Retinoid X receptor α controls innate inflammatory responses through the up-regulation of chemokine expression

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The retinoid X receptor α (RXRα) plays a central role in the regulation of many intracellular receptor signaling pathways and can mediate ligand-dependent transcription by forming homodimers or heterodimers with other nuclear receptors. Although several members of the nuclear hormone receptor superfamily have emerged as important regulators of macrophage gene expression, the existence in vivo of an RXR signaling pathway in macrophages has not been established. Here, we provide evidence that RXRα regulates the transcription of the chemokines *Ccl6* and *Ccl9* in macrophages independently of heterodimeric partners. Mice lacking RXRα in myeloid cells exhibit reduced levels of CCL6 and CCL9, impaired recruitment of leukocytes to sites of inflammation, and lower susceptibility to sepsis. These studies demonstrate that macrophage RXRα plays key roles in the regulation of innate immunity and represents a potential target for immunotherapy of sepsis.

nuclear hormone receptors | macrophages | innate immunity | sepsis

Nuclear receptors are ligand-dependent transcription factors that regulate diverse aspects of development and homeostasis (1, 2). Several members of the nuclear receptor superfamily have emerged recently as key regulators of inflammation and immune responses (2, 3). Retinoid X Receptors (RXRs) occupy a central position in the nuclear receptor superfamily because they form heterodimers with many other family members and hence are involved in the control of a variety of physiologic processes (4, 5). RXRs are also able to activate transcription from cognate reporter genes as homodimers (6, 7). There are three RXR isotypes, RXRα (NR2B1), RXRβ (NR2B2), and RXRγ (NR2B3), which show tissue-specific differences in expression (4, 8). Previous studies suggest that the most abundant RXR in myeloid cells, or at least the most functionally important, is RXRα (9). RXRs are receptors for ligands such as 9-*cis*-retinoic acid and endogenous fatty acids (10, 11), and for a variety of synthetic agonists (called rexinoids), such as LG100268 (12). Selective RXR ligands are being developed for cancer therapy and are promising agents for the treatment of metabolic diseases (13).

Innate immunity is an ancient form of host defense that is activated rapidly to enable, through a multiplicity of effector mechanisms, defense against a broad spectrum of foreign substances (14). Inflammation, one of the first responses of the immune system to infection, is mediated by immune system cells, whose accumulation in injured tissues triggers the removal of the foreign agent, prevents subsequent infections, and promotes tissue repair (15). Normally, inflammation is self-controlled in intensity and duration (16). However, when this process is dysregulated, as in sepsis, excessive proinflammatory mediators are released into the bloodstream, resulting in multiple organ failure. Sepsis-induced multiorgan failure has a high death rate in humans and is one of the leading causes of death in intensive care units (17).

Chemokines and their receptors have been implicated in the modulation of leukocyte trafficking, immune/inflammatory responses, sepsis, and multiorgan failure (18, 19). Clinical studies have also identified elevated levels of chemokines associated with human sepsis and acute lung injury (20).

We have examined the role of RXRα in the innate immune system by conditionally disrupting RXRα in myeloid cells. We show that chemokines *Ccl6* and *Ccl9* are novel target genes for RXRα in primary peritoneal macrophages. RXRα deletion also results in decreased levels of CCL6 and CCL9 in vivo, correlating with impaired leukocyte recruitment to inflammatory sites and prolonged survival in sepsis induced by cecal ligation and puncture (CLP) or lipopolysaccharide (LPS). These results establish that RXR is involved in the regulation of the innate immune response and provide evidence for the existence of RXR signaling in macrophages in vivo.

Results

RXRα Controls Chemokine Gene Expression in Macrophages. To investigate the role of RXRα in inflammation and in macrophage function, we generated mice lacking RXRα specifically in myeloid cells (RXRα KO) (Fig. S1 *A–D*). Gene expression profiling of WT and RXRα KO peritoneal macrophages showed that *Ccl6* (*C10, Mrp-1, Scya6*) and *Ccl9* (*Mip-1γ, Mrp-2, Scya9*) are down-regulated in the KO mice. Reduction in *Ccl6* and *Ccl9* gene expression in RXRα KO macrophages was confirmed by real-time quantitative PCR (Q-PCR) (Fig. 1*A*). Q-PCR further showed that the RXR ligands 9-*cis*-retinoic acid (RA) and LG100268 induced *Ccl6* and *Ccl9* gene expression in WT macrophages, but expression was not induced by LG100754 (Fig. 1*B*). LG100268 and 9-*cis*-RA are RXR pan-agonists, whereas LG100754 is an agonist of PPAR/RXR and RAR/RXR heterodimers but an antagonist of RXR homodimers (12). *Ccl6* and *Ccl9* mRNA levels were maximally induced after 24 h stimulation with 9-*cis*-RA (Fig. S2*A*), and the induction of *Ccl6* and *Ccl9* by LG100268 and 9-*cis*-RA was inhibited by LG100754 (Fig. S2*B*). The effect of RXR ligands on protein expression was examined by ELISA. Treatment of macrophages with 9-*cis*-RA or LG100268 significantly increased CCL6 and CCL9

