The Journal of Immunology, 2014, 192: 000–000.

Macrophages (Mφs) critically contribute to the immunologically silent disposal of apoptotic cells (ACs). A failure in this process eventually results in an aberrant immune response to AC-derived self-Ags, such as dsDNA or histones, as well as in severe autoimmune diseases like systemic lupus erythematosus (1, 2). Notably, ACs are not only silently removed, but their binding and uptake also actively repress the expression of proinflammatory cytokines within the Mφs. However, the molecular mechanisms underlying such anti-inflammatory properties of ACs are still incompletely understood (3).

Nuclear receptors (NRs) make up a superfamily of ligand-activated transcription factors that are able to recruit both transcriptional coactivators and corepressors and, thereby, positively and negatively affect transcription in a promoter-specific manner. Although NRs initially were identified as key regulators of fat and glucose metabolism (4), recent data highlighted an additional role for NRs during the regulation of the innate and adaptive immune response (5).

The Nr4a subfamily of NRs consists of three members (Nr4a1–3), which are rapidly induced in response to stimuli, such as LPS and oxidized lipoproteins. Nr4a family members were shown to exert transcriptional activity in a ligand-independent manner (6), and they have been implicated in the regulation of glucose metabolism, apoptosis, and inflammation, as well as the differentiation of monocytes and T cells (7–14).

Our present data show that the binding and uptake of ACs by tissue-resident Mφs resulted in the rapid induction of Nr4a1, which, in turn, interfered with NF-κB signaling and the expression of proinflammatory genes, such as IL-12. Furthermore, we provide evidence that this pathway contributes both to the noninflammatory disposal of ACs in vivo and to the maintenance of immunological tolerance to self-Ags.

Materials and Methods

Mice

Animal experiments were approved by the government of Mittelfranken. Mice were housed in the animal facility of the University of Erlangen-Nuremberg. All mice used were on the C57BL/6 background. Nr4a1−/− mice were on the C57BL/6 background and were obtained from The Jackson Laboratory.

Peritoneal Mφs

Resident Mφs were isolated from the peritoneum of naive 8–10-wk-old mice. Inflammatory Mφs were isolated from peritoneitis exudates, after i.p. injection of 3% Brewer’s thioglycollate (Sigma-Aldrich) into wild-type (WT) and Nr4a1−/− mice at the age of 8–10 wk. Both elicited and resident peritoneal Mφs were obtained by peritoneal lavage with ice-cold 4% FCS/PBS and cultured in RPMI 1640 (Invitrogen) supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin/streptomycin. To purify the elicited Mφs, the cells collected after peritoneal lavage were incubated with Tim4 (PE) Ab and purified with anti-PE MicroBeads (Miltenyi Biotec).

Generation of ACs

To generate ACs, thymocytes isolated from 4–6-wk-old C57BL/6 mice were incubated in RPMI 1640, supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin/streptomycin, in the presence of 1 μM dexmethasone (Sigma-Aldrich). After 6 h, the thymocytes were washed several times with PBS, spun through a FCS cushion to eliminate the dexmethasone, and resuspended in RPMI 1640 containing 10% FCS, 1% L-glutamine, and 1% penicillin/streptomycin. The frequency of ACs was checked by annexin V/PI staining. Mφs were incubated with ACs at a 1:5 ratio at 37°C for the indicated times.
Phospholipid preparation
1-palmitoyl-2-achidonoyl-sn-glycerol-3-phosphocholine and 1,2-dimyristoyl-
3-sn-phosphoserine were purchased from Avanti Polar Lipids. Phospholipids
were dissolved in chloroform and stored at ~80°C until their use. For each
experiment, the indicated lipids were evaporated under a stream of argon
and resuspended in culture medium by vigorous vortexing for ~30 s. For
the generation of phosphatidylserine-containing liposomes, 1-palmitoyl-
2-achidonoyl-sn-glycerol-3-phosphocholine and 1,2-dimyristoyl-3-sn-phos-
phoserine were mixed at a 4:1 ratio.

In vitro phagocytosis assay
A mixture of WT resident and thioglycollate-elicited peritoneal Mψs was
isolated 5 d after i.p. injection of 1 ml 3% Brewer’s thioglycollate, plated
on coverslips in 24-well plates, and incubated with CFSE-labeled apoptotic
thymocytes at a 1:5 ratio, in 10% FCS in RPMI 1640 for 30 min at 37°C.
Unbound cells were washed away with ice-cold PBS, and the Mψs were
stained as indicated and mounted on glass slides. Microscopy was per-
formed with an Eclipse-80i microscope and a monochromatic camera (DS-
Qi1MC; both from Nikon). Photomicrographs are shown with indicated
pseudocolors produced by NIS elements software BR3.0 (Nikon).

