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# CC chemokine receptor 4 modulates Toll-like receptor 9-mediated innate immunity and signaling

*Makoto Ishii, Cory M. Hogaboam, Amrita Joshi, Toshihiro Ito, Daniel J. Fong and Steven L. Kunkel*

Immunology Program, Department of Pathology, University of Michigan Medical School, Ann Arbor, MI, USA

**The present study addressed the modulatory role of CC chemokine receptor 4 (CCR4) in Toll-like receptor (TLR) 9-mediated innate immunity and explored the underlying molecular mechanisms. Our results demonstrated that CCR4-deficient mice were resistant to both septic peritonitis induced by cecal ligation and puncture (CLP) and CpG DNA/D-galactosamine-induced shock. In bone marrow-derived macrophages (BMMΦ) from CLP-treated CCR4-deficient mice, TLR9-mediated pathways of MAPK/AP-1, PI3K/Akt, and IκB kinase (IKK)/NF-κB were impaired compared to wild-type (WT) cells. While TLR9 expression was not altered, the intensity of internalized CpG DNA was increased in CCR4-deficient macrophages when compared to WT macrophages. Pharmacological inhibitor studies revealed that impaired activation of JNK, PI3K/Akt, and/or IKK/NF-κB could be responsible for decreased proinflammatory cytokine expression in CCR4-deficient macrophages. Interestingly, the CCR4-deficient BMMΦ exhibited an alternatively activated (M2) phenotype and the impaired TLR9-mediated signal transduction responses in CCR4-deficient cells were similar to the signaling responses observed in WT BMMΦ skewed to an alternatively activated phenotype. These results indicate that macrophages deficient in CCR4 impart a regulatory influence on TLR9-mediated innate immunity.**

**Key words:** Chemokines · Macrophages · Toll-like receptors

## Introduction

The innate immune system is the first line of defense for protecting the host against invading microbial pathogens and involves a number of host defense systems, including chemokine-directed movement of inflammatory cells and pathogen detection *via* pattern recognition receptors (PRR) [1–3]. Chemokines bind to chemokine receptors, which are cell-surface G protein-coupled receptors (GPCR), leading to subsequent inflammatory signaling events involving various second messengers [4, 5]. Chemokine receptor ligation ultimately leads to cell signaling events *via* PI3K, MAPK/AP-1, and the NF-κB pathways [5–7]. Toll-like receptors (TLR) are major PRR that have evolved to recognize pathogen-associated molecular patterns (PAMP), which are conserved molecules of invading pathogens. After ligand binding, the majority of TLR recruit the adaptor protein MyD88, which together forms a complex with IL-1 receptor-associated kinase (IRAK) 4, IRAK1, and TNF receptor-associated factor 6 (TRAF6). TRAF6 subsequently activates TGF-β-activated kinase 1 (TAK1), which in turn activates MAPK/AP-1 and the IκB kinase (IKK)/NF-κB signaling pathways, resulting in a rapid innate immune response [1, 3]. Clearly, these two disparate groups of receptors evoke similar signaling pathways, but the manner in which chemokine receptors and TLR interact during innate immunity is poorly understood.

CC chemokine receptor 4 (CCR4), a specific receptor for CCL17 and CCL22, is generally recognized as a receptor expressed on Th2 cells [8–10]. However, increasing evidence has confirmed that CCR4 is expressed not only in Th2 cells but also in $CD4^+CD25^+$ T regulatory cells [11, 12], NK cells [13], platelets [14], DC [15], and macrophages [16, 17]. Additionally, CCR4 appears to be a key receptor in the innate immune response as

*Correspondence:* Dr. Steven L. Kunkel
e-mail: slkunkel@med.umich.edu

 

CCR4$^{-/-}$ mice are more resistant to LPS-induced sepsis [16, 18]. However, the cellular signal transduction events accounting for the protection and resistance in CCR4$^{-/-}$ mice have not been fully elucidated.

TLR9 selectively binds unmethylated CpG DNA motifs, which are common in the prokaryote genomes of most bacteria and DNA viruses and possess strong immunostimulatory activities [1, 19–21]. Unlike most PRR, TLR9 is mainly located in the endosome and cells must internalize CpG DNA into acidified endosomes in order to bind/activate TLR9. Further, IFN-γ-inducible protein 10 (IP-10 or CXCL10), a type 1 (Th1) chemokine, can also be produced by CpG DNA stimulation [22, 23]. TLR9 activation *via* CpG DNA ligand has been reported to play a pivotal role in the pathophysiology of sepsis [24] and severely injured patients with sepsis showed higher surface expression of TLR9 in B lymphocytes compared to healthy controls [25]. In a corollary animal model of experimental sepsis, elevated TLR9 expression in NKT cells and macrophages was associated with organ injury [26].

Macrophages can be differentially activated to express a specific protein phenotype with specialized functionalities in response to exposure to microenvironmental signals such as cytokines and microbial products. In the same fashion of the Th1/Th2 paradigm, the polarized macrophages can be divided into at least two groups, classically activated (or M1) and alternatively activated (or M2) macrophages [27–29]. Classical activation (M1) is mediated by the priming stimulus IFN-γ either alone or in concert with microbial trigger such as LPS. One of the hallmarks of M1 activation is the generation of NO by iNOS [27]. On the other hand, alternative activation (M2) is generally mediated by Th2 cytokines such as IL-4 and/or IL-13. Found in inflammatory zone-1 (FIZZ1), Arginase-1, and Mannose receptor are major markers of alternative macrophage activation. M2 macrophages have been shown to be correlated with resolution of excessive inflammation, wound repair, and promoting tumor progression, while M1 macrophages appeared to be significantly involved in pathogen defense [27]. We previously reported that peritoneal macrophages from CCR4$^{-/-}$ mice possess features of an M2 phenotype. However, intracellular activation circuits that M2 macrophages rely upon for signal transduction are not well understood.

In the present study, we explored the modulation of TLR9-mediated innate immunity in CCR4$^{-/-}$ mice and investigated the molecular mechanisms of this regulation. Our studies demonstrate that CpG DNA activation of bone marrow-derived macrophages (BMMΦ) recovered from CCR4$^{-/-}$ septic mice possess a dramatically altered signal transduction profile, as compared to their WT counterpart. Further, the signal-transduction pathways that are altered in the CpG DNA-challenged BMMΦ recovered from the CCR4$^{-/-}$ mice are nearly identical to the signaling modifications observed in alternatively activated BMMΦ derived from WT mice.

## Results

### CCR4$^{-/-}$ mice are resistant to polymicrobial sepsis and CpG DNA-induced shock

To specifically examine the role of CCR4 on TLR9-mediated innate immunity *in vivo*, we compared the susceptibility of WT and CCR4$^{-/-}$ mice to cecal ligation and puncture (CLP)-induced sepsis and to CpG/D-galactosamine (D-GalN) induced acute systemic inflammatory response. As shown in Fig. 1A, all WT mice died within 3 days after the initiation of polymicrobial sepsis induced by CLP, whereas 80% of CCR4$^{-/-}$ mice were alive after 7 days and were long-term survivors. In addition, all WT mice challenged intraperitoneally with CpG DNA (20 nmol) and D-GalN (20 mg) died within 24 h, whereas only one of seven CCR4$^{-/-}$ mice injected with CpG/D-GalN died (86% survival) (Fig. 1B). We also investigated the serum level of proinflammatory cytokines TNF-α and IL-6 after CpG/D-GalN stimulation and found that challenged CCR4$^{-/-}$ mice displayed significant suppression in serum levels of both TNF-α and IL-6 (Fig. 1C). Our results indicate that in the absence of CCR4 a protective effect on CLP-induced sepsis and CpG/D-GalN-induced shock was induced *in vivo* and correlated with the suppression of circulating proinflammatory cytokines.

