Measure an array of cytokines in COVID-19 samples with our multiplex flow cytometry assays.

Read our app note

BioLegend®

The Journal of Immunology

CCR4 Is a Key Modulator of Innate Immune Responses

Traci L. Ness, Jillian L. Ewing, Cory M. Hogaboam and Steven L. Kunkel

J Immunol 2006; 177:7531-7539; ;
doi: 10.4049/jimmunol.177.11.7531
http://www.jimmunol.org/content/177/11/7531

This information is current as of February 24, 2022.

References This article **cites 30 articles**, 14 of which you can access for free at:
http://www.jimmunol.org/content/177/11/7531.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

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

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2006 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

CCR4 Is a Key Modulator of Innate Immune Responses[1]

Traci L. Ness, Jillian L. Ewing, Cory M. Hogaboam, and Steven L. Kunkel[2]

CCR4 is recognized as a key receptor in Th2-associated immune processes, although very little is known about its role in innate immunity. Previous studies reported increased resistance to LPS-induced lethality in CCR4$^{-/-}$ mice compared with wild-type mice. This study demonstrates that CCR4$^{-/-}$ mice are similarly resistant to challenge with other TLR agonists, as well as bacterial peritonitis. Resistance was associated with enhanced early leukocyte recruitment, increased TLR expression, a skewed type 2 cytokine/chemokine profile, and improved bacterial clearance. Macrophages from CCR4$^{-/-}$ mice exhibited many features consistent with alternative activation, including elevated secretion of type 2 cytokines/chemokines and the found in inflammatory zone 1 (FIZZ1) protein. MyD88-dependent NF-κB signaling was significantly down-regulated in CCR4$^{-/-}$ macrophages, whereas p38 MAPK and JNK activation were conversely increased. These data stress the importance of CCR4 in macrophage differentiation and innate immune responses to pathogens, as well as the involvement of chemokine receptor expression in TLR signaling regulation. ***The Journal of Immunology,*** **2006, 177: 7531–7539.**

It is becoming increasingly clear that the successful operation of the host defense system is the culmination of several interactive processes that work together to eliminate foreign pathogens. Coordinated innate and acquired immune responses are required, and many secreted and cell-associated factors have been recognized as important mediators regulating and bridging these two arms of host defense. For many of these factors, the specific mechanism of action has yet to be fully elucidated.

Chemokines are a family of secreted proteins known primarily for their roles in leukocyte activation and chemotaxis. Their class-specific interaction with G protein-coupled receptors on target cells triggers complex signaling cascades resulting in degranulation, inflammatory mediator release, changes in cell shape, and cellular migration. Although required for innate and acquired host defenses against infection and trauma, chemokines and their receptors have been repeatedly implicated in the pathogenesis of chronic inflammatory diseases including allergy and autoimmune disease.

CCR4 is a high affinity receptor for CCL17 and CCL22 found on dendritic cells, macrophages, NK cells, platelets, and basophils, but it is predominantly known for its expression on T cells, especially of the Th2 phenotype (1). Increased expression of CCR4 and its ligands is associated with the pathogenesis of several diseases including pulmonary fibrosis (2, 3), eosinophilic pneumonia (4), hepatic inflammation (5), granuloma development (6), and diabetes (7). Each of these is characterized by the infiltration of CCR4^{+} T cells into affected sites. Neutralization of CCR4 ligands prevents T cell-mediated airway inflammation, liver injury, granuloma formation, and development of insulitis and diabetes in appropriate disease models (5–9). The role of CCR4 is less certain, as specific neutralizing Abs or antagonists have not been described in the literature.

CCR4$^{-/-}$ mice were derived in an effort to define the role of CCR4 in the development of Th2-mediated disease (10). Although CCR4 deletion has no apparent effect on Th2 differentiation in acute allergic models (10), it does affect differentiation in chronic disease (11). In the context of type 1 responses, CCR4$^{-/-}$ mice have diminished inflammatory responses. For example, cardiac allograft survival is significantly improved in CCR4$^{-/-}$ mice. A skewed population of infiltrating cells is comprised of increased numbers of CD8^{+} T cells and decreased CD4^{+} T and NK1.1^{+}CD3^{+} cells (12). CCR4$^{-/-}$ mice are also more resistant to the effects of LPS challenge, exhibiting reduced proinflammatory cytokines and peritoneal macrophage recruitment (10).

The purpose of this study was to further define the mechanism of increased LPS resistance, as well as resistance to other TLR agonists. Peritoneal macrophages from CCR4$^{-/-}$ mice exhibited many features characteristic of alternatively activated macrophages (aaMφ),[3] including skewed type 2 cytokine/chemokine and found in inflammatory zone 1 (FIZZ1) synthesis. MyD88-dependent NF-κB signaling was significantly inhibited in CCR4$^{-/-}$ macrophages, whereas p38 MAPK and JNK pathways were increased. These studies provide further evidence that despite its well-recognized role as a director of adaptive T cell driven responses, CCR4 is also an essential player in innate immunity.