In vivo phagocytosis assay
Five days after i.p. injection of 1 ml 3% Brewer’s thioglycollate, 15 x 10^6
CFSE-labeled apoptotic thymocytes were injected i.p. into WT mice. After
30 min, peritoneal lavage was performed, and the cells were stained for Tim4
(PE), CD11b (allophycocyanin), and F4/80 (Pacific Blue) and analyzed with
a Gallios cytofluorometer (Beckman Coulter) and FlowJo software.

In vivo AC uptake
A total of 5 x 10^7 CFSE-labeled apoptotic thymocytes was injected i.v. into
10–12-wk-old WT and NR4A1 mice. The mice were sacrificed after 18 h. The
spleens were collected, and one part was embedded in OCT Tissue-Tek compound, snap-frozen, and stored at
80°C until their use. For each experiment, the indicated lipids were evaporated under a stream of argon
and resuspended in culture medium by vigorous vortexing for ~30 s. The
results were normalized to the controls (= 1).

Reagents
Murine IL-12p40, TNF-α, IL-6, and CXCL1 were measured by specific
ELISA kits (R&D Systems). LPS was purchased from Sigma-Aldrich
(ESER-2630; Escherichia coli O111:B4). All inhibitors were used at 10 μM.
The PI3K inhibitor (wortmannin) was purchased from Sigma-Aldrich, the
PKC inhibitor (Bisindolylmaleimide I, Hydrochloride) was purchased from
Cell Signaling, the PKA inhibitor (KT 5720) was purchased from Santa
Cruz, and the NF-κB activation inhibitor, the p38 inhibitor (SB203580), and
the p44/p42 inhibitor (PD98059) were purchased from Merck Millipore.

FIGURE 1. Apoptotic cells are preferentially engulfed by resident Mψs. (A) Five days after
injection of thioglycollate, 15 x 10^6 CFSE-
labeled apoptotic thymocytes were injected into
the inflamed peritoneum, and flow cytometric
analysis was performed to determine the uptake
of apoptotic cells by Tim4+ resident Mψs and
Tim4+ inflammatory Mψs. After exclusion of
the doublets, the cells were gated on CD11b^+ cells, and the intensity of CFSE was determined
in both populations of Mψs. (B) Immunofluorescence microscopy analysis and immunofluorescence-based quantification of the phagocytosis
of ACs (green) by Mψs isolated 5 d after initiation
of thioglycollate-induced peritonitis. Mψs were
stained for TIM4 (red) and CD68 (blue) to
differentiate between TIM4+ resident peritoneal
Mψs and TIM4+ inflammatory monocyte-derived
Mψs. Scale bar, 10 μm. (C) Immunofluorescence microscopy analysis of the phagocytosis of ACs (green) by a pure population of resident perito-
neal Mψs. (D) After induction of thioglycollate-
induced peritonitis, Tim4+ Mψs and Tim4- Mψs were separated by MicroBeads, as described in
Materials and Methods, and relative mRNA ex-
pression of the indicated receptor was determined
by real-time PCR in unstimulated resident Mψs
and Tim4- inflammatory peritoneal Mψs. The
data shown are representative of at least three
independent experiments. Data are mean ± SEM.
*p < 0.05, **p < 0.01, ***p < 0.001.
Latex beads (size 1 μm, 0.01%) and CFSE were purchased from Sigma-Aldrich, and cycloheximide (CHX) was purchased from Merck Millipore. The polyclonal anti-F4/80 (labeled with either PE or Pacific Blue), anti-TIM4 (PE), anti-CD68 (allophycocyanin), and anti-CD115 (allophycocyanin) Abs were purchased from BioLegend, and anti-SIGNR1 (allophycocyanin) was from eBioscience.

Statistical analysis

Data are mean ± SEM. Group mean values were compared using the two-tailed Student t test. The data shown are representative of at least three experiments producing similar results.

Results

Tissue-resident MΦs perform the noninflammatory clearance of ACs

Recent studies revealed a surprising dichotomy among the various MΦ subsets encountered in vivo. In addition to classical monocyte-derived MΦs, which are recruited during inflammation, a large group of tissue-resident MΦs, such as Kupffer cells, splenic MΦs, alveolar MΦs, and resident peritoneal MΦs settle the tissues during embryogenesis. Notably, such resident MΦs sustain their numbers via local proliferation throughout adulthood independent from monocyte-derived precursors (15–17). However, little is known about the differential function and specific properties of inflammatory (monocyte-derived) and resident MΦs.