### Impaired MAPK/AP-1 and PI3K/Akt pathways in BMMΦ from CLP-treated CCR4$^{-/-}$ mice

Macrophages have been shown to play a pivotal role during the evolution of a septic response [30] and studies have identified that severe sepsis induces a shift in these cells to an alternatively activated (M2) macrophage that possesses a dramatically changed

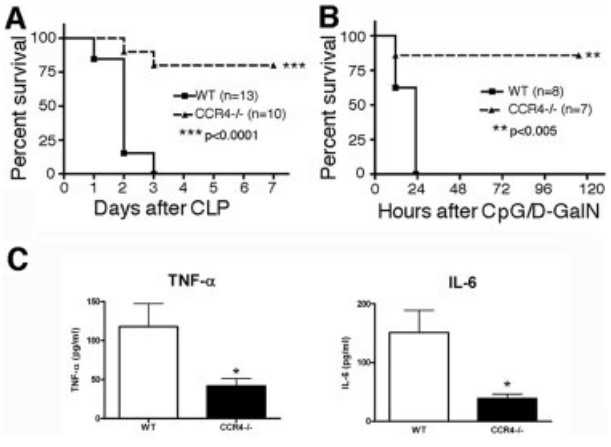


**Figure 1.** Resistance of CCR4$^{-/-}$ mice to both CLP-induced sepsis and CpG DNA-induced shock. (A) Age-matched WT ($n$ = 13) and CCR4$^{-/-}$ ($n$ = 10) mice were subjected to CLP surgery and survival was monitored for 7 days. (B) Age-matched WT ($n$ = 8) and CCR4$^{-/-}$ ($n$ = 7) mice were intraperitoneally injected with CpG DNA (20 nmol) and D-galactosamine (D-GalN) (20 mg) and survival was monitored for 5 days. (C) Serum concentration of TNF-α and IL-6 2 h after CpG DNA/D-GalN stimulation was measured by ELISA ($n$ = 5 per group). $^{*}p<0.05$; $^{**}p<0.005$; $^{***}p<0.0001$.

 

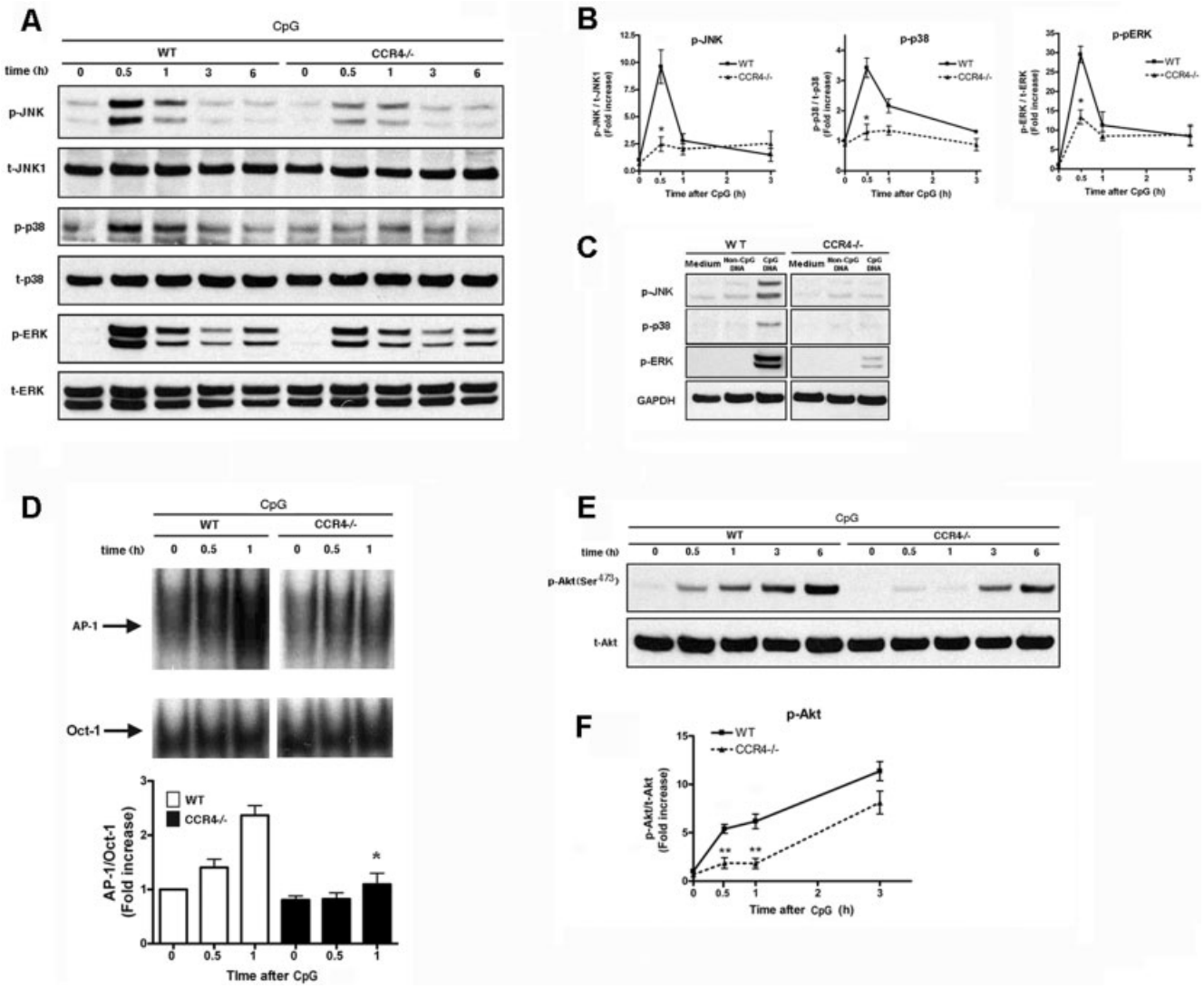


**Figure 2.** Impaired CpG DNA-induced MAPK/AP-1 and PI3K/Akt activation in CCR4$^{-/-}$ BMMΦ. BMMΦ from WT or CCR4$^{-/-}$ mice subjected to 48 h of CLP-induced sepsis were stimulated by CpG DNA (1 μM) for the indicated time and then whole-cell lysates or nuclear extracts were prepared. (A) The whole-cell lysates were subjected to immunoblotting using antibody for p-JNK, p-p38 MAPK, and p-ERK. Total (t)-JNK1, p38 MAPK, and t-ERK levels are shown as loading controls, respectively. The immunoblots are representative of three independent experiments. (B) The data of (A) at 0–3 h after CpG DNA stimulation were quantified by densitometric analysis. Data are shown as mean ± SEM of three independent experiments. (C) The whole-cell lysates of cells stimulated with CpG DNA or non-CpG DNA (1 μM) at 30-min time point were subjected to immunoblotting using antibody for p-JNK, p-p38 MAPK, and p-ERK. GAPDH levels are shown as loading controls. (D) The nuclear extracts were analyzed by EMSA with an AP-1-specific probe. The ubiquitous nuclear protein Oct-1 was used as a loading control. Data were quantified by densitometric analysis and are shown as mean ± SEM of three independent experiments. (E) The whole-cell lysates were subjected to immunoblotting using an antibody for p-Akt (pAkt Ser$^{473}$). Akt was used for loading control. (F) The data of (E) at 0–3 h after CpG stimulation were quantified by densitometric analysis. Data are shown as mean ± SEM of three independent experiments. *$p<0.05$; **$p<0.01$ as compared with WT BMMΦ at the same time point.

phenotype [16, 31]. Interestingly, CCR4$^{-/-}$ macrophages express an alternatively activated phenotype [16], which formed the basis to assess the protein expression system subsequent to TLR9 activation in BMMΦ from CLP-treated CCR4$^{-/-}$ mice. We assessed the signal-transduction pathways that potentially could be altered in these cells and initially focused on MAPK/AP-1 and PI3K/Akt pathways because of their crucial role in the induction of cytokines involved in severe acute inflammation [32, 33]. As compared to the 0 time point, the levels of phospho (p)-JNK, p-p38 MAPK, and p-ERK were increased in both WT and CCR4$^{-/-}$ BMMΦ at 0.5 h after CpG DNA challenge. However, the levels of these proteins were significantly lower in CCR4$^{-/-}$ BMMΦ compared to WT BMMΦ (Fig. 2A and B). These differences were more pronounced in BMMΦ from septic mice than from naive mice (data not shown). To exclude the TLR9-independent effects of CpG DNA, BMMΦ from CLP-treated WT andCCR4$^{-/-}$ mice were stimulated with control non-CpG DNA for 30 min. The levels of p-JNK, p-p38 MAPK, and p-ERK were not dramatically changed in non-CpG DNA-treated group as compared with non-stimulated group (Fig. 2C). The expression of AP-1, a downstream transcription

