Macrophages Engulfing Apoptotic Cells Produce Nonclassical Retinoids To Enhance Their Phagocytic Capacity

Zsolt Sarang, Gergely Joós, Éva Garabuczi, Ralph Rühl, Christopher D. Gregory and Zsuzsa Szondy

*J Immunol* published online 21 May 2014
http://www.jimmunol.org/content/early/2014/05/21/jimmunol.1400284

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Macrophages Engulfing Apoptotic Cells Produce Nonclassical Retinoids To Enhance Their Phagocytic Capacity

Zsolt Sarang,*1 Gergely Joós,*1 Éva Garabuczi,* Ralph Rühl,† Christopher D. Gregory,*§ and Zsuzsa Szondy*

Previous work in our laboratory has shown that transglutaminase 2 (TG2) acting as a coreceptor for integrin β3 is required for proper phagocytosis of apoptotic cells. In the absence of TG2, systemic lupus erythematosus–like autoimmunity develops in mice, similarly to other mice characterized by a deficiency in the clearance of apoptotic cells. In this study, we demonstrate that increasing TG2 expression alone in wild-type macrophages is not sufficient to enhance engulfment. However, during engulfment, the lipid content of the apoptotic cells triggers the lipid-sensing receptor liver X receptor (LXR), which in response upregulates the expression of the phagocytic receptor Mer tyrosine kinase and the phagocytosis-related ABCA1, and that of retinaldehyde dehydrogenases leading to the synthesis of a nonclassical retinoid. Based on our retinoid analysis, this compound might be a dihydro-retinoic acid derivative. The novel retinoid then contributes to the upregulation of further phagocytic receptors including TG2 by ligation retinoic acid receptors. Inhibition of retinoid synthesis prevents the enhanced phagocytic uptake induced by LXR ligation. Our data indicate that stimulation of LXR enhances the engulfment of apoptotic cells via regulating directly and indirectly the expression of a range of phagocytosis-related molecules, and its signaling pathway involves the synthesis of a nonclassical retinoid. We propose that retinoids could be used for enhancing the phagocytic capacity of macrophages in diseases such as systemic lupus erythematosus, where impaired phagocytosis of apoptotic cells plays a role in the pathogenesis of the disease. The Journal of Immunology, 2014, 192: 000–000.

Efficient execution of apoptotic cell death followed by efficient clearance mediated by professional macrophages is a key mechanism in maintaining tissue homeostasis. Every day billions of our cells die, and as they become apoptotic, they undergo dramatic changes in the composition of their surface, which allows their recognition by phagocytes and subsequent removal (1). Because there is no one single characteristic cell-surface change on the apoptotic cells, macrophages seem to recognize multiple cell-surface alterations by using a number of phagocytic receptors acting simultaneously (2, 3). The redistribution of phosphatidylserine on the surface of the apoptotic cell is the best characterized mark that distinguishes cellular life from death (4). Many of the macrophage receptors recognize phosphatidylserine or its oxidized form binding to it directly or indirectly via bridging molecules, such as milk fat globule–epidermal growth factor-8 (MFG-E8) (5), thrombospondin-1 (6), or complement 1q (C1q) (7). Macrophages are capable of both expressing the cell-surface receptors and releasing the bridging molecules for the recognition and engulfment of apoptotic cells. Some of these receptors, such as CD14 (8) or Tim4 (9), mediate tethering, whereas other receptors, such as CD36 (10), integrin β3 (6), integrin β3 (11), Mer tyrosine kinase (Mertk) (12), stabilin-2 (13), and ABCA1 (14) seem to activate two evolutionally conserved parallel signaling pathways promoting cytoskeletal reorganization via the activation of the low m.w. GTPase Rac1, which is obligatory for phagocytosis (15).

Macrophages are exposed to varying numbers of apoptotic cells in vivo; therefore, it is likely that they have a sensing mechanism that prepares them, when required, for increased levels of apoptotic cell uptake. Increasing evidence suggests that the receptors that sense the amount of apoptotic cell material engulfed and enhance the phagocytic capacity of macrophages in response are the lipid-sensing receptor liver X receptor (LXR) (16) and the peroxisome proliferator-activated receptors (PPARs) γ (17) and δ (18). Triggering of these receptors leads to enhanced expression of phagocytic receptors and bridging molecules such as Mertk, C1q, CD36, and ABCA1 (16–18). Lipid-sensing receptors belong to the nuclear retinoid X receptor (RXR) heterodimer family and can be triggered independently by both the lipid content of the engulfed cells on the LXR or PPAR and by a vitamin A (retinol) derivative on the RXR ligand binding sites (19). It is believed that the RXR ligand in tissues is 9-cis retinoic acid (9cRA), which is thought to be produced by a nonenzymatic reaction from all-trans retinoic acid receptor; RXR, retinoid X receptor; siRNA, small interfering RNA; SREBP, sterol response element binding protein; TG2, transglutaminase 2.