Our previous studies indicate that clearance of ACs by tissue-resident MΦs maintains self-tolerance, whereas uptake of ACs by inflammatory monocytes and monocyte-derived MΦs eventually results in an immune response to AC-derived Ags (18). To better understand the underlying molecular mechanisms, we studied the phagocytosis of ACs by inflammatory and resident MΦs of the peritoneal cavity. By using Tim4 as a specific marker for resident peritoneal MΦs (19, 20), we were able to distinguish the two populations of MΦs during flow cytometry and via immunofluorescence (Fig. 1A, 1B). In accordance with our previous data, we observed that the phagocytosis of CFSE-labeled ACs was enhanced in the population of resident MΦs, whereas inflammatory MΦs bound and ingested ACs to a lesser extent (Fig. 1A, 1B). Even within an inflammatory infiltrate, where monocyte-derived inflammatory MΦs were the prevalent cell type, the remaining resident MΦs

FIGURE 2. Clearance of ACs induces expression of the Nr4a1 family of NRs. (A) ELISA-based analysis of the cytokine profile of resident MΦs after preincubation (1 h) with ACs (ratio 1:5) and stimulation (overnight) with LPS (100 ng/ml). (B) Resident peritoneal MΦs were incubated for 30 min with 10 μg/ml of CHX and then incubated with ACs (ratio 1:5) for 1 h before being stimulated with 100 ng/ml of LPS for 4 h. The relative mRNA expression of IL-12p40 was analyzed by real-time PCR. Resident peritoneal MΦs were incubated with ACs (ratio 1:5) for the indicated times, and relative mRNA expression of the indicated early response genes (C) and Nr4a family members (D) was determined by real-time PCR. Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.01.

FIGURE 3. ACs induce expression of Nr4a1 via the p38MAPK and ERK 1/2 (p44/p42) pathway in a PS-dependent manner. (A) Resident peritoneal MΦs were incubated (1 h) with either ACs (1:5) or latex beads (2%) before Nr4a1 mRNA expression was determined. (B) Analysis of Nr4a1 mRNA expression after stimulation (1 h) of resident MΦs with ACs or ACs coupled with annexin V (AC+AxV). (C) Resident MΦs were treated with PS-containing phospholipid liposomes (PS-lipo.) for the indicated times, and relative mRNA expression of Nr4a1 was determined by real-time PCR. (D) Resident peritoneal MΦs were preincubated with the indicated inhibitors (inh.) for 30 min before addition of ACs. The expression of Nr4a1 was analyzed after an additional hour by real-time PCR. Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.01.
exerted AC removal that outpaced the clearance performed by inflammatory MΦs. Accordingly, uptake of ACs by a pure population of resident MΦs was highly efficient and homogenous (Fig. 1D). mRNA expression analysis of purified populations of resident and inflammatory MΦs showed that the enhanced capacity of resident MΦs to ingest ACs was paralleled by a differential expression of certain receptors for ACs (Fig. 1D, Supplemental Fig. 1).

These data supported a concept whereby ACs are preferentially cleared by resident, but not monocyte-derived, MΦs. However, previous studies addressing the clearance of ACs focused on the phagocytosis by monocyte-derived MΦs or MΦ cell lines. The molecular details of the phagocytosis of ACs by tissue-resident MΦs have received little attention.

**Phagocytosis of ACs by tissue-resident MΦs results in the induction of a unique pattern of immediate early genes and the differential blockade of cytokine expression**

We subsequently sought to gain mechanistic insight into the effects that ACs exert on this specialized subset of MΦs. Resident MΦs responded with a differential inhibition of the LPS-induced cytokine release after phagocytosis of ACs, and we observed an almost complete inhibition of the expression of distinct proinflammatory cytokines, such as IL-12p40, whereas expression of other cytokines, such as CXCL1, was not affected by the uptake of ACs (Fig. 2A). Because these AC-induced changes in the cytokine response of MΦs were observed with a delay of several hours, we hypothesized that the regulation of cytokine expression in this type of MΦ relied on earlier transcriptional events that are directly initiated upon the ingestion of ACs. In accordance, CHX-induced inhibition of ribosomal protein translation resulted in abrogation of the AC-induced block of IL-12 expression (Fig. 2B), suggesting that ACs induced the rapid synthesis of proteins that negatively regulated inflammatory cytokine production by MΦs. To screen for potential candidates, we subsequently performed an analysis of the MΦs’ immediate early gene expression, which showed vigorous transcriptional changes in response to the ingestion of ACs (Fig. 2C). These experiments resulted in the identification of the Nr4a family of NRs, and in particular, Nr4a1, as one of the most robustly induced set of genes (Fig. 2D, Supplemental Fig. 2). Because Nr4a1 was recently implicated as a key factor regulating the inflammatory response (8), we subsequently addressed its role during the effects that ACs exerted on resident MΦs.