 

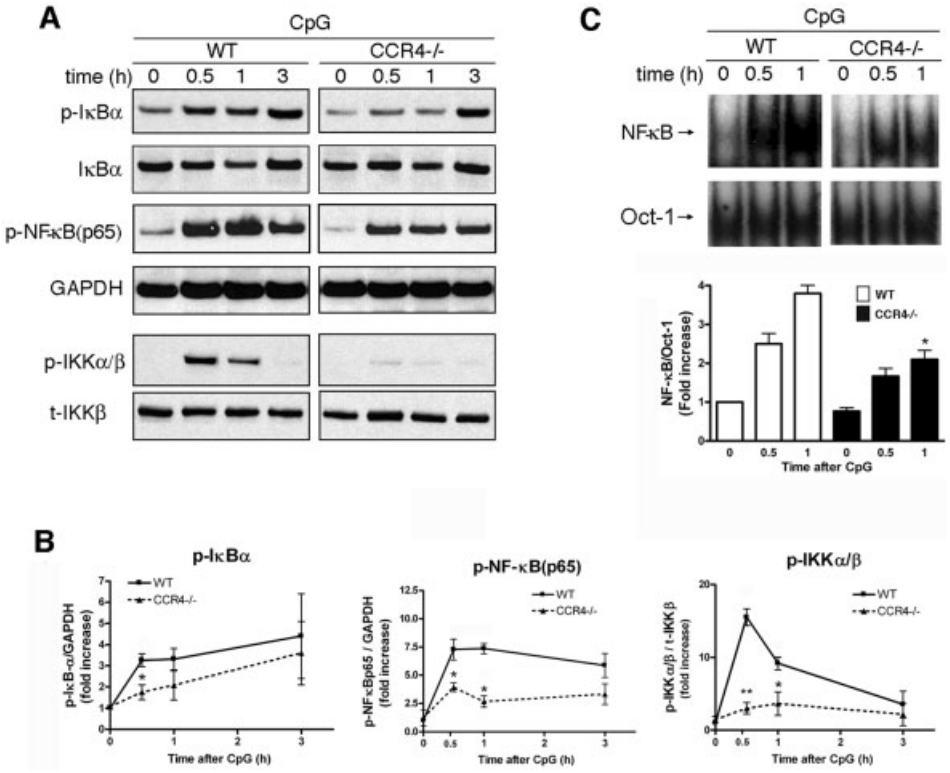


**Figure 3.** Impaired CpG DNA-induced activation of IKK/NF-κB pathway in CCR4$^{-/-}$ BMMΦ. BMMΦ from WT or CCR4$^{-/-}$ mice subjected to 48 h of CLP-induced sepsis were stimulated by CpG DNA (1 μM) for the indicated time and the whole-cell lysates or nuclear extracts were prepared. (A) The whole-cell lysates were subjected to immunoblotting using antibody for p-IκB-α, IκB-α, p-NF-κB (p65), and p-IKKα/β. GAPDH was used as a loading control for p-IκB-α, IκB-α, and p-NF-κB (p65), and total (t)-IKKβ was the loading control for p-IKKα/β. (B) The data of (A) were quantified by densitometric analysis. Data are shown as mean ± SEM of three independent experiments. (C) The nuclear extracts were analyzed by EMSA with an NF-κB-specific probe. Oct-1 was used as loading control. Data were quantified by densitometric analysis and are shown as mean ± SEM of three independent experiments. $^{*}p<0.05$; $^{**}p<0.01$ as compared with WT BMMΦ at the same time point.

factor of JNK, p38 MAPK and ERK, was consistently lower in CCR4$^{-/-}$ BMMΦ than in WT BMMΦ (Fig. 2D) (mean relative intensities at 1 h were 1.1 and 2.4, respectively). Interestingly, it has been previously shown that the unregulatedd expression of p-Akt in neutrophils from septic patients can correlate with the severity of sepsis-induced acute lung injury [32]. Here, we show that p-Akt was up-regulated after CpG DNA challenge and was significantly higher in BMMΦ from CLP-treated WT mice compared to CLP-treated CCR4$^{-/-}$ mice (Fig. 2E and F). Our results indicate that both MAPK/AP-1- and PI3K/Akt-signaling pathways were suppressed in CpG DNA-challenged BMMΦ recovered from CCR4$^{-/-}$ CLP-treated mice, as compared to WT mice.

## IKK/NF-κB pathway is altered in BMMΦ from CLP-treated CCR4$^{-/-}$ mice

We next focused on IKK/NF-κB pathway, another important pathway leading to cytokine expression during acute inflammation [32]. In this set of studies, we found that IκB-α expression levels were not dramatically altered in either macrophage population after CpG DNA stimulation. However, the expressions of p-IKKα/β, p-IκB-α, and p-NF-κB (p65) were observed in WT and CCR4$^{-/-}$ BMMΦ following CpG DNA stimulation and these levels were significantly lower in CCR4$^{-/-}$ BMMΦ (Fig. 3A and B). Consistent with these latter data, the NF-κB-DNA binding activities were increased in both WT and in CCR4$^{-/-}$ BMMΦ and the binding activity was lower in CCR4$^{-/-}$ BMMΦ from CLP-treated mice compared to WT BMMΦ from CLP-treated mice (Fig. 3C) (mean relative intensities at 1 h were 2.1 and 3.8, respectively). Together, these results suggest that the IKK/NF-κB pathway is impaired in macrophages from CLP-treated CCR4$^{-/-}$ mice.

## Modulated TLR9-mediated signaling in BMMΦ from CLP-treated CCR4$^{-/-}$ mice

Following CpG DNA recognition, TLR9 recruits the intracellular adapter molecule MyD88 that ultimately leads to the activation of MAPK/AP-1 and IKK/NF-κB pathways [2]. Since we showed that TLR9-mediated MAPK/AP-1 and IKK/NF-κB pathways were impaired in BMMΦ from CLP-treated CCR4$^{-/-}$ mice, we hypothesized that signaling molecules upstream of MAPK/AP-1 and IKK/NF-κB pathways might also be impaired in BMMΦ from these mice, including TLR9 itself. To investigate this hypothesis, MyD88-

 