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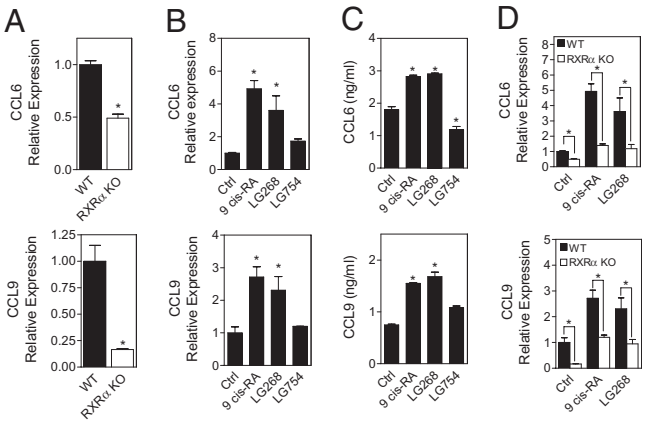

Fig. 1. Loss of RXRα in macrophages reduces *Ccl6* and *Ccl9* chemokine expression. (*A*) Q-PCR analysis of *Ccl6* and *Ccl9* mRNA expression in RXRα KO peritoneal macrophages. *, $P \leq 0.05$ compared with WT. (*B*) Q-PCR analysis of *Ccl6* and *Ccl9* gene expression in peritoneal macrophages treated for 24 h with the RXR ligands 9-*cis*-RA (1 μM), LG268 (50 nM), or LG754 (1 μM). *, $P \leq 0.05$ compared with control (Ctrl). (*C*) ELISA of CCL6 and CCL9 protein in macrophages treated as in *C* for 48 h. *, $P \leq 0.05$ compared with control (Ctrl). (*D*) Q-PCR analysis of *Ccl6* and *Ccl9* mRNA expression in WT and RXRα KO macrophages treated with LG268 or 9-*cis*-RA for 24 h. *, $P \leq 0.05$ compared with WT. Data are means ± SEM of at least three independent experiments.

accumulation in the culture medium (Fig. 1*C*). The ligand-induced mRNA expression of these chemokines was substantially lower in RXRα KO macrophages (Fig. 1*D*); the residual effect of the ligands is consistent with the presence of RXRβ.

***Ccl6* and *Ccl9* Are Transcriptionally Regulated by RXRα.** The induction of *Ccl6* and *Ccl9* mRNA by treatment with RXRα ligands raised the possibility that these genes might be direct targets of RXRα. To test this, we generated luciferase reporter constructs (pGL3 vector) driven by the proximal 1.1 kb of the mouse *Ccl6* or *Ccl9* promoter. These constructs were separately cotransfected into the mouse macrophage cell line RAW 264.7 together with empty (pCMX) or pCMX-RXRα expression vectors. After transfection, cells were treated with vehicle or the RXR-specific ligands 9-*cis*-RA or LG100268. RXRα activated transcription from the proximal promoters of *Ccl6* and *Ccl9* genes in a ligand-dependent manner (Fig. 2 *A* and *B*). The requirement for transfected RXRα suggests that these cells do not express sufficient endogenous RXRα to support transactivation (Fig. 2 *A* and *B*). Ligands specific for PPARs and LXRs could not activate these promoters (Fig. S2*C*), suggesting that RXRα could regulate *Ccl6* and *Ccl9* gene transcription independently of these heterodimeric partners.

Analysis of the two promoter regions identified sequences, between −67 and −54 (*Ccl6*) and between −80 and −67 (*Ccl9*), with homology to a DR-1 (direct repeat with one nucleotide spacer) type hormone response element. To determine the contribution of the putative DR-1 sites to RXRα-dependent transactivation, we generated shorter (~200 bp) promoter-luciferase reporter constructs spanning these sites (−200 to +14 for *Ccl6* and −187 to +3 for *Ccl9*), together with versions containing point mutations (Fig. 2*C*). The 200-bp *Ccl6* and *Ccl9* promoter constructs both retained the ability to be activated by RXRα ligands, whereas the mutant versions showed minimal responsiveness to RXRα, showing that these DR-1-like sites are rexinoid response elements (RXREs) (Fig. 2 *D* and *E*). Ligand-dependent binding of RXRα to the DR-1-like RXREs in macrophages in vivo was shown by chromatin immunoprecipitation (ChIP) assays (Fig. 2*F*). Gel-shift analysis confirmed the ability of RXRα homodimers to bind to oligonucleotides spanning the *Ccl6* and *Ccl9* DR-1-like RXREs, but not to mutated versions (Fig. 2*G*).