Abbreviations used in this article: ATRA, all-trans retinoic acid; BMDM, bone marrow–derived macrophage; CFDA, carboxyfluorescein diacetate succinimidyl ester; CMTMR, 5-(and-6)-[(4-chloromethyl]benzoylamino)tetramethylrhodamine; 9cRA, 9-cis retinoic acid; C1q, complement 1q; DEAB, diethylaminobenzaldehyde; Dex, dexamethasone acetate; HPLC-MS/MS, HPLC tandem mass spectrometry; LXR, liver X receptor; Mertk, Mer tyrosine kinase; MFG-E8, milk fat globule–epidermal growth factor-8; PPAR, peroxisome proliferator-activated receptor; qRT-PCR, quantitative RT-PCR; RALDH, retinaldehyde dehydrogenase; RAR, retinoic acid receptor; RXR, retinoid X receptor; siRNA, small interfering RNA; SREBP, sterol response element binding protein; TG2, transglutaminase 2.

Copyright © 2014 By The American Association of Immunologists, Inc. 0022-1767/14/$16.00.
acids (ATRA). Interestingly, however, 9cRA has not yet been detected in vivo. ATRA is an oxidative derivative of retinol and is generated from it by two subsequent dehydrogenase steps. In addition to the lipid-sensing receptors, ATRA and 9cRA can activate the retinoic acid receptors (RARs) as well. RAR/ RXRs, unlike the lipid-sensing receptors, cannot be activated via the RXR ligand binding site alone (23).

After ligation, lipid-sensing receptors regulate the transcription of various genes including those controlling lipid homeostasis (24). Thus, LXRαs can directly promote transcription of sterol response element binding protein (SREBP)-1c through two LXR response elements in the mouse SREBP-1c promoter (25), and also that of RARαs (26) and ABCA1 (27). In addition, Mertk was also found to be a direct LXR target gene (16). LXR binding sites also exist in the promoter of LXR; thus, after ligation, LXRαs promote their own transcription via an autoregulatory loop mechanism (28).

Previous work in our laboratory has shown that transcriptional activity of 2 (TG2) acting as a coreceptor for integrin β1, is required for proper phagocytosis of apoptotic cells (29). In the absence of TG2, systemic lupus erythematosus (SLE)-like autoimmunity develops in mice (30), similarly to other mice characterized by a deficiency in the clearance of apoptotic cells (31). In this study, we demonstrate that macrophages engulfing apoptotic cells produce derivatives that may be nonclassical retinoids with a suggested activation potential for both RARαs and RXRs to enhance their phagocytic capacity, and we show that TG2 is one of the novel retinoid-regulated genes.

Materials and Methods

Reagents

All reagents were obtained from Sigma-Aldrich (Budapest, Hungary) except when indicated otherwise.

Experimental animals

The experiments were carried out with 4-week-old or 2- to 4-mo-old C57B6 mice. To study the effect of loss of TG2 or CD14 on apoptotic cell phagocytosis, bone marrow–derived macrophages (BMDMs) derived from TG2−/− (30) and CD14−/− (8) mice, respectively, were used. In some experiments, RARE-hsp68-lacZ reporter transgenic mice (32) were used to indicate in vivo retinoid acid production. For determining gene expression in the thymus, 4-week-old C57B6 mice were injected i.p. with either 0.3 mg dexamethasone acetate (Dex) dissolved in DMSO or vehicle alone. RARE-hsp68-lacZ mice were injected i.p. with 20 mg/kg GW3965 for 2 d, to measure the in vivo production of RAR-activating derivatives. On the third day, peritoneal cells were harvested by physiological saline lavage. Mice were maintained in specific pathogen-free condition in the Central Animal Facility, and all animal experiments were approved by the Animal Care and Use Committee of University of Debrecen.