**ACs induce Nr4a1 in a phosphatidylserine-dependent manner via activation of p38MAPK and MAP/ERK**

Notably, we did not observe expression of Nr4a1 in response to the phagocytosis of other particles, such as latex beads (Fig. 3A), indicating that signals initiated by the specific binding of ACs, and...
not the process of phagocytosis per se, triggered the expression of this NR in resident MΦs. Exposure of the phospholipid phosphatidylycerine (PS) on the surface of cells undergoing apoptosis was shown to serve as a major “eat me” signal for phagocytes (21), and our previously obtained data showed that resident MΦs were well equipped with PS-specific receptors for ACs, such as Tim4. To address a potential involvement of PS exposure during the effects exerted by ACs, we subsequently performed phagocytosis assays in the presence of annexin V, a protein that masks the PS moieties on the surface of ACs. Addition of annexin V blocked the AC-induced expression of Nr4a1 in resident MΦs (Fig. 3B), whereas the addition of PS-containing liposomes induced the expression of Nr4a1, also in the absence of ACs (Fig. 3C). Together, these data suggested a key role for PS recognition by resident MΦs during the AC-mediated induction of Nr4a1 expression in this MΦ subpopulation. To further address the underlying signaling pathways, we applied different inhibitors of the p38MAPK, MAP/ERK 1/2 (p44/p42), protein kinase C, protein kinase A, and NF-κB pathway in our assays. We observed a significant inhibition of AC-induced Nr4a1 expression after blockade of both the p38MAPK and the MAP/ERK kinase 1/2 pathways, whereas the other inhibitors did not interfere with the effects exerted by ACs, or they even slightly increased Nr4a1 expression (Fig. 3D).

Nr4a1 supports the maintenance of an anti-inflammatory milieu during the clearance of ACs

Nr4a family members act as crucial regulators of the inflammatory response (8, 10, 22). Consequently, we sought to determine a potential role for Nr4a1 during the maintenance of an anti-inflammatory cytokine milieu in response to the clearance of ACs. Nr4a1 supports the maintenance of an anti-inflammatory milieu during the clearance of ACs by resident MΦs. In contrast to the expression of IL-12, expression of Nr4a1 was synergistically induced by ACs and LPS (Fig. 4A). Notably, ACs exhibited reduced anti-inflammatory effects in the absence of this NR (Fig. 4B), which resulted in the restoration of LPS-induced IL-12p40 production in Nr4a1−/− MΦs after phagocytosis of ACs. These Nr4a1-mediated effects were specific for cytokines such as IL-12p40, because neither phagocytosis of ACs nor the presence of Nr4a1 affected the expression of other factors, such as CXCL1. Our previous data indicated a key role for AC-derived PS during the regulation of Nr4a1. Accordingly, we observed that PS-containing liposomes blocked production of IL-12p40 but not of CXCL1 (Fig. 4C). Again these anti-inflammatory effects involved Nr4a1, because we observed reduced inhibitory effects of PS-containing liposomes in Nr4a1−/− resident MΦs (Fig. 4C).

The NF-κB pathway acts as a master regulator of the inflammatory response in MΦs (23), and uptake of ACs was reported to interfere with NF-κB activity in various cell types (24). Notably, analysis of the NF-κB-signaling cascade revealed a block of the LPS-induced phosphorylation of NF-κB subunit p65 at serine 536 in resident MΦs ingesting either ACs or PS-containing liposomes (Fig. 4D, 4E). Notably, these effects were abrogated in Nr4a1−/− resident MΦs, indicating an AC-induced and Nr4a1-mediated block of the phosphorylation of p65 (Fig. 4D, 4E).