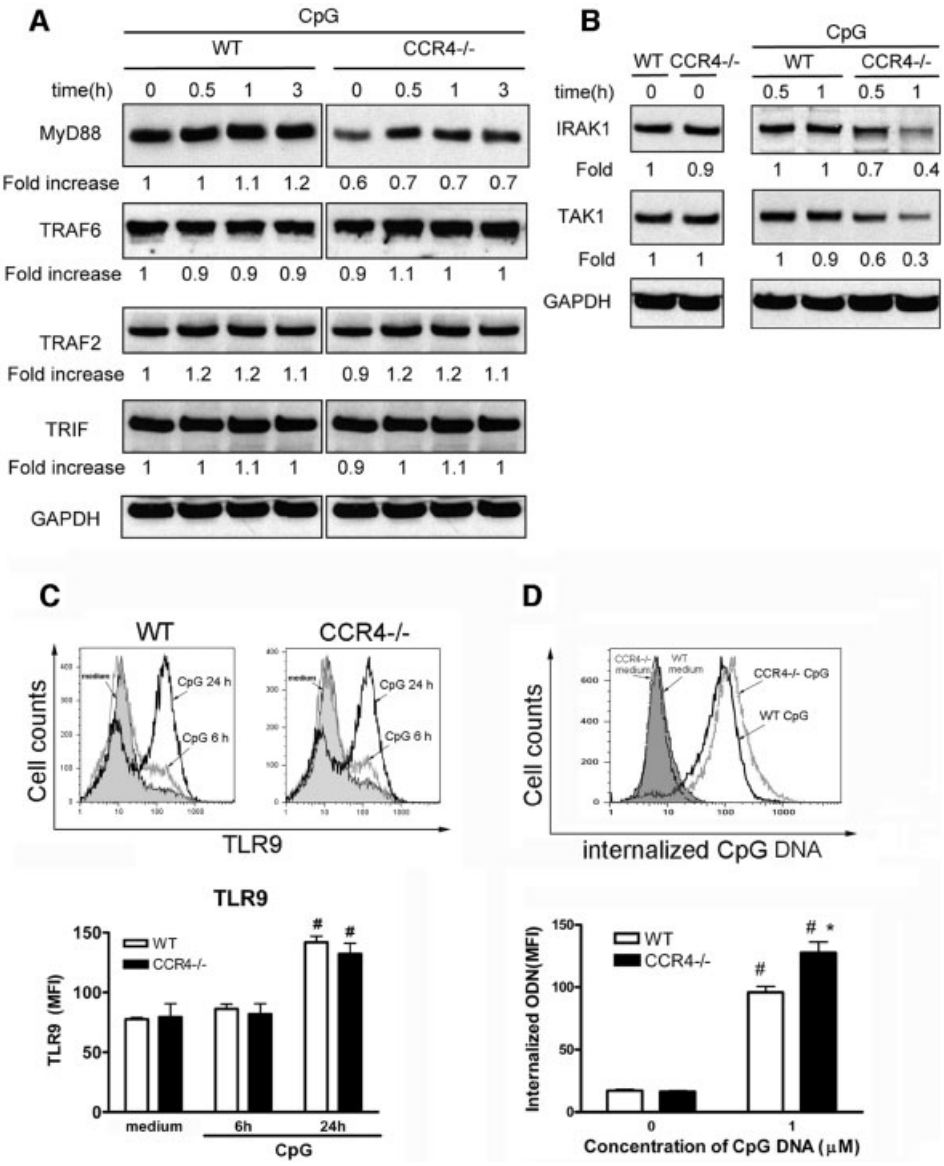


**Figure 4.** Modulated activation of TLR9-mediated signaling molecules in CCR4$^{-/-}$ BMMΦ. BMMΦ from WT or CCR4$^{-/-}$ mice subjected to 48 h of CLP-induced sepsis were stimulated by CpG DNA (1 μM) for the indicated times. (A, B) The whole-cell lysates were subjected to immunoblotting using antibody for MyD88, TRAF6, TRAF2, TRIF, IRAK1, and TAK1. GAPDH was used as a loading control. The immunoblots are representative of two independent experiments. The data of (A) and (B) were quantified by densitometric analysis. (C) After stimulation with CpG DNA for 6–24 h, the cells ($1 \times 10^6$) were stained with TLR9 antibody and the mean fluorescent intensity (MFI) of TLR9 was measured by flow cytometry ($n = 5$ per group). Representative histograms of three independent experiments are shown. $_{\#}p<0.05$ as compared with untreated cells (shown as medium) in same group. (D) After stimulation with FITC-labeled CpG DNA for 1 h, the cells ($1 \times 10^6$) were washed and FITC bounded on cell surface was quenched by trypan blue. Representative histograms of three independent experiments are shown. MFI of internalized CpG ODN was measured by flow cytometry ($n = 4$ per group). $_{\#}p<0.05$ as compared with untreated cells (shown as 0 μM CpG DNA) in the same group. $^{*}p<0.05$ as compared with WT BMMΦ in the same condition.

dependent signaling molecules in BMMΦ from CLP-treated WT and CCR4$^{-/-}$ mice were examined after stimulation with CpG DNA (Fig. 4). While the basal level of MyD88 in BMMΦ from CCR4$^{-/-}$ mice was lower than that from WT mice, stimulation with CpG DNA had no dramatic effect on subsequent MyD88 levels in either CCR4$^{-/-}$ or WT BMMΦ (Fig. 4A). Interestingly, the expression levels of IRAK1 and TAK1, but not of TRAF6, TRAF2 or Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF), were decreased in CLP-treated CCR4$^{-/-}$ BMMΦ after 1 h of CpG DNA stimulation (Fig. 4A and B). We next examined the extracellular and intracellular expression of TLR9 by flow cytometry (Fig. 4C). The mean fluorescent intensity of TLR9 significantly increased 24 h after CpG DNA stimulation in both WT and in CCR4$^{-/-}$ BMMΦ. However, the expression levels were not significantly different between WT and CCR4$^{-/-}$ BMMΦ. These results suggest that the CpG DNA-mediated MyD88-dependent signaling mole-

 

cules IRAK1 and TAK1 are decreased in CCR4$^{-/-}$ BMMΦ from CLP-treated mice compared to CLP-treated WT mice and this response is not due to altered expression of TLR9.

To determine whether the impairment of TLR9-mediated MAPK/AP-1, IKK/NF-κB, and PI3K/Akt pathways in CCR4$^{-/-}$ BMMΦ was due to decreased CpG DNA internalization, WT and CCR4$^{-/-}$ BMMΦ were stimulated with FITC-labeled CpG oligonucleotide (ODN), and the internalized FITC-CpG ODN was quantified (Fig. 4D). Interestingly, the level of internalized FITC-CpG ODN was higher in CCR4$^{-/-}$ BMMΦ than in WT BMMΦ, suggesting that the defects in TLR9-mediated signaling pathways in CCR4$^{-/-}$ mice were not due to inadequate amounts of CpG DNA internalization.

## Altered cytokine expression by CpG DNA in BMMΦ from CLP-treated CCR4$^{-/-}$ mice

As TLR9-mediated signaling pathways were impaired in CCR4$^{-/-}$ BMMΦ (Fig. 2 and 3), we hypothesized that TLR9-mediated expression of proinflammatory cytokines should be altered in CCR4$^{-/-}$ BMMΦ. To examine the role of CCR4 on CpG DNA-induced cytokine/chemokine expression, BMMΦ from CLP-treated WT and CCR4$^{-/-}$ mice were stimulated by CpG DNA for 6 and 24 h. The level of IL-12 p70 at 6 h was lower in CCR4$^{-/-}$ BMMΦ when compared to WT BMMΦ (Fig. 5). In addition, concentrations of TNF-α and CCL3, two other proinflammatory cytokines, were also suppressed in CCR4$^{-/-}$ BMMΦ at 24 h compared to levels in WT BMMΦ. The level of CXCL10, a Th1 cytokine, at 24 h was suppressed in CCR4$^{-/-}$ BMMΦ. Moreover, the level of CCL22, one of the CCR4 ligands, at 24 h was also suppressed in CCR4$^{-/-}$ BMMΦ (Fig. 5) as compared with WT BMMΦ, but CCL17, the other CCR4 ligand, was not detectable (data not shown).

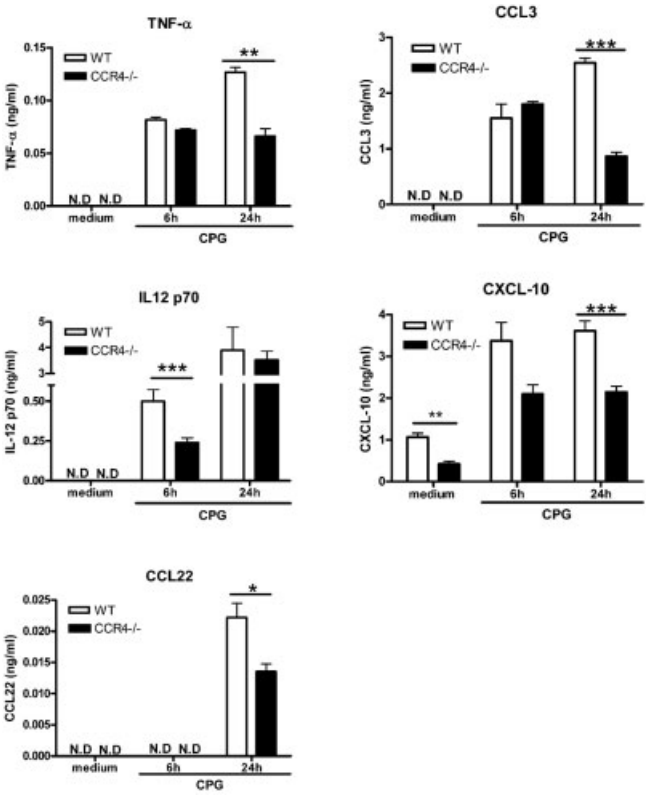


**Figure 5.** Impaired cytokine expression in CCR4$^{-/-}$ BMMΦ after CpG DNA treatment. Cell-free supernatants were obtained from the culture medium of BMMΦ from CLP-treated WT and CCR4$^{-/-}$ mice 6 and 24 h after CpG DNA stimulation or unstimulated (medium). The levels of TNF-α, CCL3, IL-12 p70, CXCL10, and CCL22 in the supernatants were measured by ELISA ($n$ = 4 per group). N.D: not detected. The results shown are representative of three individual experiments. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