Materials and Methods

In vivo studies

Male wild-type (WT) C57BL/6 mice (6–8 wk) were purchased from Taconic Farms. CCR4$^{-/-}$ mice were provided by Tularik and were generated as previously described (10). Mice were bred and housed under specific pathogen-free conditions, and all animal experiments were approved by the Animal Use Committee at the University of Michigan (Ann Arbor, MI).

Mice received a single dose of LPS (*Escherichia coli* O55:B5; Sigma-Aldrich) i.p. and survival was monitored. In other studies, mice were given 10 mg/kg LPS, 50 μg of Pam_3Cys-Ser-$(Lys)_4$ (Pam_3Cys; EMC Microcollections), or 4×10^8 *E. coli* (O86a:K61; American Type Culture Collection) i.p. and later bled and subjected to 2 ml of peritoneal lavage. Serum

Department of Pathology, University of Michigan, Ann Arbor, MI 48109

Received for publication July 10, 2006. Accepted for publication September 4, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants HL74024, HL31237, and HL35276 (to S.L.K.).

[2] Address correspondence and reprint requests to Dr. Steven L. Kunkel, University of Michigan, 109 Zina Pitcher Place, Biomedical Sciences Research Building, Room 4698, Ann Arbor, MI 48109-2200. E-mail address: slkunkel@med.umich.edu

[3] Abbreviations used in this paper: aaMφ, alternatively activated macrophage; ALT, alanine transaminase; AST, aspartate transaminase; FIZZ1, found in inflammatory zone 1; IRAK1, IL-1R associated kinase 1; Pam_3Cys, Pam_3Cys-Ser-$(Lys)_4$; TAM, tumor-associated macrophage; TRAF6, TNFR-associated factor 6; WT, wild type.

Copyright © 2006 by The American Association of Immunologists, Inc.

0022-1767/06/$02.00

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

Table I. *Custom primer and probe sequences used for real time RT-PCR analysis*[a]

Target	Primers (5′–3′)	Probes (5′-FAM and 3′-TAMRA)
TLR2	Fwd - GCCACCATTTCCACGGACT Rev - GGCTTCCTCTTGGCCTGG	TGGTACCTGAGAATGATGTGGGCGTG
TLR4	Fwd - CAGAACTTCAGTGGCTGGATTTATC Rev - GGGTTTCCTGTCAGTATCAAGTTTG	SYBR Green
TLR6	Fwd - GAATGTGACCCTCCAGCACAT Rev - AGTTTAACCGAGCACTTCCAGG	SYBR Green
TLR9	Fwd - AGCTGAACATGAACGGCATCT Rev - TGAGCGTGTACTTGTTGAGCG	SYBR Green
FIZZ1	Fwd - TCCAGCTAACTATCCCTCCACTGT Rev - GGCCCATCTGTTCATAGTCTTGA	CGAAGACTCTCTCTTGC
T1/ST2	Fwd - GCAATTCTGACACTTCCCATGTAT Rev - CCAGACCCCAGGACGATTTA	SYBR Green

[a] Fwd, Forward; Rev, reverse.

aspartate transaminase (AST) and alanine transaminase (ALT) were measured by the Clinical Pathology Laboratory at the University of Michigan. CFU levels and cytokine/chemokine concentrations were determined from serum and lavage fluid, as previously described (13). After RBC lysis, RNA was isolated from peritoneal cells using the TRIzol (Invitrogen Life Technologies) method and analyzed by quantitative RT-PCR (14). Custom primers and probes are listed in Table I, whereas all others were obtained from Applied Biosystems. In the absence of a specific probe, SYBR Green was used. In some experiments, peritoneal cells were stained with Abs to CD11b (BD Pharmingen), F4/80 (Serotec), TLR2 (eBioscience), TLR4 (Imgenex), TLR9 (Abcam; extracellular and intracellular staining), and ST2 (MD Biosciences) using standard procedures. Analysis was performed with a Beckman Coulter Cytomics FC 500 and FlowJo 6.3 software (Tree Star).

Macrophage stimulation

Peritoneal macrophages were collected from mice and cultured at 10^6/ml DMEM (with 5% FCS, L-glutamine, and penicillin/streptomycin) as previously described (13), after which nonadherent cells were removed at 1 h. RNA was isolated or cells were rested overnight. Macrophages were treated with medium or 1 μg/ml LPS for 4–24 h or 100 ng/ml IL-4 (R&D Systems), IL-13, or IL-4 plus IL-13 for 4–48 h. Cytokine/chemokine production in cell-free supernatants was measured by ELISA, whereas RNA was subjected to quantitative RT-PCR as described above.