Of the 49 nuclear receptors encoded by the mouse genome, systematic quantitative PCR analysis has demonstrated that 28 are expressed in macrophages (21). Of these, PPARγ, PPARδ, and Nurr1 have been demonstrated to form heterodimers with RXRs on DR-1 elements. RXR also forms heterodimers with LXRs, TRs, RARs, and VDR on DR-4, DR-2/5, and DR-3 elements, respectively (4). To investigate whether RXR induces *Ccl6* and *Ccl9* transcription as a homodimer or by forming a heterodimer, we tested the ability of potential heterodimeric partners to influence expression of these genes in macrophages. Expression of *Ccl6* and *Ccl9* was upregulated in cells treated with the RXR ligand LG100268 but not the PPAR ligands rosiglitazone, GW327647, or GW610742; moreover, we observed no additive or synergistic effect when PPAR and RXR ligands were both present (Fig. 2*H*). PPAR ligands were, however, able to induce the expression of the PPAR- and RXR-target genes *Abcg1* and *Adrp* (Fig. S2*D*). Furthermore, no effect was observed upon treatment with the Nurr1/RXR selective ligand XCT0135980, the LXR ligand T1317, the RAR ligand TTNPB, the VDR ligand Vit D3, or the TR ligand T3 (Fig. S2*E*). Collectively, these findings provide evidence for an RXRα signaling pathway in macrophages that is independent of RXR heterodimeric partners.

Decreased CCL6 and CCL9 Expression Impairs Leukocyte Recruitment in Myeloid RXRα Knockout Mice. We next investigated the functional consequences of RXRα-mediated chemokine regulation in an in vivo model of acute inflammation. We induced peritonitis by injecting thioglycollate or zymosan into the mouse peritoneal cavity and monitored the accumulation of CCL6 and CCL9 in cell-free peritoneal lavage fluid. Levels of CCL6 and CCL9 peaked at 24 h and remained high 48 h after injection (Fig. S3*A*). Peritoneal exudates collected from RXRα KO mice 24 or 48 h after the initiation of peritonitis contained significantly less CCL6 and CCL9 than did time-matched WT exudates (Fig. 3*A*). Similarly, the exudate levels of IL-6 and MCP-1 in response to thioglycollate- or zymosan-induced peritonitis, respectively, were lower in RXRα KO mice (Fig. S3*B*). There were no significant differences in the quantity of MIP-1α, GROα, Rantes, IL-12, and TNFα (Fig. S3*B*).

Because chemokines are chemoattractants that direct leukocytes to inflammation sites, we next investigated whether decreased CCL6 and CCL9 levels in peritoneal exudates had an impact on leukocyte recruitment. Thioglycollate or zymosan injection induced strong recruitment of monocytes/macrophages and granulocytes from 24 h up to 48 h into the abdominal cavity of WT mice (Fig. S3 *C* and *D*). In contrast, the influx induced by these agents in RXRα KO mice was significantly lower (Fig. 3 *B* and *C*). The number of resident monocytes/macrophages and granulocytes was, however, unaffected by deletion of RXRα (Fig. S4*A*). The impaired ability of RXRα KO mice to mount a proper leukocytic response was not due to any overt leukocyte defect, as shown by the similar sizes and composition of leukocyte subpopulations in blood and bone marrow from WT and RXRα KO mice (Table S1 and Fig. S4 *B* and *C*). In addition, RXRα KO mice challenged with i.p. thioglycollate showed normal leukocyte population profiles in bone marrow and peripheral blood (Fig. S4*D*), strongly indicating that the low number of leukocytes infiltrating the inflamed peritoneum in the absence of RXRα is the result of impaired leukocyte migration.

To explore the mechanistic link between decreased peritoneal exudate levels of CCL6 and CCL9 and impaired leukocyte migration, we conducted a series of in vitro migration assays. Peritoneal exudates from WT mice were as effective as recombinant CCL6 and CCL9 inducing monocyte/macrophage migration; in contrast, exudates from RXRα KO mice were significantly less chemoattractive (Fig. 3*D*). We next tested the chemoattractant potential of the conditioned media obtained from peritoneal macrophages stimulated in culture with the RXRα ligands LG100268 or 9-*cis*-RA for 72 h. These conditioned media significantly induced the migration of bone marrow mononuclear cells across activated endothelial cells