## Modulated cytokine expression by inhibition of signaling molecules in WT BMMΦ

We demonstrated above that impaired TLR9-mediated signaling of the MAPK/AP-1, PI3K/Akt, and IKK/NF-κB pathways was accompanied by suppressed cytokine expression in CCR4$^{-/-}$ macrophages. Next, we determined if any of these pathways contribute to the decreased cytokine expression in CLP-treated CCR4$^{-/-}$ macrophages. BMMΦ from CLP-treated WT mice were preincubated with the relevant pharmacological inhibitors, stimulated with CpG DNA, and the subsequent levels of TNF-α, CCL3, and IL-12 p70 were measured. Inhibitors of Akt, JNK, and IKK, but not p38 MAPK suppressed the expression of TNF-α, CCL3, and IL-12 p70. By pretreatment with ERK inhibitor, the expression level of TNF-α was decreased, while CCL3 was not significantly changed, and IL-12 p70 was increased (Fig. 6A–C). These results suggest that p38 MAPK and ERK do not significantly contribute to impaired CCL3 and IL-12 p70 expression, and p38 MAPK does not contribute to impaired TNF-α expression. On the other hand, Akt, JNK, and/or IKK/NF-κB may be responsible for the impaired cytokine expression observed in CCR4$^{-/-}$ macrophages.

We demonstrated that PI3K/Akt appears to be one of the important pathways in TLR9-mediated signaling, however, little is known about the relationship between PI3K/Akt and other signaling pathways. To investigate the relationship between PI3K/Akt, MAPK, and IKK/NF-κB in macrophages, BMMΦ from CLP-treated WT mice were preincubated with medium (containing control levels of DMSO) or with the pharmacological inhibitor of Akt (LY294002), stimulated with CpG DNA, and the level of p-JNK, p-38 MAPK, p-ERK, p-IKKα/β, and p-IκB-α were measured. We first confirmed that p-Akt was suppressed by the Akt inhibitor (Fig. 6D) and the expression level of p-IκB-α, but not p-IKKα/β, was suppressed in LY294002-treated macrophages (Fig. 6E). On the other hand, the expression levels of p-JNK and p-p38 MAPK in LY294002-treated macrophages were comparable to the control level (Fig. 6F). The p-ERK level in the LY294002-treated macrophages was slightly higher or comparable to control level. These results suggest that PI3K/Akt pathway is an upstream positive regulator of IκB-α/NF-κB but not IKK, JNK, p38 MAPK, or ERK in macrophages from CLP-treated mice.

 

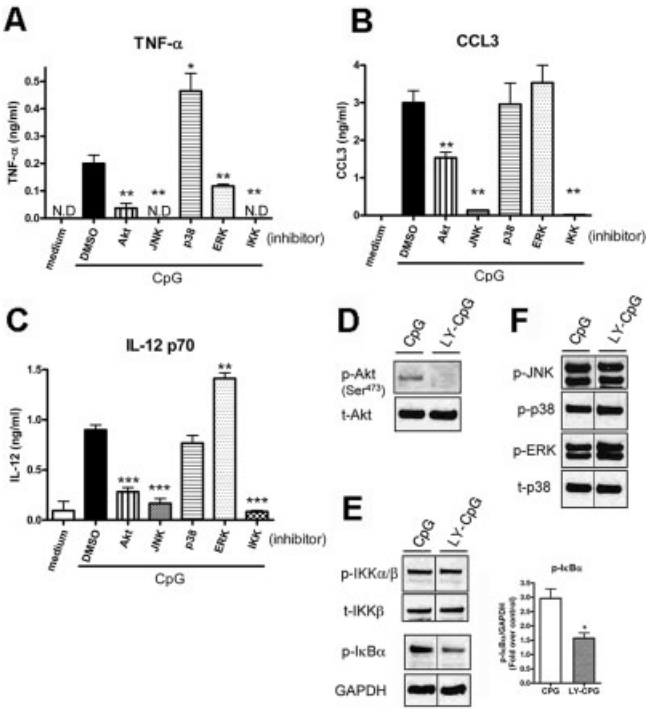


**Figure 6.** Pharmacological inhibitors alter CpG-induced cytokine expression in WT BMMΦ. (A-C) BMMΦ isolated from WT mice 48 h post-CLP surgery were treated with pharmacological inhibitors of Akt (LY294002, 10 μM), JNK (SP600125, 10 μM), p38 MAPK (SB203580, 10 μM), ERK (U0126, 10 μM), or IKK (Wedelolactone, 50 μM) for 30 min prior to stimulation with CpG DNA (1 μM) for 6 or 24 h. The levels of IL-12 p70 at 6 h, TNF-α and CCL3 at 24 h, were measured by ELISA. All inhibitors were diluted in DMSO. The samples treated with DMSO and stimulated with CpG DNA were used as a control (DMSO) group. Medium represents cells subjected to CLP-induced sepsis and then cultured in medium alone. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ as compared with the DMSO group. (D-F) BMMΦ from WT mice subjected to 48 h of CLP-induced sepsis were treated with DMSO (shown as CpG) or a pharmacological inhibitor of Akt (LY294002, 10 μM, shown as LY-CpG) for 30 min. Then the cells were stimulated with CpG DNA (1 μM) for 0.5–1 h (1 h for p-Akt and p-IκB-α, 0.5 h for others). The whole-cell lysates were subjected to immunoblotting using antibodies for p-Akt, p-IKKα/β, p-IκB-α, p-JNK, p-p38 MAPK, and p-ERK. Total (t)-Akt was used as loading control for p-Akt, p38 MAPK for each p-MAPK (p-JNK, p-p38 MAPK, and p-ERK), GAPDH for p-IκB-α, and t-IKKβ for p-IKKα/β. Data of p-IκB-α were quantified by densitometric analysis and are shown as mean ± SEM of three independent experiments. $^{*}p<0.05$.

### M2 macrophages show similar TLR9 signaling response as CCR4$^{-/-}$ macrophages

We previously showed that peritoneal macrophages from CCR4$^{-/-}$ mice exhibited a feature of alternatively activated (or M2) protein phenotype, but did not understand the mechanism [16]). To determine if the impaired TLR9-mediated signaling response in CLP-treated CCR4$^{-/-}$ macrophages is due to skewing toward an M2 phenotype, we examined whether BMMΦ from CLP-treated CCR4$^{-/-}$ mice express an alternatively activated phenotype. An assessment of the M2 protein markers, FIZZ1, Arginase-1, and Mannose receptor, were all markedly higher in CCR4$^{-/-}$ BMMΦ, as compared to WT BMMΦ (Fig. 7A). Previous study has shown that CCL17 and IL-10 inhibit classically activated macrophages generation from resident macrophages stimulated with CpG DNA [34]. To examine whether the cocktail of IL-4, IL-13, IL-10, and CCL17 can skew WT macrophages to M2 phenotype more efficiently than the cocktail of IL-4 and IL-13, BMMΦ from CLP-treated mice were incubated with IL-4 and IL-13, or IL-4, IL-13, IL-10, and CCL17, and the mRNA levels of M2 markers were measured. The mRNA levels of FIZZ1, Arginase-1, and Mannose receptor, were significantly higher in IL-4, IL-13, IL-10, and CCL17-treated BMMΦ than in IL-4 and IL-13-treated BMMΦ (Fig. 7B). Next, BMMΦ from WT CLP-treated mice were incubated with or without IL-4, IL-13, IL-10, and CCL17, and TLR9-mediated signaling responses were examined. The levels of p-JNK, p-p38 MAPK, p-ERK, p-Akt, p-IκB-α, p-NF-κB (p65), and p-IKKα/β were lower in M2-skewed BMMΦ compared to non-treated BMMΦ, and the levels of IRAK1 and TAK1 were totally suppressed (Fig. 7C). On the other hand, MyD88 was slightly increased. These results suggest that the impaired-TLR9-mediated signaling response in BMMΦ from CCR4$^{-/-}$ mice is, at least partially, because of their M2 phenotype.