Signaling pathway analyses

NF-κB. Thioglycolate-elicited cells were obtained as previously described (13). Macrophages were plated, rested overnight, and treated with medium, 2.5 μg/ml Pam_3Cys, or 1 μg/ml LPS for 4 h (time of peak NF-κB activation; data not shown). Nuclear extracts were prepared and analyzed using a TransAM NF-κB p65 kit (Active Motif) as previously described (13).

p38 MAPK/JNK. Resident peritoneal macrophages were isolated, plated (10^5/well), rested overnight, and treated with medium, 2.5 μg/ml Pam_3Cys, or 1 μg/ml LPS. Reactions were stopped at 30 min (peak phosphorylation; data not shown), and processed according to manufacturer's instructions. Fast activated cell-based ELISA kits (Active Motif) measured p38 MAPK and JNK phosphorylation. Absorbance was normalized to the number of cells per well.

Signaling intermediates. Resident peritoneal macrophages were stimulated in vitro with 1 μg/ml LPS for 0–60 min. Some were pretreated with 100 ng/ml IL-4 plus IL-13 for 48 h before the addition of LPS. Lysates were made (50 mM HEPES, 1 mM $MgCl_2$, 10 mM EDTA, 1% Triton X-100 (pH 6.4), and Complete proteinase inhibitors (Roche Diagnostics)) and subjected to SDS-PAGE with immunostaining. Abs against murine MyD88 (Abcam), IL-1R-associated kinase 1 (IRAK1; Santa Cruz Biotechnology), TNFR-associated factor 6 (TRAF6; Abcam), IκBα (Santa Cruz Biotechnology), and pSer32-IκBα (Cell Signaling) were used with anti-rabbit IgG-HRP (Jackson ImmunoResearch Laboratories) and a SuperSignal West Pico chemiluminescent substrate kit (Pierce) to measure expression. β-Actin was used as a loading control. Quantity One software (Bio-Rad) was used to measure band intensity. Expression was reported as fold difference relative to untreated WT cells.

Statistical analyses

For survival studies, the log-rank test was used. All other data are shown as the mean ± SEM and were subjected to the unpaired Student's *t* test. Values of $p < 0.05$ (*), $p < 0.005$ (**), and $p < 0.0005$ (***) are indicated for significant differences between WT and $CCR4^{-/-}$ treatment groups.

Results

$CCR4^{-/-}$ mice are more resistant to TLR agonist and bacterial challenge

As our laboratory has demonstrated phenotypic differences for $CCR4^{-/-}$ mice in other murine models of disease, we were interested in the mechanism of LPS resistance previously reported for these mice (10). WT and $CCR4^{-/-}$ mice were challenged i.p. with 1–100 mg/kg LPS (Fig. 1*A*). High-dose challenge was lethal to WT and $CCR4^{-/-}$ mice; however, mortality was significantly delayed in $CCR4^{-/-}$ mice. An intermediate dose (10 mg/kg LPS) killed all WT mice by 72 h, whereas only 20% of $CCR4^{-/-}$ mice were affected at this time. After 6 days, 60% of $CCR4^{-/-}$ mice survived, indicating that the protection was long-lasting. A 1 mg/kg LPS dose was not lethal for either strain. The intermediate dose (10 mg/kg) was used for further studies involving in vivo LPS administration. In an extension of these studies, challenge with Pam_3Cys, a synthetic TLR2 agonist, induced tissue pathology in WT mice. Serum levels of AST and ALT were significantly elevated in WT but not $CCR4^{-/-}$ mice 24 h after i.p. injection (Fig.

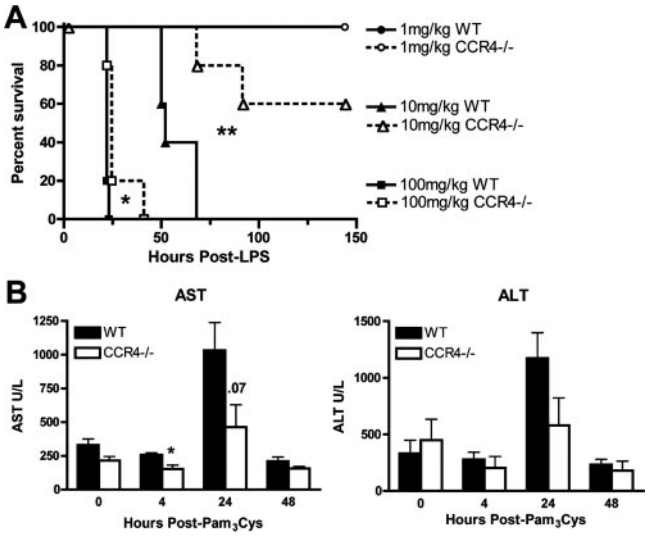

FIGURE 1. $CCR4^{-/-}$ mice are protected against the detrimental effects of in vivo TLR agonist challenge. *A*, WT and $CCR4^{-/-}$ mice were given 1, 10, or 100 mg/kg LPS i.p. and survival was followed for 6 days. *B*, Mice received 50 μg of Pam_3Cys i.p. and blood was collected at 0, 4, 24, or 48 h after challenge. Serum AST and ALT levels were measured. All experiments had 4–6 mice per group. Data represent similar results from two sets of independent studies. *, $p < 0.05$; **, $p < 0.005$.