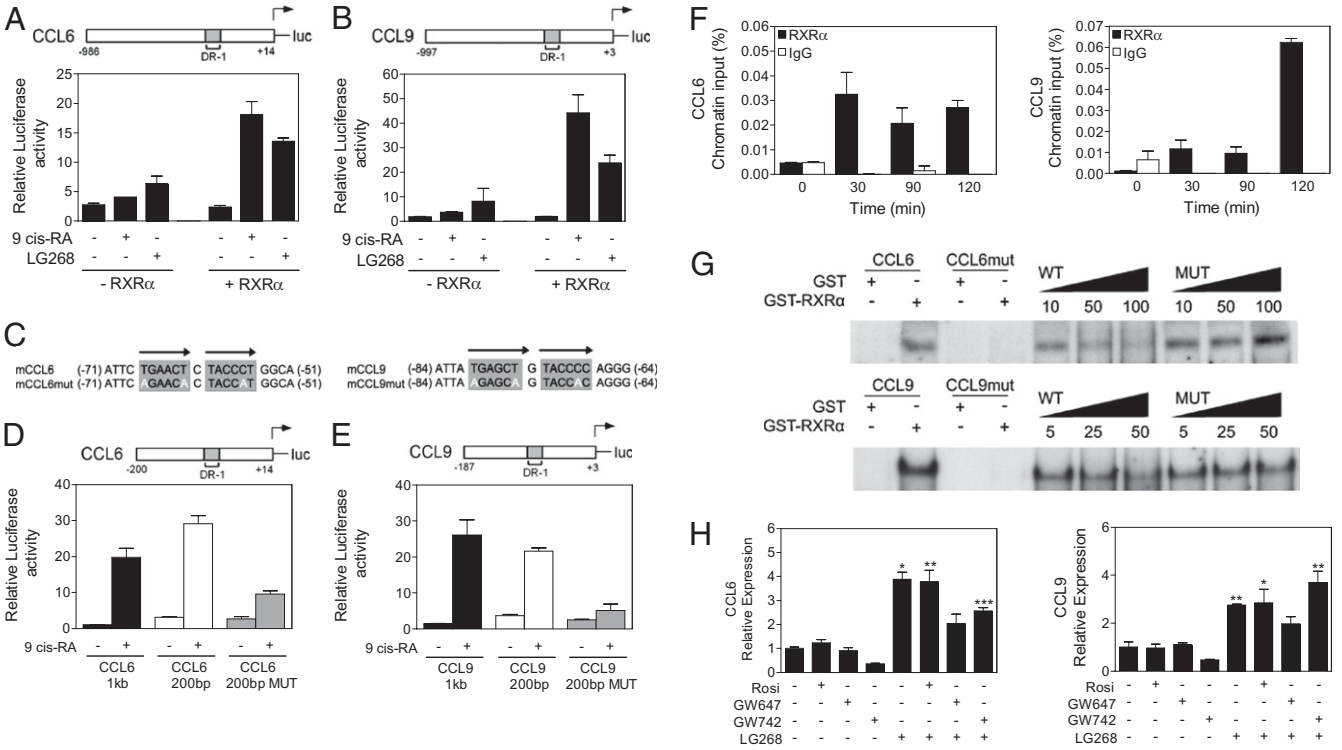

Fig. 2. Transcription of *Ccl6* and *Ccl9* is regulated by RXRα via DR-1 rexinoid response elements. (*A* and *B*) RAW 264.7 macrophages were cotransfected with a luciferase reporter plasmid under the transcriptional control of either the *Ccl6* (pGL3-CCL6-luc) or the *Ccl9* (pGL3-CCL9-luc) promoters together with an RXRα expression plasmid (pCMX-RXRα) or empty vector, as indicated. Cells were treated with LG268 (50 nM) or 9-*cis*-RA (1 μM) and analyzed for reporter activity 24 h later. (*C*) Mutations introduced into the *Ccl6* and *Ccl9* DR-1 motifs. (*D* and *E*) RAW 264.7 cells were transfected with 1 kb, 200 bp, or DR-1-mutated 200 bp (MUT) proximal sequences from the *Ccl6* or *Ccl9* promoters. Cells were treated with LG268 (50 nM) or 9-*cis*-RA (1 μM) and analyzed for reporter activity 24 h later. Values are means ± SEM from at least three experiments. (*F*) ChIP analysis of the binding of RXRα to *Ccl6* and *Ccl9* proximal promoter regions in macrophages treated for the indicated times with LG268 (50 nM). ChIP assays were performed with antibodies against RXRα and control IgG. Immunoprecipitated DNA was analyzed by Q-PCR. Values are means ± SEM. (*G*) Recombinant GST-labeled RXRα homodimers were bound to ^{32}P-labeled oligonucleotides corresponding to the RXRE from the *Ccl6* and *Ccl9* promoter or a version mutated as in C (mut). Competition assays were conducted with unlabeled WT and mutant RXRE oligonucleotides. (*H*) Q-PCR analysis of mRNA expression in peritoneal macrophages treated with ligands for PPARα (GW7647, 0.1 μM), PPARβ (GW0742, 0.1 μM), PPARγ (Rosiglitazone, 1μM), or RXR (LG268, 50 nM), alone or in the indicated combinations. Values are means ± SEM *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

(Fig. 3*E*); this transmigration was significantly inhibited by preincubation of the media with anti-CCL6 and anti-CCL9 antibodies, indicating that CCL6 and CCL9 are key chemoattractants in this setting. Further experiments showed that WT and RXRα KO bone marrow mononuclear cells have the same capacity to transmigrate toward recombinant CCL6 and CCL9 (Fig. S5*A*). Consistently, there was no difference between the two genotypes in the expression of CCR1 mRNA (the receptor for CCL6 and CCL9) in peritoneal macrophages or in the expression of CCR1 protein in bone marrow, blood leukocytes, and peritoneal macrophages (Fig. S5*B*).

To exclude the possibility that the defects in leukocyte migration involved RXRα heterodimerization with PPARγ, we performed thioglycollate-induced peritonitis experiments in myeloid PPARγ KO mice. In contrast to RXRα KO mice, the PPARγ KO mice showed no defect in leukocyte recruitment (Fig. S6 *A* and *B*), no significant differences in the expression levels of CCL6, CCL9, MCP-1, or IL-6 in peritoneal exudates after thioglycollate injection (Fig. S6*C*), and no changes in *Ccl6* and *Ccl9* gene expression in peritoneal macrophages in vitro (Fig. S6*D*).These results suggest that the regulation of chemokine production and leukocyte migration by RXRα is independent of PPARγ.