## Discussion

Increasing evidence suggests that chemokine receptors and TLR work in a synergistic manner during innate immune responses. Previously, others and we have examined this linkage in CCR4$^{-/-}$ mice, which are resistant to LPS (TLR4 ligand) and $Pam_3Cys$ (TLR2 ligand) challenge [16, 18]. In our previous study, peritoneal macrophages from CCR4$^{-/-}$ mice exhibited an alternatively activated (M2) phenotype, and TLR4- and TLR2-mediated NF-κB signaling pathways were suppressed whereas p-p38 MAPK and p-JNK were conversely increased in peritoneal CCR4$^{-/-}$ macrophages [16]. Since TLR9 plays a key role in sepsis [24–26], we hypothesized that CCR4 could also modulate TLR9-mediated innate immune response in experimental sepsis and shock. In our study, we showed CCR4$^{-/-}$ mice were highly resistant to CLP-induced sepsis, which seems to be inconsistent with our previous study that administration of CCL22, one of the CCR4 ligands, is protective and inhibition of CCL22 is detrimental to CLP-induced sepsis [35]. However, previous studies have shown that both CCL22 and CCL17 may bind to different chemokine receptors in addition to CCR4 [36, 37]. Indeed, CCR8 gene level was 4.5-fold higher in CCR4$^{-/-}$ BMMΦ as compared with the level in WT BMMΦ (data not shown). Therefore, CCR4 deficiency does not necessarily result in the inhibition of CCL22 or CCL17 bioactivity. We also confirmed that CCR4$^{-/-}$ mice were resistant to CpG/D-GalN-induced shock likely *via* the suppression of certain proinflammatory cytokines, which contribute to the pathophysiology of acute systemic inflammatory response. However, the mechanisms that direct cytokine regulation in this system are not well understood.

We observed that TLR9-mediated cytokine expression and lethality in CCR4$^{-/-}$ mice were modulated in our experimental models of severe systemic inflammation, which led us to

 

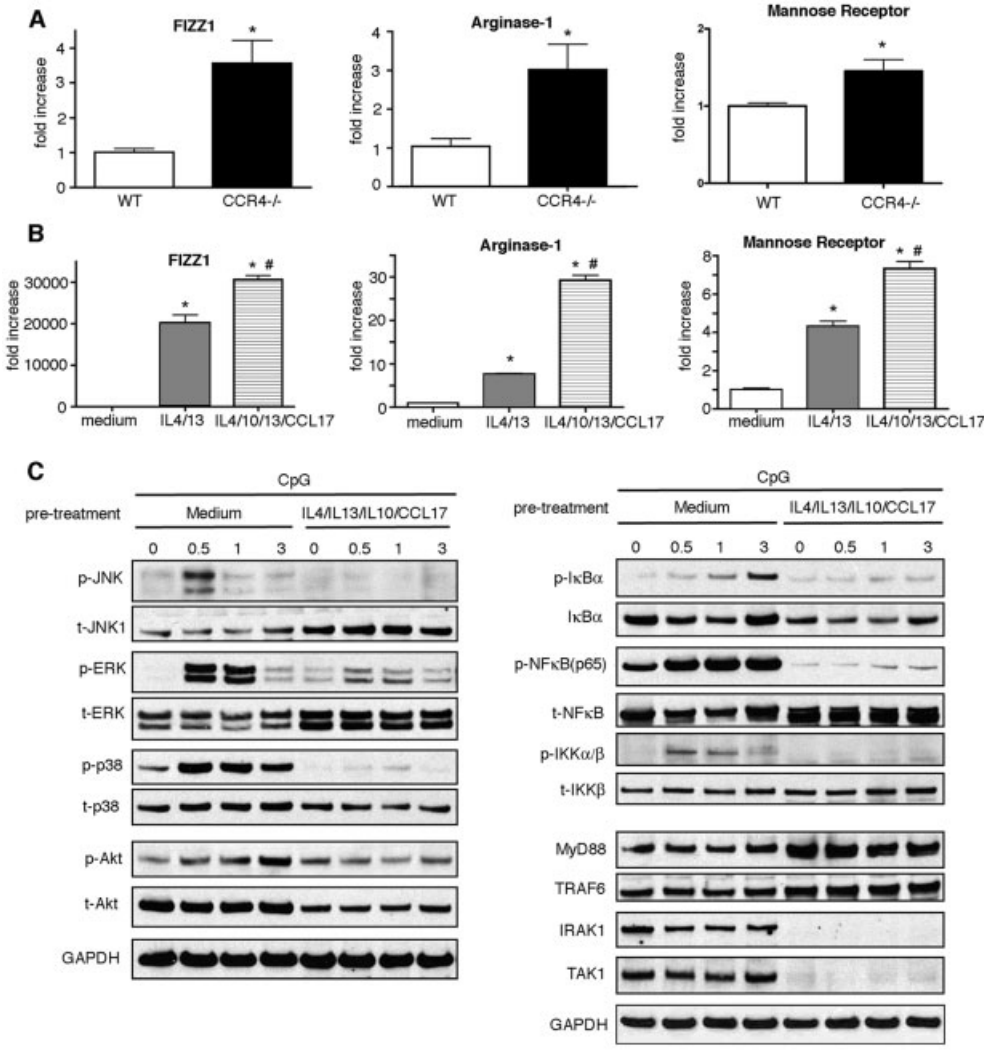


**Figure 7.** TLR9-mediated signaling responses in CCR4$^{-/-}$ BMMΦ and M2-skewed WT BMMΦ. (A) Gene expressions of FIZZ1, Arginase-1, and Mannose receptor [proteins expressed by alternatively activated (M2-skewed) cells] in BMMΦ from WT and CCR4$^{-/-}$ mice subjected to 48 h of CLP-induced sepsis. Each group: $n = 5$. The results shown are representative of three individual experiments. $^{*}p<0.05$. (B) BMMΦ from WT mice subjected to 48 h of CLP-induced sepsis were challenged with IL-4 and IL-13, or IL-4, IL-13, IL-10, and CCL17 for 48 h. Gene expression of FIZZ1, Arginase-1, and Mannose receptor were analyzed by quantitative real-time PCR. Each group: $n = 5$. The results shown are representative of three individual experiments. $^{*}p<0.05$ as compared with untreated BMMΦ (medium group). $_{\#}p<0.05$ as compared with the IL-4/IL-13-treated group. (C) BMMΦ from WT mice subjected to 48 h of CLP-induced sepsis were incubated with or without IL-4, IL-13, IL-10, and CCL17 for 48 h and then stimulated with CpG DNA (1 μM) for the indicated times. The whole-cell lysates were subjected to immunoblotting using antibody for the indicated molecules. GAPDH was used as a loading control. Alterations in signal-transduction pathway proteins from the WT macrophages skewed by cytokines to an M2 phenotype were similar to the alterations found in cells recovered from the CCR4$^{-/-}$ mice in the absence of the cytokine skewing.

hypothesize that TLR9-mediated intracellular signaling could be altered in cells recovered from CCR4$^{-/-}$ mice subsequent to experimental sepsis. In contrast to DC, which are well studied in TLR9 signaling [2, 3, 20], we focused on signaling pathways in macrophages, key cells in sepsis-induced pathology [30]. Since ligand binding to TLR9 results in activation of MAPK/AP-1- and IKK/NF-κB-signaling pathways [2], we initially focused on these intracellular activation events. MAPK pathways are highly conserved signaling pathways, which regulate various cellular functions [38]. The MAPK signaling pathways can be activated by many stimuli, such as an environmental stress, cytokines, and TLR ligands [2, 38, 39]. In this study, activation of JNK, p38 MAPK, and ERK were all significantly impaired in CLP-treated CCR4$^{-/-}$ macrophages after CpG DNA stimulation. Activity of AP-1, a downstream transcription factor of JNK, p38 MAPK, and ERK, was consistently suppressed in CCR4$^{-/-}$ macrophages. These results suggest that the absence of CCR4 attenuates TLR9-mediated MAPK/AP-1 signaling, which would help control the excessive inflammatory response that accompanies sepsis.

In our study, both the expression levels of p-IKKα/β, p-IκB-α, and p-NF-κB (p65) and the NF-κB DNA-binding activity were consistently suppressed in BMMΦ from CLP-treated CCR4$^{-/-}$ mice compared to CLP-treated WT mice. Thus, we would expect to find less IκB-α in the BMMΦ from WT mice than from CCR4$^{-/-}$ mice,

 

but instead we found comparable levels. The reason for this result is unclear, but possibly the peak of IκB-α degradation occurs at time points we did not measure or other IκB proteins such as IκB-β, IκB-ε, or IκBNS may participate in regulating NF-κB activity. Our results clearly suggest that the absence of CCR4 attenuates TLR9-mediated IKK/NF-κB signaling, which would also help control the excessive proinflammatory response that accompanies early sepsis.