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

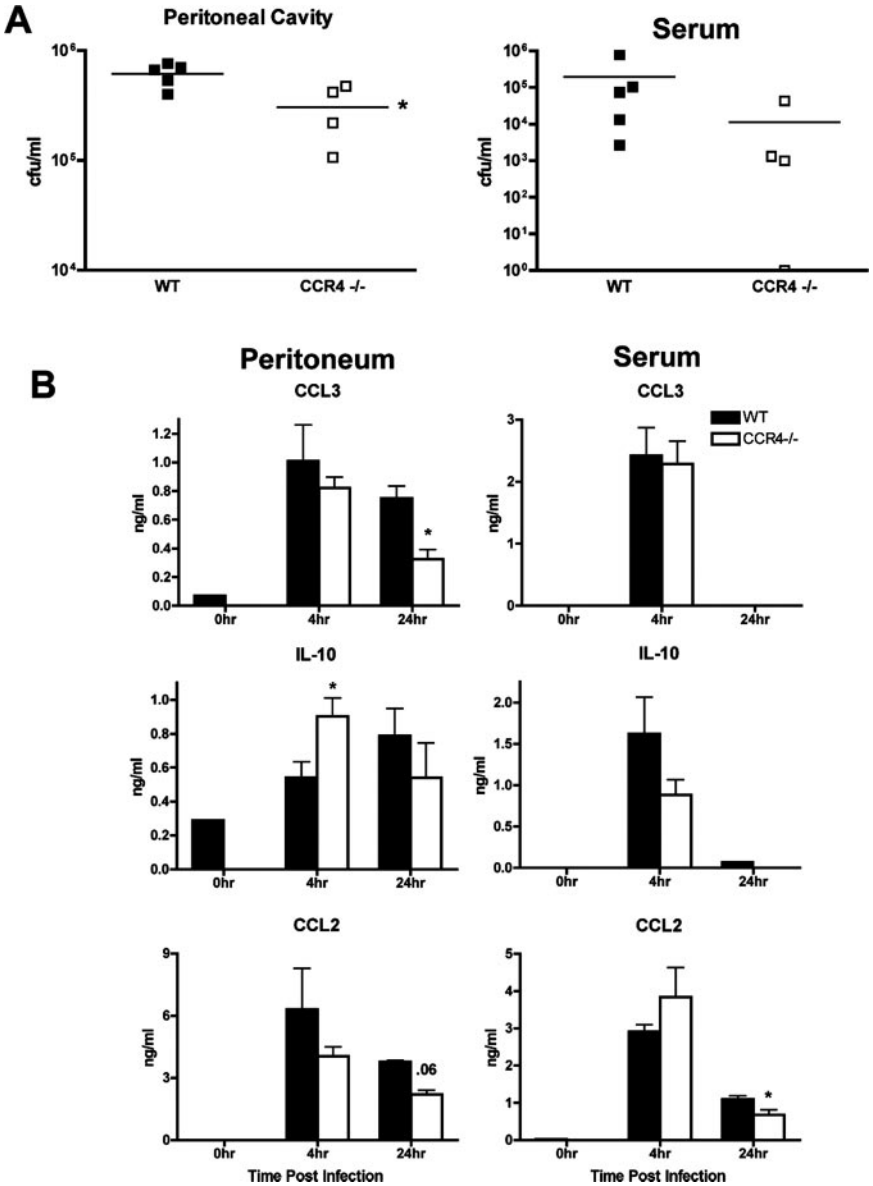

FIGURE 2. CCR4$^{-/-}$ mice are more resistant to bacterial peritonitis. WT and CCR4$^{-/-}$ mice were infected i.p. with 4×10^8 *E. coli*. Blood and peritoneal lavages were collected from mice at 0, 4, or 24 h after infection. *A*, Bacterial load was measured and expressed as CFU per milliliter. The horizontal bar indicates the mean for each group. *B*, Cytokine and chemokine concentrations were measured by ELISA. Each group contained 4–6 mice. *, $p < 0.05$.

1*B*), indicating that knockout mice have unique resistance to multiple bacterial Ags.

LPS administration initiates a "cytokine storm" characterized by high levels of inflammatory cytokines, especially TNF-α (15). rTNF-α alone can recapitulate many of the physiologic changes induced by LPS or sepsis, including fever, alterations in blood pressure, and weight loss (16). Thus, it was of interest to determine whether resistance to TLR agonist challenge in CCR4$^{-/-}$ mice was related to hyporesponsiveness to TNF-α. A 50-μg TNF-α i.v. challenge was equally lethal to WT and CCR4$^{-/-}$ mice (data not shown), suggesting that the protection against TLR agonists was not due to a defect in TNF-α responsiveness.