X-gal staining of peritoneal macrophages

RARE-hsp68-lacZ reporter transgenic mice were injected with 20 mg/kg GW3965 or vehicle. Twenty-four hours later, peritoneal cells were collected by lavage. Cells were allowed to adhere for 3 h; then the nonadherent cells were washed away. Adherent cells were fixed with 0.25 mM glutaraldehyde for 5 min on ice. Fixed cells were incubated at 37°C for 24 h in X-gal staining solution (35 mM potassium ferricyanide, 35 mM potassium ferrocyanide, 2 mM MgCl2, 0.02% Nonidet P-40, 1 mg/ml X-gal). Images were taken using an AMG EVOs inverted microscope at original magnification ×20.

BMDM cell culture and treatment

Bone marrow progenitors were obtained from the femurs of 2- to 4-mo-old mice lavage with sterile physiological saline. Cells were allowed to differentiate for 6 d in DMEM supplemented with 10% FBS, 1% conditioned medium derived from L929 cells, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO2. Nonadherent cells were washed away every second day. BMDMs were treated with 1 μM GW3965 (Glaxo Smith Kline, Budapest, Hungary), a synthetic LXR agonist, 1 μM LG268 (kind gift of John Schwabe, University of Leicester), a synthetic RXR agonist, 1 μM AM580 (Tocris Bioscience), a synthetic pan RAR agonist, 500 nM AGN193109, a pan RAR antagonist, 300 nM ATRA, a natural pan RAR agonist, or 300 nM 9cRA, a natural RXR and RAR agonist for the indicated periods. In some experiments, 25 μM diethylaminobenzaldehyde (DEAB) was used to block aldehyde dehydrogenase enzyme activities.

In vitro apoptotic cell phagocytosis

BMDMs were stained for 24 h with 5 μM CellTracker Orange (5-(and-6)-[1,4-chloromethyl]benzoylamino)tetratetramethylrhodamine [CMTMR]; Invitrogen). To generate apoptotic thymocytes, thymus were collected from 4- wk-old C57B6 mice, thymocytes were isolated and cultured for 24 h (106 cells/ml) in RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and 10 μM carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Invitrogen) in the absence of serum. CFDA-stained apoptotic thymocytes were added to the BMDMs in 10:1 (apoptotic cells/macrophage) ratio for 30 min. After coculture, apoptotic cells were washed away and macrophages were detached by trypsinization. Cells were analyzed on a Becton Dickinson FACScalibur.

Analysis of mRNA expression

Total RNA was isolated from BMDMs, peritoneal cells of RARE-hsp68-lacZ reporter transgenic mice, and from thymic samples by TRI reagent according to the manufacturer’s guidelines. Total RNA was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Budapest, Hungary) according to the manufacturer’s instructions. Quantitative RT-PCR (qRT-PCR) was carried out in triplicate using predesigned FAM-labeled MGB assays (Life Technologies, Budapest, Hungary) on a Roche LightCycler LC 480 real-time PCR instrument. To detect lacZ mRNA, we used the following primers and FAM-TAMRA-labeled TaqMan probes (designed and ordered from Eurogentec, Seraing, Belgium): forward, 5′-TCG-CTG-ATT-TTG-ACC-TQA-G-3′; reverse, 5′-CCG-CCA-CAT-ATC-CTG-ATC-TTC-C-3′; probe, FAM-ACT-CCA-ACG-CAG-CAC-CAT-CAC-GCG-TAMRA. Relative mRNA levels were calculated using the comparative cycle threshold method and were normalized to GAPDH mRNA.

Western blot analysis

BMDMs were homogenized in ice-cold lysis buffer containing 0.5% Triton X-100. Protein concentration of samples was diluted to 2 mg/ml; then the samples were boiled with an equal volume of Laemmli buffer. Electrophoresis was performed in 10% SDS-polyacrylamide gel. Separated proteins were transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA). The membrane was probed with rabbit polyclonal anti-TG2 (Santa Cruz, Heidelberg, Germany), anti-mouse LXRXβ (ABGENT), GAPDH, or mouse anti–β-actin (Sigma-Aldrich, Budapest, Hungary). Protein bands were visualized by Immobilon Western Chemiluminescent HRP substrate (Millipore).

Immunofluorescence staining and confocal microscopy

Bone marrow progenitors from WT mice were plated in 8-well chamber slides (5 × 104/well) and allowed to mature for 6 d. Phagocytosis assay was carried out as described previously. After coculturing macrophages with apoptotic cells for 30 min, cells were washed and fixed in 4% paraformaldehyde. For integrin β1 and CD14 staining, macrophages were blocked with 50% PBS for 30 min at 37°C, then washed with ice-cold HEPES buffer and stained with PE-conjugated anti-mouse β1 integrin (BD Biosciences) or FITC-conjugated anti-CD14 (BD Biosciences) Abs for 15 min on ice. After washing, cells were fixed in 4% paraformaldehyde. Images were taken with a Zeiss LSM 510 or Olympus FX1000 confocal laser-scanning microscope.

