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

Natacha Ipseiz,*,‡ Stefan Uderhardt,*,‡ Carina Scholtysek,*,‡ Martin Steffen,*,‡ Gernot Schabbauer,*,‡ Aline Bozec,*,‡ Georg Schett,*, and Gerhard Kro¨nke*,‡

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 inhibition 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 (MoFs) 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 MoFs. 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 MoFs 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 MoFs

Resident MoFs were isolated from the peritoneum of naive 8–10-wk-old mice. Immunofluorescence experiments were done using MoBs isolated from the peritoneal lavage of mice. The cells were collected by plastic adherence, 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. To purify the elicited MoFs, the cells collected after peritoneal lavage were incubated with Tim4 (PE) Ab and purified with anti-PE MicroBeads (Miltenyi Biotec).

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Abbreviations used in this article: AC, apoptotic cell; CHX, cycloheximide; MoF, macrophage; NR, nuclear receptor; PS, phosphatidylserine; WT, wild-type.

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Phospholipid preparation

1-palmitoyl-2-arachidonoyl-sn-glycero-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 at room temperature, and resuspended in culture medium by vigorous vortexing for 30 s. For the generation of phosphatidylserine-containing liposomes, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine and 1,2-dimyristoyl-3-sn-phosphoserine were mixed at a 4:1 ratio.

In vitro phagocytosis assay

A mixture of WT resident and thioglycollate-elicted peritoneal Mφs 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 Mφs were stained as indicated and mounted on glass slides. Microscopy was performed 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 × 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), CD115 (allophycocyanin), and F4/80 (Pacific Blue) and analyzed with a Gallios cytometer (Beckman Coulter) and FlowJo software.

In vivo AC uptake

A total of 5 × 10^6 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; the other part was used for RNA analysis.

Pristane model

Autoimmunity was induced in NR4a1−/− mice and C57BL/6 mice with a single i.p. injection of 0.5 ml pristane oil (Sigma-Aldrich) at 10 wk of age. Mice were sacrificed 4 mo later. Spontaneous autoimmune phenomena were assessed in mice without any stimulus or challenge at 26 wk of age. For detection of autoantibodies, microtiter plates were coated overnight with 50 μg/ml histone of calf thymus (Roche Diagnostics) dissolved in ethanol. For detection of anti-dsDNA Abs, plates were coated with 20 μg/ml poly-c-lysine before coating with 20 μg/ml calf thymus DNA (both from Sigma-Aldrich). Sera were added at a 1:100 dilution in 1% BSA/PBS for 1 h at room temperature. Bound IgG was detected with an HRP-conjugated goat anti-mouse IgG (Dianova).

Real-time PCR

In brief, total RNA was isolated from cells using peqGOLD TriFast (PEQLAB). A total of 700 ng total RNA was reverse transcribed with human leukemia virus reverse transcriptase using the Gene Amp RNA PCR kit (Applied Biosystems) and oligo(dT) primers. Total RNA from the spleen was isolated by the same procedure, but DNA digestion took place prior to transcribing the RNA (DNase I, #EN0521; Fermentas). mRNA levels were quantified using a LightCycler instrument and SYBR Green I kit (Roche Diagnostics). The gene expression values were normalized to β-actin expression. The sequence of the primers can be found in Supplemental Table I.

Western blotting

After lysis of the cells, the protein extracts were separated on 10% SDS–polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane, and immunoblotted with the following Abs: phospho–NF-κB-p65 (#3033; Cell Signaling) and β-actin (clone AC-74; Sigma-Aldrich). Quantitative analyses were performed with Photoshop CS5 software, and 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 (#L-2630; Escherichia coli 0111:B4). All inhibitors were used at 10 μM. The PK3 inhibitor (wortmannin) was purchased from Sigma-Aldrich, the P38 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.
Latex beads (size 1 μm, 0.01%) and CFSE were purchased from Sigma-Aldrich, and cycloheximide (CHX) was purchased from Merk 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 self-Ags (18). To better understand the underlying molecular mechanisms, we studied the phagocytosis of ACs by inflammatory and resident MΦs in 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.](http://www.jimmunol.org/)

FIGURE 2. Clearance of ACs induces expression of the Nr4a 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 < 0.001.
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. 1C). 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 MΦ’s 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

![FIGURE 4](http://www.jimmunol.org/)

Nr4a1 contributes to the anti-inflammatory effects of ACs. (A) Real-time PCR of Nr4a1 mRNA expression in resident MΦs after preincubation (2 h) with ACs and treatment (1 h) with 100 ng/ml of LPS. (B) ELISA-based analysis of the IL-12p40 and CXCL1 expression of resident Nr4a1+/+ and Nr4a1−/− peritoneal MΦs after preincubation (1 h) with ACs (ratio 1.5) and overnight treatment with 100 ng/ml of LPS. (C) ELISA-based analysis of the IL-12p40 and CXCL1 expression of resident Nr4a1+/+ and Nr4a1−/− peritoneal MΦs after preincubation (1 h) with PS-liposomes (PS-lipo.) and overnight treatment with 100 ng/ml of LPS. Western blot analysis of phosphorylation of the NF-κB subunit p65 (Ser536) in Nr4a1+/+ and Nr4a1−/− peritoneal MΦs after preincubation (2 h) with ACs (D) or PS-containing liposomes (E) and stimulation with 100 ng/ml LPS for the indicated times (D) or for 5 min (E). Data are mean ± SEM. *p < 0.05.
not the process of phagocytosis per se, triggered the expression of this NR in resident MΦs. Exposure of the phospholipid phosphatidylserine (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-kB 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 milieu during the clearance of ACs that resemble another type of resident MΦs, indicating an AC-induced and Nr4a1-mediated block of the phosphorylation of p65 (Fig. 4D, 4E).

The NF-kB pathway acts as a master regulator of the inflammatory response in MΦs (23), and uptake of ACs was reported to interfere with NF-kB activity in various cell types (24). Notably, analysis of the NF-kB–signaling cascade revealed a block of the LPS-induced phosphorylation of NF-kB 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+SIGNR1+ 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 MΦs.
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 Φ 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 PS is essential for the anti-inflammatory effects of ACs (30, 31); and during the last several years several PS-binding receptors, such as

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