The next question we addressed was whether immune responses protecting CCR4$^{-/-}$ mice against the effects of Ag challenge also protected these mice against a live infection. Accordingly, WT and CCR4$^{-/-}$ mice were given 4×10^8 CFU of a pathogenic strain of *E. coli* i.p., and bacterial loads were measured 24 h after infection (Fig. 2*A*). CCR4$^{-/-}$ mice had significantly less bacteria at the site of infection and more than a log reduction in systemic levels as compared with WT mice. Similarly, bacterial levels in the liver and lung were lower in CCR4$^{-/-}$ vs WT mice (data not shown). Infection altered the local and systemic concentrations of several cytokines and chemokines. Fig. 2*B* illustrates the cytokines/chemokines that had differential WT and CCR4$^{-/-}$ expression. Compared with WT mice, CCR4$^{-/-}$ mice produced significantly more peritoneal IL-10 early after infection and less proinflammatory CCL3 at later times. Serum CCL2 was higher in CCR4$^{-/-}$ mice at 4 h but significantly less than WT levels at 24 h. Reductions in CCL2 and CCL3 secretion from CCR4$^{-/-}$ mice were likely due to both decreased bacterial load and the anti-inflammatory effects of enhanced IL-10 production.

Early recruitment of TLR-expressing cells is enhanced in LPS-treated CCR4$^{-/-}$ mice

TLRs are a pivotal group of pattern recognition receptors responsible for sensing the presence of pathogens and initiating immune responses. Therefore, TLR expression was evaluated on cells recruited to the peritoneal cavity in response to 10 mg/kg LPS (Fig. 3*A*). Basal levels of TLR transcripts were similar between strains, with reduced TLR4 expression in CCR4$^{-/-}$ cells. TLR2 was significantly up-regulated on WT peritoneal cells at 1–2 h after LPS treatment, whereas no changes were seen for other TLRs assayed.