LXR receptor silencing

Five-day–matured BMDMs were transfection with TransiT-siQUEST Transfection Reagent (Mirus Bio LLC) according to user’s instruction. In brief, 1.5 × 106 BMDMs/well in 6-well plate were cultured in 1.25 ml DMEM supplemented with 10% FBS, 1% conditioned medium derived from L929 cells, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Transfection mixture containing 250 μl OptiMem, 6 μl TransIT-siQUEST reagent, and 37.5 μl (1 μM) scrambled or LXRα-specific small interfering RNA (siRNA) was added drop-wise to the cells. Medium was replaced after 3 h of incubation with complete DMEM.

Retinoid measurement by HPLC tandem mass spectrometry

Four-week-old C57B6 mice were injected i.p. with either 0.3 mg Dex dissolved in DMSO alone or with DEAB (0.24 mg/ body weight) or...
vehicle. Twenty-four hours later, thymi were removed in the dark, snap-frozen in liquid nitrogen, and stored on −70°C. Concentrations of retinolic acids were determined in mouse thymi by our HPLC tandem mass spectrometry (HPLC-MS/MS) method (21). In summary, 100 mg of the thymic samples (if samples were under 100 mg, water was added to attain 100-mg sample) was diluted with a 3-fold volume of isopropanol, the tissues were minced using scissors, vortexed for 10 s, put in an ultrasonic bath for 5 min, shaken for 6 min, and centrifuged at 13,000 rpm in a Heraeus BIOFUGE Fresco at +4°C. After centrifugation, the supernatants were dried in an Eppendorf concentrator 5301 (Eppendorf, Germany) at 30°C. The dried extracts were resuspended with 60 μl methanol, vortexed, shaken, diluted with 40 μl of 60 mM aqueous ammonium acetate solution, transferred into the autosampler, and subsequently analyzed using HPLC MS-MS equipment. In addition, we focused on detecting novel dihydro-retinoic acid derivatives like the ones we described previously (33), and we switched our MS-MS to single ion recording mode, focusing on 303 m/z signals in relative intensity. Relative retinoid concentrations were given as area under the curve and normalized to thymic weight.

Statistical analyses

All the data are representative of at least three independent experiments carried out on three different days. Values are expressed as mean ± SD. The p values were calculated by using unpaired Student t test. Statistical significance is indicated by a single asterisk (*p < 0.05).

Results

Upregulation of TG2 alone in wild-type macrophages is not sufficient to promote phagocytosis of apoptotic cells

Previous studies have shown that both ATRA (34) and IFN-γ (35) are capable of inducing TG2 expression in macrophages. In our experiments, we decided to test whether induction of TG2 expression is sufficient to enhance phagocytosis of apoptotic cells by macrophages. Although both compounds increased the expression of TG2 in macrophages (Fig. 1A), only ATRA was capable of significantly inducing the engulfment of apoptotic cells (Fig. 1B and 1C). However, ATRA could significantly induce phagocytosis of TG2 null macrophages as well, indicating that, besides TG2, ATRA must also affect the expression of other phagocytosis-related genes (Fig. 1D).

Indeed, when the expression of various other phagocytic receptors were tested by quantitative PCR analysis after 24 h of retinoid treatment, we found that, in addition to TG2, the expression of six other phagocytosis-related molecules, namely, Mertk, Stabilin-2, Tim4, ABCA1, CD14, and C1q, were also significantly enhanced by retinoids (Fig. 1E). From the additional phagocytosis-related molecules tested, retinoids had no significant effect on the expression of integrin β1, MFG-E8, thrombospondin, CD91, or CD36, whereas the expression levels of scavenger receptor A, integrin αc, βa, and β2 were decreased (Fig. 1E and 1F).

Besides ATRA, a natural pan RAR agonist, which, however, can be converted to 9cRA in cells (20), all the retinoids tested (9cRA, a pan RAR and RXR agonist; Am580, a synthetic pan RAR agonist; and LG268, a synthetic RXR agonist) could induce both the expression of TG2 (Fig. 2A) and the percentage phagocytosis (Fig. 2B). Among these retinoids, ATRA and 9cRA were found to be the most effective, indicating that, although both RXR and RAR receptors can contribute to TG2 induction and the enhancement of phagocytosis, the effect is more pronounced when both receptors are stimulated.