Absence of RXRα from Macrophages Prolongs Mouse Survival in CLP- and LPS-Induced Sepsis. To investigate the role of RXRα in the innate immune response to experimental sepsis, we performed cecal ligation and puncture (CLP) in WT and RXRα KO mice. After 24 h, plasma levels of CCL6 and CCL9 and peritoneal levels of CCL6 in RXRα KO mice were significantly lower than in WT animals (Fig. 4*A*). In addition, levels of MCP-1 were lower in RXRα KO peritoneal exudate, whereas they were unaltered in plasma, and there was also no significant difference in peritoneal GRO-α, MIP-1α, or Rantes, or in the peritoneal and plasma concentrations of IL-6, IL-1α, IL-12, and TNFα (Fig. S7 *A* and *B*). We also found that RXRα KO mice survived CLP-induced sepsis for significantly longer than WT (Fig. 4*B*). Consistently, the severity of CLP, scored by quantification of liver histological parameters (hemorrhage, fibrin accumulation, inflammatory infiltrate, and degenerated tissue), was lower in RXRα KO mice (Fig. 4 *C* and *D*).

RXRα KO mice were also less susceptible than WT mice to septic shock induced by LPS (Fig. 4*E*), and the increase in plasma CCL9 after LPS challenge (90 min) was significantly lower in RXRα KO mice (Fig. 4*F*). The absence of RXRα did not, however, affect the plasma concentrations of IL-6, IL-1α, IL-12, TNFα, and MCP-1 (Fig. S7*C*). We did not find significant differences in the plasma levels of CCL9, MCP-1, and IL-6 after LPS injection in PPARγ KO mice (Fig. S7*D*). Next, we studied the effect of RXR signaling on LPS-induced CCL9 production by peritoneal macrophages in vitro. In WT macrophages, LPS induced the expression of *Ccl9* mRNA, peaking at 4 h, and a strong accumulation of CCL9 protein in culture supernatants; in contrast, RXRα KO macrophages showed significantly lower *Ccl9* mRNA expression and CCL9 protein secretion (Fig. S8 *A* and *B*). No changes in LPS-induced *Ccl9* expression were found in PPARγ KO

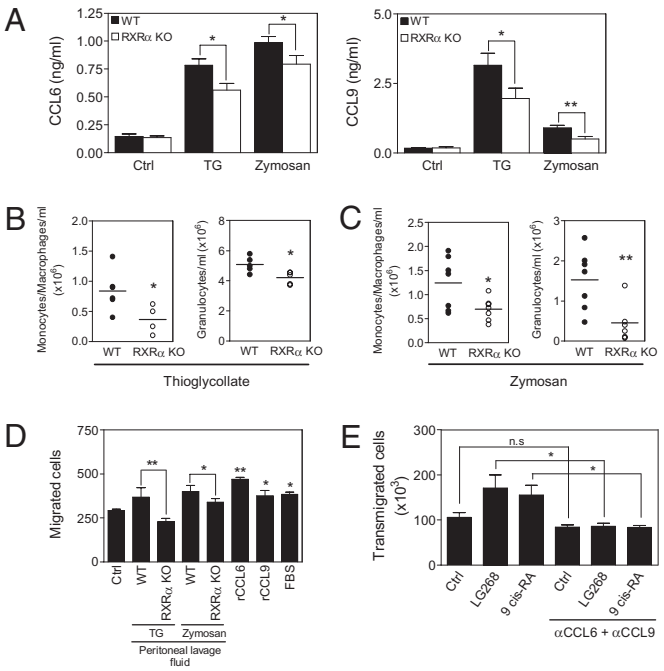

Fig. 3. RXRα deficiency impairs leukocyte recruitment and decreases CCL6 and CCL9 expression during peritonitis induced by thioglycollate or zymosan. (*A*) ELISA analysis of CCL6 and CCL9 in peritoneal exudates collected from WT and RXRα KO mice after the initiation of peritonitis by i.p. injection with thioglycollate (48 h) or zymosan (24 h). Values are means ± SEM; $n = 6–7$ for each genotype. (*B* and *C*) Total numbers of monocytes/macrophages and granulocytes recovered from WT and RXRα KO mice 24–48 h after i.p. injection of thioglycollate (*B*) or zymosan (*C*). $n = 4–7$ mice per genotype. (*D*) Chemotaxis of peritoneal monocytes/macrophages toward rCCL6 (50 ng/mL), rCCL9 (5 ng/mL), FBS, or peritoneal lavage fluid from thioglycollate (TG)- or zymosan-injected WT and RXRα KO mice. Each condition was determined in duplicate, and data are the means ± SEM of three experiments. (*E*) Transmigration of bone marrow mononuclear cells across activated human endothelial cell monolayers toward conditioned medium from WT macrophages treated for 72 h with LG268 (50 nM) or 9-*cis*-RA (1 μM). Where indicated, anti-CCL6 (16 μg/mL) and anti-CCL9 (2 μg/mL) antibodies were added to the conditioned media. Data represent the total number of cells collected in the lower chamber, and are the means ± SEM of three experiments run in duplicate. *, $P \leq 0.05$; **, $P \leq 0.01$; n.s., non significant.

macrophages (Fig. S8*C*). *Ccl9* expression in primary cultures of WT macrophages was further significantly increased by treatment with 9-*cis*-RA or LG100268 together with LPS, and this increase was again lower in RXRα KO macrophages (Fig. S8*D*). *Ccl6* expression was not up-regulated by LPS treatment (Fig. S8*E*).