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

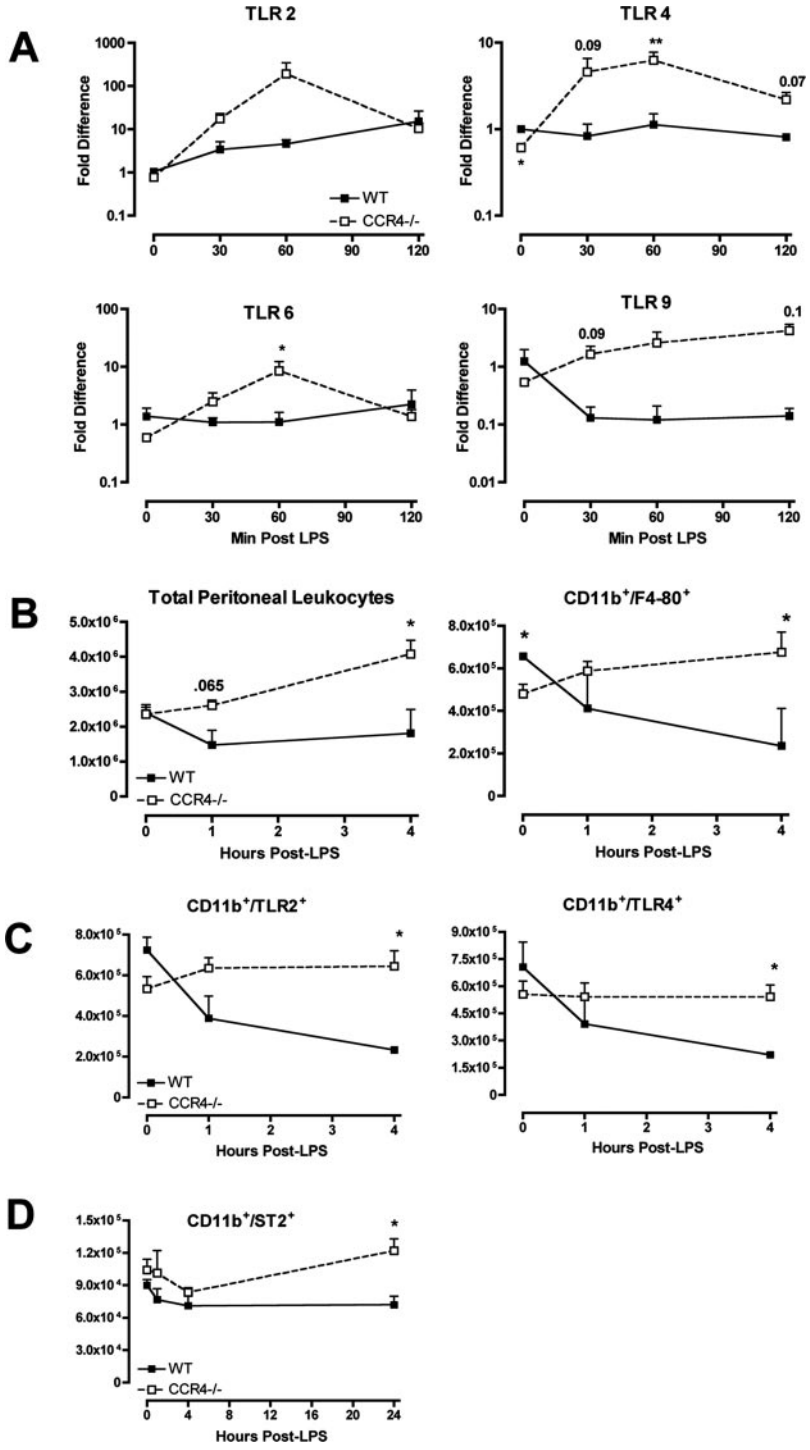

FIGURE 3. CCR4$^{-/-}$ mice have increased numbers of TLR-expressing cells after LPS challenge in vivo. WT and CCR4$^{-/-}$ mice ($n = 3$–5) were given 10 mg/kg LPS i.p. Peritoneal cells were collected at 0–120 min or 0–4 h post-LPS challenge for RNA (*A*) and flow cytometry (*B–D*) analyses, respectively. *A*, Real-time quantitative PCR was used to analyze TLR expression after LPS challenge. TLR expression was normalized to individual GAPDH levels and converted to fold difference relative to basal WT expression. *B–D,* Peritoneal cell totals were determined for each mouse. Cells (5×10^5) were double stained with Abs to murine CD11b and one of the following: F4–80 (*B*), TLR2 or TLR4 (*C*), or ST2 (*D*). Macrophage-like cells were gated and isotype Abs were used as controls. The results shown represent data from one of at least three independent experiments. *, $p < 0.05$; **, $p < 0.005$.

Conversely, significant increases in TLR2, 4, 6, and 9 expression were noted in CCR4$^{-/-}$ cells as early as 30 min post-LPS treatment, with peak expression at 1 h. Peak expression in CCR4$^{-/-}$ cells was significantly higher than that observed for cells from WT mice. These data suggest that TLRs are immediately induced in CCR4$^{-/-}$ cells, contributing to a rapid response to pathogen challenge.

To characterize the cellular response, we assessed the kinetics of leukocyte recruitment into the peritoneum after LPS challenge. Equal numbers of resident cells were detected in untreated WT and CCR4$^{-/-}$ mice (Fig. 3*B*). Significantly higher numbers of leukocytes were seen in CCR4$^{-/-}$ mice as compared with WT mice early after treatment (4 h), but no differences were observed at later times (24–48 h; data not shown). The CD11b$^+$F4-80$^+$ cell population was higher in CCR4$^{-/-}$ than WT mice at 4 h, and CCR4$^{-/-}$ mice had significantly more infiltrating CD11b$^+$TLR2$^+$ and CD11b$^+$TLR4$^+$ cells (Fig. 3*C*). Our findings demonstrate that CCR4$^{-/-}$ mice mobilize LPS-responsive leukocytes to the site of challenge more rapidly and aggressively than WT mice. Increases in TLR expression and leukocyte recruitment typically lead to a robust inflammatory response, yet CCR4$^{-/-}$ mice are LPS resistant, prompting further investigation of the phenotype of CCR4$^{-/-}$ peritoneal cells.

ST2, the receptor for IL-33 (17) and a negative regulator of TLR4 (18), has been mainly studied in association with its specific

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

expression on Th2 cells; however, it is also found on fibroblasts, mast cells, and macrophages. Interestingly, significantly more $CD11b^+ST2^+$ cells were recruited to the peritoneal cavities of $CCR4^{-/-}$ vs WT mice 24 h post-LPS (Fig. 3*D*). ST2 is critical to Th2-mediated responses, as neutralization increases IFN-γ and suppresses IL-4 and IL-5 production (19). Because aaMφ secretes several Th2-related cytokines/chemokines, ST2 may also be a specific marker for these cells (20).

$CCR4^{-/-}$ macrophages exhibit features of an alternatively activated phenotype

TLR expression was significantly higher on peritoneal cells from LPS-treated $CCR4^{-/-}$ mice as compared with similarly treated WT mice. Elevated expression was partially due to increased cellular recruitment, but TLR transcript levels were also increased in total peritoneal cells. Because resident macrophages are key to initiating an immediate innate response, peritoneal macrophages were isolated from naive mice and analyzed in vitro. $CCR4^{-/-}$ macrophages expressed higher basal levels of TLR2 and TLR4 transcripts than WT macrophages (Fig. 4*A*). Furthermore, these cells secreted significantly more CCL2 (Fig. 4*B*) and CCL6 (data not shown) than WT cells. Following in vitro LPS challenge, macrophages up-regulated several cytokines and chemokines. No differences were noted in CCL3 production, but $CCR4^{-/-}$ macrophages produced significantly more CCL2 than WT cells. Higher CCL17 concentrations were also detected, which may have been due to the absence of CCR4-mediated feedback regulation. FIZZ1 secretion was significantly higher in $CCR4^{-/-}$ cells than in WT cells but was not affected by LPS treatment.