These retinoids affected the expression of the other ATRA-sensitive phagocytic receptors as well (Fig. 2C). Although, however, all the ATRA-sensitive phagocytic receptors could be induced also by 9cRA or LG268, with LG268 being less effective, expression of Mertk and ABCA1 were not Am580-sensitive, indicating that, unlike the expression of others, theirs was not regulated via RAR/RXRs. These data implied that retinoids might facilitate phagocytosis via triggering both RAR/RXR and other RXR heterodimers in macrophages.

Similar to the loss of TG2, the loss of CD14 alone had no significant effect on the ATRA responsiveness of phagocytosis tested in CD14 null macrophages (Fig. 2D), indicating that very likely upregulation of all the ATRA-sensitive phagocytic receptors together results in the enhanced engulfment induced by ATRA.

Ligation of LXRα induces retinoid production in peritoneal macrophages

Previous studies in our laboratory have shown that the in vivo apoptosis induction of thymocytes is coupled to an enhanced retinoid production in the thymus, and the cells that expressed retinaldehyde dehydrogenases (RALDHs) responsible for retinoic acid production were the engulfing macrophages (36). Apoptotic cell uptake in vitro also triggered the expression of RALDHs in macrophages, indicating that the uptake of apoptotic cells is coupled to retinoid synthesis. Because the three lipid-sensing nuclear receptors (LXR, PPARγ, and PPARδ) have been implicated in the macrophage response to engulfed apoptotic cells (16–18), we have also tested whether triggering of these receptors affects the expression of RALDH1. As we have reported previously, we found that agonists of all these three receptors promoted the mRNA expression of RALDH1 in macrophages (data published in Ref. 36).

Because the LXR agonist, GW3965, was the most effective in inducing RALDHs (36), we decided to check in vivo, using the RARE lacZ mice (32), whether triggering LXR indeed leads to enhanced retinoid production. For this purpose, mice were injected i.p. with 20 mg/kg GW3965, and both the RALDH1 and the β-galactosidase mRNA expression were determined in the freshly isolated peritoneal macrophages 24 h later. In these mice, lacZ expression demonstrates the in vivo retinoid-dependent transcriptional activity. As shown in Fig. 3A, LXR ligation in vivo induced the expression of both RALDH1 and lacZ, indicating that LXR signaling is coupled to both enhanced RALDH synthesis and enhanced retinoid production in macrophages. In addition, we could detect the increased expression of TG2 as well. We could also demonstrate the induced expression of β-galactosidase protein by detecting its enzymatic activity, which results in blue staining of macrophages (Fig. 3B). Although in vivo we could not apply DEAB, an inhibitor of RALDHs, at optimum concentration because it in higher concentrations killed the mice, induction of both lacZ and TG2 could be attenuated by pretreating mice with DEAB administered at the highest tolerable concentration, proving further that the induction is the result of in vivo synthesis of retinoic acid (Fig. 3A).

Ligation of LXR enhances phagocytosis of apoptotic cells in a retinoid-dependent manner

Because previous studies indicated that LXR signaling enhances phagocytosis of apoptotic cells (16), we decided to investigate whether LXR-induced retinoid synthesis contributes to the enhancement of phagocytosis by macrophages. Administration of GW3965 for 2 h had no effect on the in vitro phagocytosis of macrophages, and addition of DEAB had no effect on it (Fig. 3C). However, exposure to GW3965 for 24 h significantly enhanced the engulfment capacity of peritoneal macrophages, and inhibition of retinoid synthesis by DEAB nearly completely prevented this effect (Fig. 3D).

Although 2 h of LXR stimulation had no effect on the phagocytosis of apoptotic cells, it already affected the expression of those two phagocytic genes (Mertk and ABCA1), the induction of which could not be induced by the pan RAR agonist Am580 (Fig. 1C),
and that of LXRα, which together with Merkt and ABCA1 is a known LXR target gene (16, 26, 27) (Fig. 3E). RALDHs, which were also induced, are not known LXR target genes, but they are known to be induced by SREBP-1c (37), which contains two LXR response elements in its promoter (25). Thus, we checked the expression of SREBP-1c as well, and detected its induction by GW3965 within 2 h. However, the genes (TG2, C1q, stabilin-2, Tim4, and CD14) found to be induced by the pan RAR agonist AM580 (Fig. 1A and 1C), were not induced by GW3965 at 2 h. In addition, at this time point, the expression of none of the LXR-induced genes was affected by simultaneous administration of DEAB, an inhibitor of all aldehyde dehydrogenases including RALDHs.