Discussion

Nuclear hormone receptors are important modulators of the immune response (2), and nuclear receptors such as PPARγ and LXRα/β play important roles in macrophage biology, affecting innate and adaptive immunity (3, 22). These receptors heterodimerize with RXR to regulate gene transcription (4). Previous studies have suggested that activation of RXR promotes T helper 2 (Th2) differentiation, establishing a role for RXR in adaptive immunity through its interaction with VDR and RAR (23). In addition, various stimuli, including IL-1β and LPS, have been shown to regulate RXRα gene expression or subcellular localization in liver (24–26), and several reports have indicated possible roles of RXRα in skin and liver inflammation and viral immune responses (24, 25, 27). However, the role of macrophage RXRα in innate immunity and inflammation is not established. Our results show that RXR is important for the proper control of genes involved in the innate immune responses to inflammatory stimuli. Mice lacking RXRα in myeloid cells show impaired recruitment of leukocytes to sites of inflammation and are less susceptible than control mice to sepsis. These defects are associated with decreased levels in vivo of the chemokines CCL6 and CCL9, which are shown to be transcriptional targets of RXRα.

Chemokines are required for leukocyte trafficking during inflammatory responses and for host responses during sepsis (18–20). CCL6 and CCL9 are CC chemokines that serve as chemoattractants for macrophages, $CD11b^+$ cells, B cells, $CD4^+$ and $CD8^+$ T lymphocytes, and eosinophils (28–30). These chemokines are present in a variety of inflammatory and remodeling disorders, but their transcriptional regulation is not well characterized (29, 31, 32). *Ccl6* and *Ccl9* are paralogous genes, separated by only 9.4 kb on chromosome 11, and show a high degree of sequence similarity (28). Few reports have shown chemokines to be direct target genes for nuclear receptors. Retinoic acid (RA) induces *Ccl2* (*Mcp-1*) expression in human monocytic cells lines and peripheral blood monocytes *in vitro*, but the relevance of this finding in vivo is unclear (33).

A few genes have been reported to be regulated by RXR homodimers signaling through specific RXREs (DR-1 type elements): rat cellular retinol-binding protein type II (CRBPII), human apo A-I, human Apo A-II, and rat growth hormone (rGH) (34). However, many of these gene regulations were identified by in vitro transient transfection assays (35), and the possibility that these genes might be activated by binding of RXR heterodimers to DR-1 elements in vivo was not explored. More recently, IJpenberg et al. (7) have shown that RXR homodimers are able to regulate metabolic pathways in vivo. By excluding roles of other nuclear receptors that are expressed in macrophages and have the ability to heterodimerize with RXRα on the *Ccl6* and *Ccl9* RXREs, we provide evidence for an RXR-selective signaling pathway in primary macrophages. We have identified the RXRE in the *Ccl6* and *Ccl9* proximal promoters as a DR-1 *cis*-element, consisting of two imperfect AGGTCA sequences separated by a single nucleotide. RXRα deletion thus results in a significant reduction of *Ccl6* and *Ccl9* gene expression in primary mouse macrophages, although the residual induction of *Ccl6* and *Ccl9* in KO macrophages indicates a possible compensatory effect of RXRβ.

The role of RXRα in inflammation was investigated in the present study using four mouse models of peritonitis, all characterized by leukocyte influx into the peritoneal cavity and the release of inflammatory mediators (cytokines/chemokines): i.p. administration of zymosan or thioglycollate produces a sterile peritonitis, LPS-endotoxemia mimics bacterially induced sepsis, and CLP produces a polymicrobial bacterial peritonitis that closely mimics human sepsis. Using these models, we have shown that RXRα signaling occurs in vivo. Our results show that the impaired leukocyte migration in response to inflammatory stimuli in RXRα KO mice is associated with the lower secretion of CCL6 and CCL9 in the peritoneal cavity in these animals. Reduced levels of CCL6 and CCL9 might also regulate the secretion of other cytokines/chemokines such as IL-6 and MCP-1, contributing to the phenotype. In contrast, although PPARγ also binds a DR-1 element, myeloid PPARγ null mice showed no defects in leukocyte recruitment to inflammatory sites and alterations in the levels of CCL6, CCL9, IL-6, or MCP-1. Although PPARγ has been shown to negatively regulate cytokines and chemokines in chronic inflammation (2, 3, 36), we did not observe any role of PPARγ in the in vivo models of acute inflammation. This unexpected result might be due to the lack of PPARγ expression in monocytes and resting peritoneal macrophages, which play key roles in the earlier phases of acute inflammatory responses (36, 37), or it may be that repression of proinflammatory genes by PPARγ does not require RXRα. Our data demonstrate the existence in vivo of an RXR signaling pathway independent of PPARγ. Moreover, given the known macrophage expression profiles of nuclear receptors able to interact