CCL2, CCL17, and FIZZ1 expression is characteristic of aaMφ (21, 22). Increased secretion of these factors from $CCR4^{-/-}$ macrophages prompted investigation of IL-4- and/or IL-13-driven alternative activation of these cells. Basal IL-10, CCL2, CCL17, and FIZZ1 transcript levels were higher in $CCR4^{-/-}$ macrophages than in WT cells (Fig. 5). With the exception of CCL17, higher protein levels were also detected. IL-4/IL-13-induced increases in CCL2, CCL17, and FIZZ1 expression confirmed alternative activation of WT macrophages, although concentrations of these factors were significantly higher from $CCR4^{-/-}$ macrophages. Although its expression was not induced by cytokine treatment, IL-10 concentrations remained significantly higher from $CCR4^{-/-}$ vs WT macrophages. These data support the concept that $CCR4^{-/-}$ mice have a resident population of peritoneal macrophages with many features of aaMφ.

NF-κB signaling is inhibited, but p38 MAPK and JNK pathways are increased in $CCR4^{-/-}$ macrophages

Most TLR-dependent inflammatory cytokine/chemokine synthesis is dependent on NF-κB. Phosphorylation of the inhibitor protein, IκBα, releases NF-κB into the nucleus where it initiates the transcription of inflammatory mediators. NF-κB p65 was measured in WT and $CCR4^{-/-}$ nuclear extracts in an effort to evaluate the overall activation of this pathway. Thioglycollate was used to augment macrophage recruitment to the peritoneal cavity to obtain sufficient numbers of cells for treatment and nuclear extract preparation. Pretreatment levels of nuclear p65 were 31% lower in $CCR4^{-/-}$ peritoneal macrophages as compared with WT (Fig. 6*A*). In vitro Pam_3Cys or LPS treatment significantly induced WT activation/translocation of NF-κB p65, but minimal NF-κB activation was measured in $CCR4^{-/-}$ peritoneal macrophages.

Because activation of the NF-κB pathway was suppressed in $CCR4^{-/-}$ macrophages, other pathways of transcription factor activation were also analyzed. Although resident peritoneal macrophages had similar levels of p38 MAPK, activated JNK

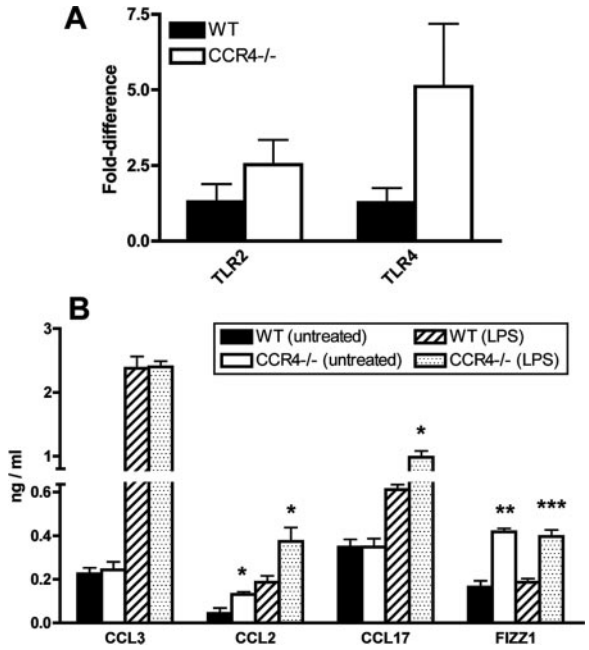

FIGURE 4. $CCR4^{-/-}$ macrophages have high basal TLR expression, and LPS triggers production of high levels of immunomodulatory chemokines. Resident peritoneal macrophages from naive mice were treated (n = 3) after an overnight rest. *A*, Basal TLR2 and TLR4 expression was analyzed by real-time quantitative PCR. *B*, Macrophages were untreated or stimulated with 1 μg/ml LPS for 24h. ELISAs were used to measure cytokines, chemokines, and FIZZ1 in cell-free supernatants. Graphs represent data from three independent studies. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

was significantly higher in untreated cells from $CCR4^{-/-}$ mice than from WT mice (83% elevation; Fig. 6*B*). Pam_3Cys and LPS induced phosphorylation of p38 MAPK and JNK; however, all $CCR4^{-/-}$ treatment groups displayed significantly more activation than their WT counterparts. These data indicate that enhanced p38 MAPK and JNK signaling may compensate for NF-κB deficiency, allowing altered cytokine/chemokine expression from $CCR4^{-/-}$ macrophages in response to TLR agonists.