If, however, we determined the expression of the same genes 24 h later (Fig. 3F), we found that all the phagocytic receptors, which were induced by ATRA or 9cRA (Fig. 1C), were induced by LXR stimulation as well. From these phagocytosis-related genes, the LXR-induced expression of those receptors (TG2, C1q, stabilin-2, Tim4, and CD14), which were inducible by the pan RAR agonist Am580, was fully prevented by coadministration of DEAB. In addition, administration of a pan RAR antagonist (AGN193109) also prevented the induction of these five genes by the LXR agonist (Fig. 3G). Taken together, these data indicate that these five phagocytosis-related molecules are true RAR-regulated genes in the LXR pathway, and their expression is fully dependent on the newly synthetized retinoid.

To test whether the RAR expression is also affected by LXR stimulation, we detected RAR expression after exposure to GW3965. From the retinoid receptors, we detected only the increase in the expression of RARα (Fig. 3E and 3F), in line with
The induction of SREBP-1c, Mertk, Tim4, and TG2 by GW3965 (Fig. 4B). In addition, silencing of LXRs also prevented the increase in phagocytosis of apoptotic cells induced by GW3965 (Fig. 4C), indicating that GW3965 acts indeed via the LXRs in macrophages. Based on these data, we propose the following model, shown in Fig. 5, for the LXR-induced enhancement of phagocytosis of apoptotic cells by macrophages.

Macrophages engulfing apoptotic cells do not produce the classical retinoic acids

Next, we decided to analyze the RAR-ligating compound produced by macrophages. Because it is difficult to isolate sufficient macrophages to detect their retinoid production, we decided to analyze whole thymus tissue. In the thymus, we have previously shown the increased RALDH expression of macrophages and that of LacZ in the thymus of RARE LacZ mice after in vivo apoptosis induction (36). LacZ expression in RARE LacZ mice indicates the production of endogenously formed RAR-activating ligands, and because its dexamethasone-induced induction was prevented by DEAB, our data suggested that these bioactive compounds were produced via an RALDH-dependent step in vivo.

Dexamethasone injected at 0.3 mg/mice induces a high rate of thymocyte apoptosis followed by effective phagocytosis with a 60% loss of total thymic weight within 24 h (38). In line with the enhanced phagocytosis, this treatment significantly induced the thymic expression of LXR, RALDH, TG2, Tim4, CD14, C1q, and Mertk (Fig. 6A).

To detect retinoic acid production, we treated 4-wk-old mice for 24 h with dexamethasone, and the thymi collected were analyzed for retinoic acid before and after the treatment. In line with our previous publication (39), neither the cis-isomers 13-cis, 9-cis RA, nor ATRA were detectable or were around the detection limit of our LC/MS/MS technique (~10^-9 M), indicating that they were present in much lower concentrations than that responsible for potential RAR activation. In addition, there was no indication that their levels were altered by administration of dexamethasone.

Thus, we decided to search for further dexamethasone-regulated peaks and found various unknown peaks, from which only one was reduced by simultaneous DEAB treatment. This compound has a potential molecular mass of 302 Da indicated by a MS-signal of 302+1 Da using atmospheric pressure chemical ionization at positive ionization mode. A representative chromatogram is shown in Fig. 6B. These data indicate that an unknown retinoid of 302 Da is present and regulated by dexamethasone in the mouse thymus. Based on the MS settings specific for this peak, it seems to be a dihydroretinoic acid derivative. The location of this dehydrogenation at the various double bonds of the retinoic–acid conjugated system and potential presence of retinoid isomers, however, cannot be decided at present because of the lack of available standard compounds.

Previous studies have reported that there exists an alternative retinoid metabolism in several tissues, in which retinol is converted to dihydro-retinol by the retinol saturase enzyme (40). Dihydroretinol can be converted further via RALDHs to dihydro-retinoic acids (41), which can also act as low-affinity RAR ligands (42). We therefore investigated whether retinol saturase is expressed in the thymus and whether its expression is enhanced after in vivo apoptosis induction. As shown in Fig. 6A, retinol saturase was expressed by the mouse thymus, and its expression was significantly increased after dexamethasone injection.