PI3K are the lipid kinases, which phosphorylate phosphoinositides, are key enzymes involved in a number of important signaling events [40]. Class I PI3K catalyzes the production of phosphatidylinositol-3, 4, 5-triphosphate by phosphorylating phosphatidylinositol-4, 5-bisphosphate, resulting in activating downstream targets including Akt. The PI3K/Akt pathway is thought to participate in the TLR9-signaling pathway, as well as in TLR2- and TLR4-signaling pathways, and can act as either a positive or a negative regulator of TLR signaling [40–44]. Akt has also been shown to play a key role in sepsis [32]. In our investigation, we observed that the level of p-Akt was suppressed in BMMΦ from CLP-treated CCR4$^{-/-}$ mice compared with WT mice. These results indicate that the absence of CCR4 attenuates TLR9-mediated PI3K/Akt-signaling pathway during experimental sepsis.

After stimulation with CpG DNA, TLR9 recruits the adapter molecule MyD88, resulting in the activation of the MAPK/AP-1- and IKK/NF-κB-signaling pathways. Since our results showed that both MAPK/AP-1 and IKK/NF-κB pathways were suppressed after CpG DNA stimulation, we asked whether upstream TLR9-mediated MyD88-dependent signaling molecules and TLR9 itself, were also modulated. Indeed, we found that the expression of MyD88-dependent signaling molecules IRAK1 and TAK1 were decreased in macrophages from CCR4$^{-/-}$ mice. On the other hand, we showed the intensity of TLR9 expression was comparable between WT and CCR4$^{-/-}$ BMMΦ after CpG DNA challenge and further, that the intensity of internalized CpG DNA increased in CCR4$^{-/-}$ macrophages compared to WT macrophages. These results indicate that impaired TLR9-mediated signaling pathways such as MAPK/AP-1, PI3K/Akt, and IKK/NF-κB pathways are not due to decreases in TLR9 expression nor decreases in internalization of CpG DNA.

Macrophages can be phenotypically polarized and classified into at least two groups, classically activated (or M1) and alternatively activated (or M2) macrophages, that are defined by the proteins they express [27]. FIZZ1, Arginase-1, and Mannose receptor are major markers of alternative macrophage activation, while iNOS is a widely used marker of classically activated macrophages. Alternatively activated macrophages have been shown to be correlated with resolution of excessive inflammation, tumor development, and wound repair, while classically activated macrophages appeared to be significantly involved in pathogen defense [27]. Our results indicate that BMMΦ from CLP-treated CCR4$^{-/-}$ mice exhibit an alternatively activated M2 phenotype, which, at least in part, lead to impaired TLR9-mediated signaling response, contributing to resolution of excessive inflammation. We also confirmed that BMMΦ recovered from CLP-treated WT mice in which CCR4 was neutralized by CCR4 antibody during macrophage maturation have features of M2-polarized phenotype (data not shown). In addition, the signal-transduction pathway proteins altered in the CCR4$^{-/-}$ macrophages were similar to those altered in WT macrophages treated with M2 skewing cytokines (IL-4, IL-13, IL-10, and CCL17). The reason we used CCL17 plus other Th2 cytokines for M2 skewing was based on previous study demonstrating that CCL17 and IL-10 inhibit classically activated macrophages generation from resident macrophages stimulated with CpG DNA [34]. However, we did not see any dramatic differences between IL-4/IL-13/IL-10-treated and IL-4/IL-13/IL-10/CCL17-treated WT BMMΦ in TLR9-mediated signaling responses (data not shown).

We also assessed the expression of inflammatory cytokines TNF-α, CCL3, and IL-12 p70, as their expression can be controlled through TLR9-mediated MAPK/AP-1 and IKK/NF-κB pathways [2, 3]. The level of these inflammatory cytokines was significantly suppressed after CpG stimulation in BMMΦ from CLP-treated CCR4$^{-/-}$ mice. Therefore, we tried to determine which pathway was responsible for this impaired expression and found that the pharmacological inhibition of Akt, JNK, or IKK resulted in decreased expression of TNF-α, CCL3, and IL-12 p70. Although our results do not evaluate how much each pathway contributes to the suppressed cytokine expression, Akt, JNK, and/or IKK/NF-κB pathway are all candidates for driving the observed cytokine suppression in CCR4$^{-/-}$ macrophages. Studies were also initiated to explore the relationship among the PI3K/Akt, MAPK/AP-1 and IKK/NF-κB signaling pathways and subsequent cytokine expression *via* the use of signaling pathway inhibitors. These investigations showed that the inhibition of Akt led to decreased expression of p-IκB-α, but not p-JNK, p-p38 MAPK, and p-IKKα/β. These results suggest that Akt is an upstream positive signaling molecule of IκB-α/NF-κB, but not IKKα/β, JNK, and p38 MAPK.

To summarize the salient findings of this research, we have shown that CCR4$^{-/-}$ mice were highly resistant to CLP-treated sepsis and CpG DNA-induced shock *in vivo*, which was accompanied by suppressed proinflammatory cytokine expression. We also demonstrated that TLR9-mediated MAPK/AP-1, PI3K/Akt, and IKK/NF-κB signaling pathways were impaired, and that the expression of several TLR9-mediated MyD88-dependent molecules was suppressed in macrophages from CLP-treated CCR4$^{-/-}$ mice. Interestingly, these signaling pathways impairments were not due to decreases in TLR9 expression or in the internalization of CpG DNA. Alterations in these signaling molecules subsequently regulated the expression of macrophage-derived inflammatory cytokines (TNF-α, CCL3, IL-12 p70, CXCL10, and CCL22) from CLP-treated CCR4$^{-/-}$ mice. A potential mechanism that may account for the alterations in TLR9-signal transduction in the CCR4$^{-/-}$ mice is that regulators of G-protein signaling (RGS) may be altered in the CCR4$^{-/-}$ mice. The biological activity of RGS proteins have previously been shown to reach beyond only regulating GPCR and can interact with other intracellular activation systems [45, 46]. One potential mechanistic explanation for our data could relate to alterations in normal RGS activity in the CCR4$^{-/-}$ mice, which would influence TLR9 signaling. This concept is worthy of further exploration.

 

## Materials and methods

### Reagents and antibodies

Mouse CpG DNA (ODN) (HC4033) and control non-CpG DNA (HC4034) were purchased from Cell Sciences (Canton, MA). Mouse FITC-labeled CpG ODN was purchased from Invivogen (San Diego, CA). D-galactosamine was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies specific for p-SAPK/JNK, p-p38 MAPK (3D7), p38 MAPK, p-p44/42 (or p-ERK), ERK, p-Akt (Ser473), Akt (Ser473), p-IκB-α (Ser32), IκB-α, p-NF-κB (p65) (Ser536), NF-κB (p65), p-IKKα/β, IKKβ (L570), TRAF2, TAK1, and HRP-conjugated rabbit and mouse IgG antibody were purchased from Cell Signaling Technology (Danvers, MA). Antibodies for JNK1 (C17), IRAK1 (H273), and TRAF6 (H274) were obtained from Santa Cruz (Santa Cruz, CA). Antibodies for TRIF and GAPDH were purchased from Abcam (Cambridge, MA). Antibody for MyD88 was purchased from Millipore (Billerica, MA). Antibody for PE-conjugated TLR9 was purchased from Imgenex (San Diego, CA). Double-stranded NF-κB, AP-1, and Octomer-1 (Oct-1) consensus ODN probes were purchased from Promega (Madison WI). LY294002, SP600125, SB203580, U0126, and Wedelolactone were purchased from Calbiochem (San Diego, CA). Recombinant mouse IL-4, IL-13, IL-10, and CCL17/TARC were purchased from R&D systems (Minneapolis, MN).

### Mice

Female WT C57BL/6 mice (6 to 10 weeks old) were purchased from Taconic (Hudson, NY). Female CCR4$^{-/-}$ mice, also female C57BL/6, were provided by Tularik (Thousand Oaks, CA) and were generated as previously described [18]. Mice were housed under specific pathogen-free conditions, and all animal experiments were approved by the Animal Use Committee at the University of Michigan.