NR4a1 mediates the anti-inflammatory effects of ACs in vivo and contributes to the maintenance of self-tolerance

To evaluate the contribution of Nr4a1 to the anti-inflammatory effects of ACs in vivo, we subsequently injected ACs i.v. into WT and Nr4a1−/− mice. ACs were predominantly scavenged by Tim4+SIGN1+ marginal zone MΦs within the spleen (Fig. 5A), which resemble another type of resident MΦs (17). Again we observed a rapid induction of Nr4a1 and (to a lesser extent) Nr4a2 expression (Fig. 5B). In accordance with our in vitro data, injection of ACs resulted in attenuated splenic mRNA expression of the gene encoding for IL-12 subunit p40 (II12b) in WT animals, whereas expression of II12b increased in the spleens of Nr4a1−/− mice in response to ACs (Fig. 5C). In contrast, Nr4a1 expression did not significantly impact on the expression of the gene encoding for CXCL1 (Fig. 3C).

To address a potential contribution of Nr4a1 to the maintenance of tolerance to AC-derived Ags in vivo, we used the pristane model of experimental murine lupus, in which i.p. injection of pristane oil results in the induction of a chronic and sterile peritonitis that is accompanied by overwhelming cell death within the peritoneal cavity. Together, these events trigger a break of tolerance to AC-derived nuclear Ags and result in the production of autoantibodies against dsDNA and histones (25). Injection of pristane oil resulted in the occurrence of autoantibodies against nuclear Ags in Nr4a1−/− mice, whereas their WT littermates developed significantly lower titers of autoantibodies (Fig. 5D), confirming a critical role for Nr4a1 in the maintenance of immunologic tolerance to AC-derived self-Ags.

Discussion

AC death constantly occurs during regular tissue turnover and, in particular, during the course of an inflammatory response. Because ACs represent a major source of autoantigens, their rapid and nonimmunogenic removal by MΦs is essential for the maintenance of self-tolerance. Notably, ACs themselves exert direct anti-inflammatory effects, which additionally increase the threshold for an immune response against AC-derived Ags.

Our data indicate that mainly tissue-resident, and not monocyte-derived, MΦs perform the clearance of ACs. These findings are in accordance with previous studies that highlighted the importance of specialized and tolerogenic MΦ subsets, such as marginal zone

FIGURE 5. Nr4a1 promotes the noninflammatory clearance of ACs and contributes to the maintenance of self-tolerance. After i.v. injection of 50 × 10⁶ CFSE-labeled ACs into WT mice, immunofluorescence and mRNA expression analysis were performed to determine the localization of ACs (A), as well as the expression of Nr4a family members (B). (C) After i.v. injection of 50 × 10⁶ CFSE-labeled ACs into Nr4a1−/− and Nr4a1−/− mice, spleens were harvested, and an mRNA expression analysis of the indicated genes was performed by real-time PCR. Data shown are representative of two independent experiments (n = 3 or 4). (D) Serum analysis of the indicated autoantibodies in Nr4a1−/− and Nr4a1−/− mice 16 wk after i.p. injection of 0.5 ml of pristane oil. Data are mean ± SEM. Scale bars, 100 μm. *p < 0.05, ***p < 0.01.
MΦs, during the noninflammatory clearance of dying cells (26–27). Upon binding of ACs, resident MΦs rapidly induced the expression of multiple immediate early genes, including the Nr4a family of NRs. Recognition of PS on the surface of ACs was a key trigger for these AC-induced transcriptional changes, and PS-induced Nr4a1 critically contributed to the immune-modulatory properties of ACs. Because PS exposure is a universal feature of AC death, it is likely that similar events occur during the uptake of apoptotic thymocytes, as well as during the clearance of other cell types, such as apoptotic neutrophils. Thus, our data support a scenario in which PS exposure on ACs serves as a crucial “eat me” signal and likewise triggers anti-inflammatory signaling cascades within the MΦ that alter its phenotype (28, 29). Indeed, exposure of PS is essential for the anti-inflammatory effects of ACs (30, 31); and during the last several years several PS-binding receptors, such as Tim4 and MerTK, were identified and shown to mediate the non-immunogenic clearance of ACs (19, 20, 24, 32–37).

On a mechanistic level, Nr4a1 interfered with the LPS-induced phosphorylation of NF-κB subunit p65, as well as with the expression of IL-12. Nr4a1 family members were shown to attenuate immunogenic clearance of ACs (19, 20, 24, 32–37). Tim4 and MerTK, were identified and shown to mediate the non-inflammatory clearance of ACs (30, 31); and within the MΦ scenario in which PS exposure on ACs serves as a crucial “eat me” key trigger for these AC-induced transcriptional changes, and PS-erythematosus or rheumatoid arthritis.


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