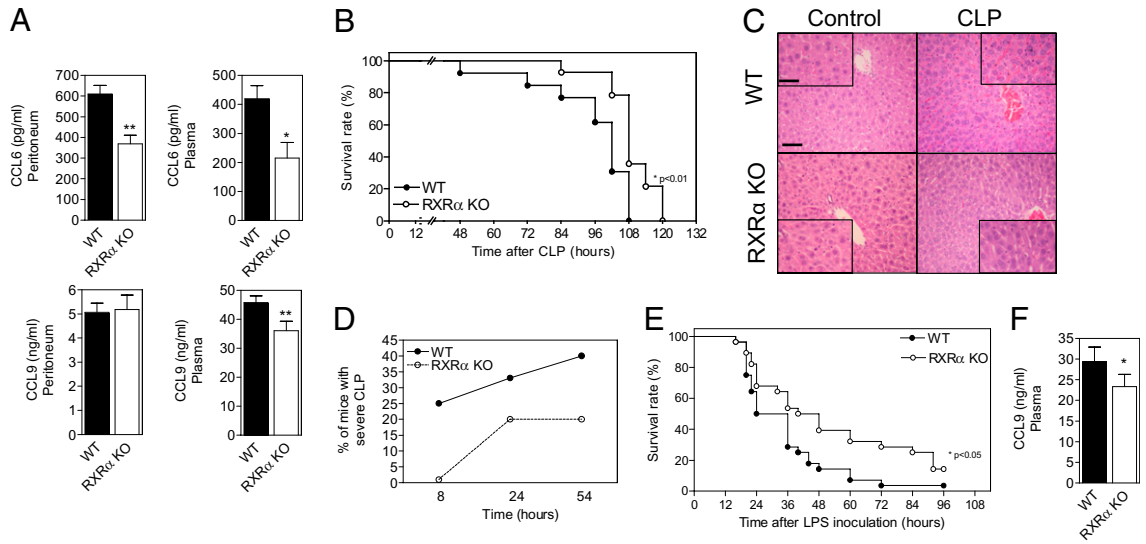

Fig. 4. Delayed CLP- and endotoxin-induced mortality in RXRα KO mice is associated with decreased levels of CCL6 and CCL9. (*A*) Decreased CCL6 and CCL9 in peritoneal exudates and plasma from WT and RXRα KO mice. Peritoneal exudates and plasma were collected 24 h after the initiation of cecal ligation and puncture (CLP), and CCL6 and CCL9 were measured by ELISA. $n = 7$–10 animals for each genotype. (*B*) Survival rates of female WT and RXRα KO mice subjected to CLP. The graph represents pooled survival data from duplicate studies showing similar results. Experiments contained 14 mice per group, and survival was monitored for 7 days after surgery. (*C*) Histological analysis of the livers of WT and RXRα KO 54 h after surgery. Representative liver sections are shown. (Scale bar: 50 μm, in inserts 15 μm.) (*D*) Percentage of mice with severe peritonitis after CLP, as determined by quantification of histological parameters (hemorrhage, fibrin accumulation, inflammatory infiltrate, and degenerated tissue) ($n = 5$–9). (*E*) Survival of WT and RXRα KO mice after i.p. injection with LPS (40 mg/kg per mouse). $n = 28$ for each genotype from three independent experiments. (*F*) Plasma concentrations of CCL9 in WT and RXRα KO mice, determined by ELISA 90 min after injection of LPS. $n = 7$ for each genotype. Values are means ± SEM; *, $P \leq 0.05$; **, $P \leq 0.01$.

with RXRs on DR-1 elements (4, 21), our findings provide evidence supporting an action of RXR homodimers. However, the variety of potential heterodimers formed by RXRs means that the possibility that a different heterodimer contributes to the observed effects cannot be completely ruled out at present. Whether other nuclear receptors are also involved in the control of acute inflammatory responses in vivo will require conditional macrophage-specific deletion of each candidate RXR partner.

Sepsis is a very complex syndrome in which the underlying inflammatory response involves the interplay of several biological systems (the complement, coagulation, and fibrinolytic cascades and the autonomic nervous system) and cell types, resulting in an imbalance of the inflammatory network (17, 38). Recent strategies to identify potential therapeutic targets have focused on these systems but with little clinical success. The finding that survival after LPS- or CLP-induced sepsis is prolonged in RXRα KO mice is therefore of possible clinical interest. Interestingly, mice deficient for CCR1, the receptor for CCL6 and CCL9, are also significantly protected against CLP-induced lethality (39). However, immunoneutralization of CCL6 enhances sepsis-related mortality (31). This discrepancy might arise because the immunoneutralization only interferes with CCL6, potentially allowing a compensatory effect of CCL9. It is also possible that the decreased MCP-1 levels in the peritoneum of RXRα KO mice after CLP-induced sepsis might contribute to the phenotype.