### *In vivo* experimental protocols

CLP surgery was performed as described [47]. Briefly, mice were anesthetized by i.p. injection of mixture of 2.25 mg of ketamine hydrochloric acid (Abbott Laboratories, Chicago, IL) and 150 μg of xylazine (Lloyd Laboratories, Shenandoah, IA). A 1-cm midline incision was made on the lower abdomen. The exposed cecum was ligated with a 3–0 silk suture, and punctured nine times using a 21-gauge needle. The cecum was returned to the peritoneal cavity, and the incision was closed with surgical staples. All mice received 1 mL of sterile saline s.c. for fluid resuscitation immediately after surgery. For the CpG DNA-induced shock model, mice were injected i.p. with CpG DNA (20 nmol) in the presence of D-GalN (20 mg).

### Bone marrow-derived macrophage culture

Bone marrow cells were collected from CLP-treated (48 h) WT and CCR4$^{-/-}$ mice by flushing their femurs and tibias with RPMI 1640 (Mediatech, Herndon, VA). Then, the cells were cultured with bone marrow medium (20% FCS, 30% L-cell supernatant, L-glutamine, penicillin/streptomycin (P/S) in RPMI 1640). On day 3, fresh bone marrow medium was added. On day 6, the cells were replated in RPMI 1640 medium. After overnight rest, cells were stimulated with CpG DNA for the indicated times. In some experiments, cells were pretreated with or without IL-4, IL-13, IL-10, and CCL17 for 48 h, and were stimulated with CpG DNA.

### Western blotting analysis

The cells were lysed at each time point after CpG DNA challenge in cell lysis buffer (Cell Signaling Technology), kept on ice for 20 min, and centrifuged at 15 000 × *g* for 15 min. The supernatant was collected and stored at –80°C until use. Total protein concentration of the samples was measured by bicinchroninic acid (BCA) protein assay (Pierce, Rockford, IL). Equal amounts (15–30 μg) of cell lysates were fractionated by SDS-PAGE (Nupage; Invitrogen, Carlsbad, CA). Then, the proteins were transferred onto nitrocellulose membrane (Invitrogen). After the overnight incubation with appropriate primary antibody, the membrane was counterstained with HRP-conjugated rabbit or mouse IgG antibody, visualized with enhanced chemiluminescence detection reagents (ECL; GE Healthcare, Piscataway, NJ). The images were analyzed using Image J 1.37v (National Institute of Health).

### Nuclear protein extraction

Nuclear protein extracts were prepared using CelLytic™ NuCLEAR™ Extraction Kit (Sigma-Aldrich) according to the manufacture's instruction. In brief, after CpG DNA stimulation, $1 \times 10^7$ cells were collected, washed with Dulbecco's PBS twice, resuspended in 1 × lysis buffer. After 15-min incubation, a detergent (0.6% IGEPAL) was added, and cells were vortexed for 10 s and centrifuged at 10 000 × *g* for 30 s. The pelleted nuclei were resuspended in extraction buffer and incubated on ice for 30 min. Samples were centrifuged at 20 000 × *g* for 5 min, and the supernatants containing nuclear protein were stored at –80°C until use.

### Electrophoretic mobility shift assay

Electrophoretic mobility shift assay was performed using the Gel Shift Assay System (Promega) as described previously [48]. The nuclear protein (5 μg) was incubated with $^{32}$P-labeled double-stranded ODN of AP-1, NF-κB, or Oct-1 (an ubiquitous transcription factor used as a loading control) in gel shift-binding buffer

 

(Promega), electrophoresed in 6% DNA Retardation Gel (Invitrogen) and visualized by autoradiography. The images were analyzed by Image J 1.37v (National Institute of Health).

### Enzyme-linked immunosorbent assay

For serum sample preparation of ELISA, mice were anesthetized by i.p. injection of mixture of 2.25 mg of ketamine hydrochloric acid and 150 μg of xylazine at 2 h after CpG DNA challenge, heart was exposed, and blood was collected by heart puncture, and was centrifuged at 7000 rpm for 8 min. The supernatants were stored at –20°C until use. Supernatants of BMMΦ for ELISA were collected at 6 and 24 h after CpG DNA challenge and stored at –20°C until use. Mouse TNF-α, IL-6, CCL3, IL-12 p70, CXCL10, and CCL22 were measured by standardized sandwich ELISA assays. The captured antibodies, detection antibodies, and recombinant cytokines were purchased from R&D Systems (Minneapolis, MN). The limits of ELISA detection for TNF-a, IL-6, and CCL22 were greater than 25, 25, and 10 pg/mL, respectively, and those for other cytokines were consistently greater than 50 pg/mL.

### Quantitative real-time PCR

RNA was isolated from BMMΦ by TRIzol according to manufacture's instruction (Invitrogen). Reverse transcription was performed to yield cDNA in a 25-μL reaction mixture containing 1X first strand (Invitrogen), 250 ng of oligo (dT) primer, 1.6 mM dNTP (Invitrogen), 5 U RNase inhibitor (Invitrogen), and 100 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen) at 38°C for 60 min and the reaction was stopped by incubating the cDNA at 94°C for 10 min. Real-time quantitative PCR analysis was performed by 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The sequences of the primers for Mannose receptor were 5′-CCATCGAGACTGCTGCTGAG-3′ (forward) and 5′-AGCCCTTGGGTTGAGGATCC-3′ (reverse) (SYBR Green). The sequences of the primers for FIZZ-1 were 5′- TCCAGCTAACTATCCCTCCACTGT-3′ (forward), 5′-GGCCCATCTGTTCATAGTCTTGA-3′ (reverse), and the probe was 6FAM-CGAAGACTCTCTCTTGC-TAMRA. Gene expression assays for Arginase-1 (Mm01190441_g1) were purchased from Applied Biosystems. GAPDH (Applied Biosystems) was used for loading control.

### Flow cytometry analysis

After fixation with 4% paraformaldehyde (Fisher Scientific), cells were permeabilized by Perm/Wash buffer (BD Biosciences) for 15 min, and then stained with PE-conjugated TLR9 antibody for 20 min. Analysis was performed with Cytomics FC500 Flow Cytometry Systems (Beckman Coulter, Fullerton, CA) and FlowJo 7.1.3 software (Tree Star, Ashland, OR).

### CpG oligodeoxynucleotide internalization

Analysis for CpG ODN internalization was performed as described in the literature [44, 49]. Briefly, cells were stimulated with FITC-labeled CpG ODN (1 μM) for 1 h, then washed with PBS, and the cell- surface FITC-CpG ODN was quenched by 0.1% trypan blue (Sigma-Aldrich). Internalized FITC-CpG ODN was analyzed with Cytomics FC500 Flow Cytometry Systems and FlowJo 7.1.3 software (Tree Star).

### Statistical analysis

For survival studies, the log-rank test was used. For other data, statistical significance was determined using the unpaired Student's *t*-test or ANOVA followed by Turkey's test for multiple comparisons as appropriate. Values are presented as mean ± SEM. The *p* values less than 0.05 were deemed statistically significant. Calculations were performed using the Prism 4.0 software program for Windows (GraphPad Software, San Diego, CA) or StatView II (Abacus Concepts, Berkeley, CA).

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*Abbreviations:* **BMMΦ:** bone marrow-derived macrophage(s) · **CCR4:** CC chemokine receptor 4 · **CLP:** cecal ligation and puncture · **D-GalN:** D-galactosamine · **FIZZ1:** found in inflammatory zone-1 · **GPCR:** G protein-coupled receptor(s) · **IKK:** IκB kinase · **IRAK:** interleukin-1 receptor associated kinase · **ODN:** oligonucleotide · **Oct-1:** Octomer-1 · **p-:** phospho · **P/S:** penicillin/streptomycin · **TAK1:** TGF-β-activated kinase 1 · **TRAF6:** TNF receptor-associated factor 6 · **TRIF:** Toll/IL-1R domain-containing adaptor-inducing IFN-β

*Full correspondence:* Dr. Steven L. Kunkel, Immunology Program, Department of Pathology, University of Michigan Medical School, Room 4017, Biomedical Sciences Research Building, 109 Zina Pitcher Place, Ann Arbor, MI 48109–2200, USA
Fax:+1–734–936–2756
e-mail: slkunkel@med.umich.edu

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