**Discussion**

Although phagocytosis of various targets requires generally the function of one definite receptor on the macrophages, uptake of apoptotic cells involves a number of phagocytic receptors, which function in a coordinate fashion to promote engulfment (2, 3).
Previous studies in our laboratory have shown that TG2, by interacting with both MFG-E8 and integrin β3 as a coreceptor (29), belongs to the cell-surface proteins required for engulfment, and the loss of it decreases the in vivo phagocytic capacity of macrophages leading to SLE-like autoimmunity (30). Similarly, the single loss of other phagocytic receptors in knockout macrophages also reduced their phagocytic capacity (5–14). In this study, we tested whether signals known to increase TG2 expression are also enhancers of engulfment of apoptotic cells. We found that an increase in the TG2 expression alone is not sufficient to enhance the phagocytic capacity of macrophages, which is in line with our previous observation, where we found that addition of recombinant TG2 significantly enhances the phagocytic capacity of TG2 null macrophages, but not that of wild-type cells (29).

However, ATRA, one of the compounds tested, was capable of significantly inducing not only the expression of TG2, but also the phagocytosis of apoptotic cells. This effect was related to a simultaneous induction of the expression of a number of phagocytic receptors or bridging molecules by ATRA, and was detected also in those macrophages that lacked one of these receptors (TG2 or CD14), indicating that simultaneous increase in the expression of all these phagocytosis-related molecules might contribute to the enhanced phagocytosis. Analysis using various retinoid receptor agonists revealed that some of the retinoid-induced phagocytosis-related proteins, such as TG2, Stabilin-2, Tim4, CD14, and C1q, were regulated by the ligation of the RARs, whereas Mertk and ABCA1 were induced by activation of other RXR heterodimers. In line with these observations, those retinoids were the most effective in enhancing phagocytosis of apoptotic cells, which were capable of transactivating both RARs and RXRs.

Previous studies have revealed that three lipid-sensing nuclear receptors from the family of RXR heterodimers respond to the lipid content of the engulfed apoptotic cells by enhancing the expression of various phagocytic receptors. As a result, they are re-
GW3965 mediates its effects on phagocytosis via stimulating LXRs. (A) BMDMs were transfected with 25 nM siLXRα/β or scrambled siRNA. Twenty-four hours later, macrophages were tested for LXRα/β expression by Western blot analysis. (B) BMDMs transfected with 25 nM siLXRα/β or scrambled siRNA were treated with 1 μM GW3965 or vehicle for 24 h. Gene expression was measured by qRT-PCR and expressed as fold inductions ± SD relative to scrambled siRNA-transfected, vehicle-treated cells after normalization of samples with GAPDH expression. (C) siLXRα/β or scrambled siRNA transfected BMDMs were treated with 1 μM GW3965 or vehicle for 24 h in the presence of 5 μM CMTMR. Phagocytosis of apoptotic thymocytes was determined by flow cytometry. Results are representative of three independent experiments (*p < 0.05, significantly different from respective control).

Because our previous studies indicated that ligation of LXR/RXR heterodimers might lead to endogenous retinoid production in macrophages (33), we tested whether the LXR-induced enhancement in phagocytosis requires retinoid production. We could confirm previous observations (16) that ligation of LXR enhances the expression of Mertk and ABCA1, two LXR-regulated phagocytic genes, but we also found that LXR stimulation leads to endogenous retinoid production. The retinoid production partly enhanced the expression of a group of phagocytosis-related genes including TG2 via activating RARs, but also contributed to the enhanced expression of LXRα, Mertk, and ABCA1 detected at 24 h after LXR ligation, very likely by acting via the RXR ligand binding site of the LXR heterodimer (Fig. 5). As a result, inhibition of retinoid production by inhibiting RALDHs prevented the LXR-induced enhancement in the phagocytosis of apoptotic cells, indicating that retinoids mediate the effect of LXR. Previously, we have reported that engulfment-coupled retinoid production of macrophages in the thymus might play a role in the upregulation of TG2 in the apoptotic thymocytes (36), as well as in the modulation of the thymic selection processes (43). It was also found that macrophage-produced retinoids contribute to regulatory T cell formation in the airway (44). However, this is the first report, to our knowledge, to indicate that retinoids also affect the phagocytic capacity of macrophages.