RXR is activated in vitro by the vitamin A metabolite 9-*cis*-RA, which binds with high affinity to the RXR ligand binding domain; however, 9-*cis*-RA has been difficult to detect in vivo (40). Recently, it has been shown that RXR can be activated by polyunsaturated long chain fatty acids (PUFA) (11), although the nature of the physiologically relevant agonists remains to be established. Interestingly, in response to LPS, RAW 264.7 cells and thioglycollate-elicited or BM-derived macrophages produce PUFA (www.lipidmaps.org), which might be important for the regulation of RXR-mediated inflammatory responses. *Ccl9* is also induced by LPS and this response is lost in RXRα KO macrophages, suggesting that natural ligands for RXR are produced in response to LPS treatment. Further studies are required to determine whether endogenous ligands for RXR are produced during inflammatory processes.

Our data support a model in which peritoneal macrophages respond to inflammatory stimuli by secreting cytokines and endogenous RXRα ligands. The activation of the RXRα-transcriptional program in macrophages will result in the production of chemokines such as CCL6 and CCL9, leading to increased leukocyte recruitment and an inflammatory response; excessive activation of this program will have deleterious consequences, as in the case of sepsis. This study outlines a previously unrecognized role for RXRα in the regulation of leukocyte migration and sepsis, and supports the existence of an RXR signaling pathway in vivo. Our data suggest that RXRs are potential targets for immunotherapy in sepsis patients and in chronic inflammatory diseases.

Materials and Methods

Cell Culture. Peritoneal macrophages were harvested from WT and RXRα KO mice as previously described (37). Further information is provided in *SI Materials and Methods*.

RNA Analysis and Chemokine Quantification. Total RNA isolation and Q-PCR were performed as described in ref. 9. Further information is provided in *SI Materials and Methods*. CCL9 (R&D Systems) and CCL6 (Antigenix) were quantified with ELISA kits.

ChIP Assays. Macrophages were cross-linked with 1% formaldehyde at room temperature for 10 min. 100-bp regions of the *Ccl6* and *Ccl9* proximal promoters were amplified by Q-PCR. Primer sequences are available upon request. Anti-RXRα (D-20, sc-553) and anti-RXRDN-197 (sc-774) (Santa Cruz Biotechnology) antibodies were used in combination. IgG was used as a control of nonspecific binding.

Gel Shift and Transient Transfection Assays. Oligonucleotides were annealed and labeled using Klenow enzyme (Roche). Purified bacterially expressed RXRα protein was incubated with labeled DNA, and protein–DNA complexes were electrophoresed and visualized by autoradiography. For competition studies, a 5- to 100-fold molar excess of unlabeled oligonucleotide was added. Oligonucleotides corresponding to RXR binding sites in *Ccl6* and *Ccl9*

promoters and their mutants are available upon request. Transient transfections of RAW 264.7 cells (ATCC) using Lipofectamine 2000 (Invitrogen) were as described (36). Promoter constructs for *Ccl6* and *Ccl9* were cloned in the pGL3-luc vector (Promega). A β-galactosidase expression vector was cotransfected as an internal control. Point mutations in *Ccl6*-luc and *Ccl9*-luc were made with the QuikChange side-directed mutagenesis kit (Stratagene).

Peritonitis Models. Eight- to 9-week-old mice were i.p. administered with 2.5 mL of 3% thioglycollate broth (Difco) or 1 mg of type A zymosan (Sigma) in 0.5 mL of sterile PBS. In other experiments, 12-week-old mice of each genotype were i.p. injected with 40 mg/kg LPS (*E. coli* 0111:B4; Sigma). Sepsis induction by cecal ligation and puncture (CLP) was performed as described elsewhere (41). Severity of CLP was quantified by histological analysis. Liver sections were H&E-stained, and three blinded observers scored six pictures per mouse for distinct parameters: hemorrhage, fibrin deposition, hepatocyte degeneration, and leukocyte infiltrate. Mice were considered severely affected if ≥3 parameters were scored higher than the group average for each time point.

Cell Migration Assay. Cell migration assays were conducted using 8.0-μm-pore transwell filters (Costar). Peritoneal monocytes/macrophages (5×10^4) were resuspended in 150 μL of 0.5% BSA in RPMI medium and added to the upper chamber of transwell filters. The lower chamber contained rCCL6 (50 ng/mL), rCCL9 (5 ng/mL), or peritoneal exudate from WT or RXRα KO mice previously injected with thioglycollate (48 h) or zymosan (24 h). After a 2-h incubation at 37 °C, the inserts were processed for fluorescence microscopy.

Endothelial Transmigration Assay. Cell transmigration assays were performed in 5-μm-pore Transwell chambers (Costar) as described in ref. 42. Further information is provided in *SI Materials and Methods*.

Statistical Analysis. The normal distribution of data were checked with the Kolmogorov–Smirnov test. Data were analyzed by unpaired Student's *t* test and nonparametric Mann–Whitney *U* test. Survival rates were represented as a Kaplan–Meier curve, and the results were analyzed with a log-rank (Mantel–Cox) *t* test.

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