We hoped also to define the nature of retinoids produced by macrophages. However, in line with our previous publication (39), none of the known classical RAs was detectable in the thymus. We could, however, detect the “apopto-phagocytosis-dependent” appearance of a novel endogenous derivative, the appearance of which was RALDH dependent and which might be a dihydroretinoic derivative or an apo-15'-lycopenoic acid derivative (33), indicated by its molecular mass of 302 Da. Besides the classical

FIGURE 4. Novel, endogenous retinoid production in the mouse thymus after apoptosis induction with dexamethasone. (A) Four-week-old mice were injected i.p. with 0.3 mg dexamethasone-acetate or vehicle. Twenty-four hours later, thymi were collected and retinoid concentration was measured by LC-MS-MS analysis. By using single ion recording at 303 m/z, we found a novel peak, which is regulated in response to Dex and Dex+ DEAB. Data were normalized in relation to sample weight to ensure comparable values and represent mean ± SD of three independent experiments. (*p < 0.05, significantly different from control. **p < 0.05, significantly different from the dexamethasone-treated macrophages). AUC, area under the curve.

FIGURE 5. Proposed model for the LXR-induced enhancement of phagocytosis of apoptotic cells by macrophages. LXR triggering leads to the upregulation of LXR target genes such as Mertk, ABCA1, SREBPc1, and RARα. LXRs also promote their own transcription via an autoregulatory loop mechanism leading to further expression of their target genes. SREBP-c1c upregulates the expression of RALDH1 resulting in a consequent retinoid production. The retinoid produced acts on RARs to upregulate the phagocytic genes TG2, Tim4, stabilin-2, CD14, and C1q, leading to enhanced phagocytosis. In addition, it might facilitate both LXR/RXR- and RAR/RXR-driven transcription via binding also to the RXR ligand binding site.
8 RETINOIDS IN THE PHAGOCYTOSIS OF APOPTOTIC CELLS

retinoic acid pathways, a novel retinoid pathway has been recently described, which is initiated by retinol saturase. This pathway also involves RALDHs and produces 13,14-dihydro-retinoic acids, which act also as low-affinity RAR ligands (40–42). We found that retinol saturase is expressed in the thymus, and its expression is induced after in vivo apoptosis induction, indicating that this alternative pathway might be involved in the formation of the novel retinoids. However, their definitive chemical nature awaits definition. Unfortunately, the expected concentrations of the retinoids are in the range of nanograms per gram of tissue, and purification may result in only picogram amounts of compounds when using milligram amounts of thymic tissue. These amounts are too small to determine a chemical structure even with the most sensitive available nuclear magnetic resonance technologies. Thus, with a range of chemist collaborators, we initiated targeted organic synthesis by considering all the possible chemical alternatives to clearly identify the novel derivative and its biological activity. But the fact that the expression of the phagocytic receptors seems to be regulated by both RAR- and RXR-dependent pathways indicates that both RAR and RXR ligands or a ligand with both RAR and RXR activating potential is formed in the LXR signaling pathway. We do not presently understand why two parallel retinol-dependent pathways can generate retinoid receptor ligands in the tissues, but the fact the retinol saturation knockout mice have phenotype indicates tissue-specific roles for this alternative pathway (45).

Increasing evidence suggests that impaired phagocytosis of apoptotic cells caused by the loss of various phagocytic receptors leads to the development of SLE in humans and in mice (5, 7, 8, 12, 16, 18, 30). It has also been suggested that SLE is characterized by impaired phagocytosis, even if the phagocytic receptors are genetically not affected, indicating that impaired primary or secondary engulfment deficiency plays a determinant role in the pathogenesis of this disease (46, 47). Thus, molecules that can enhance phagocytosis of apoptotic cells might be used in the treatment of SLE. Indeed, glucocorticoids, which are widely used in the treatment of SLE, are capable of increasing the phagocytic capacity of macrophages (48). In addition, LXR ligands were shown to decrease the symptoms of SLE in lpr mice (16). Based on our data, we propose that retinoids could also be considered as compounds in targeting phagocytosis of apoptotic cells.

Lupus nephritis is a major cause of morbidity and mortality in patients with SLE (49). Interestingly, the effect of in vivo ATRA treatment on the development of lupus nephritis has already been shown to decrease the symptoms of SLE in lpr mice (16). Based on our data, we propose that retinoids could also be related to the fact that they enhance the phagocytosis of apoptotic cells.

References

Acknowledgments
The technical assistance of Edit Komózci and Zsolt Hartmann is gratefully acknowledged.

Disclosures
The authors have no financial conflicts of interest.