In an effort to identify the TLR/NF-κB defect, naive WT and $CCR4^{-/-}$ macrophages were treated with LPS and intermediate signaling mediators were measured using Western blotting. Basal expression of TRAF6 and IκBα was similar for both strains, but $CCR4^{-/-}$ macrophages had ~2-fold less MyD88 and phosphorylated-IκBα than WT cells. LPS immediately increased MyD88, IRAK1, TRAF6, and IκBα expression and IκBα phosphorylation in WT peritoneal macrophages with peak expression between 5 and 30 min (Fig. 7, *left column*). With the exception of IRAK1, almost no induction was seen in $CCR4^{-/-}$ cells. Basal IRAK1 levels were 2-fold higher in naive $CCR4^{-/-}$ vs WT macrophages. LPS triggered rapid IRAK1 expression in $CCR4^{-/-}$ cells (5 min), whereas similar levels were not produced in WT cells until 30 min after LPS challenge.

IL-4 and IL-13 are well known for their ability to polarize T cells into a Th2 phenotype and have recently been shown to have a similar effect on macrophage development. $CCR4^{-/-}$ macrophages have defective NF-κB signaling, and we have demonstrated that these cells share many features with aaMφ. Therefore, we sought to determine whether alternative activation would alter NF-κB signaling in WT macrophages. Differentiated macrophages were stimulated with LPS, and signaling

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

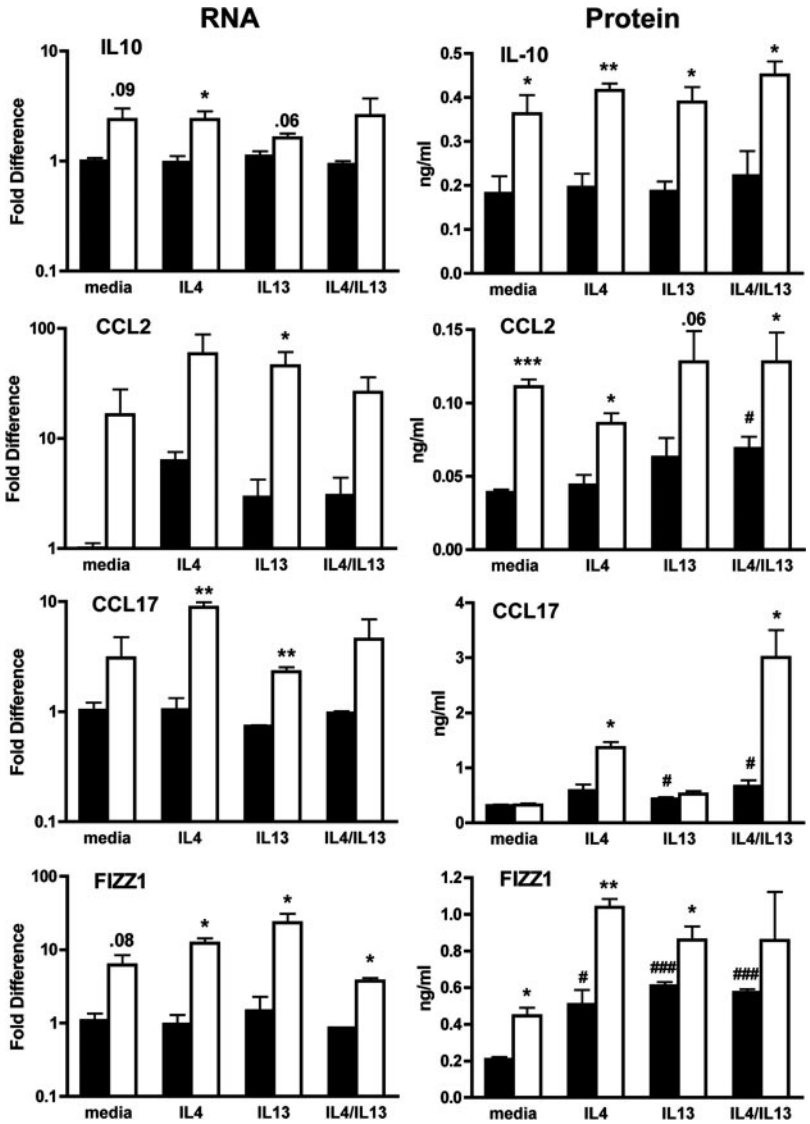

FIGURE 5. Naive and alternatively activated CCR4$^{-/-}$ macrophages secrete factors characteristic of aaMφ. Resident peritoneal macrophages were untreated or treated with 100 ng/ml murine IL-4, IL-13, or IL-4 plus IL-13. After 4 h, RNA was isolated and IL-10, CCL2, CCL17, and FIZZ1 expression was analyzed by quantitative real-time PCR. The protein expression of these mediators was measured at 48 h by ELISA. Values of $p < 0.05$ (#) and $p < 0.0005$ (###) are indicated for significant increases in WT expression after cytokine treatment. Representative data are shown for three independent studies. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

mediators were measured (Fig. 7, *right column*). Overall, LPS was unable to up-regulate expression of MyD88, IRAK1, TRAF6, or IκBα in IL-4-/IL-13-pretreated WT macrophages, and no changes were seen in the phosphorylation of IκBα. A similar profile was observed for pretreated CCR4$^{-/-}$ macrophages, indicating that the phenotype of naive CCR4$^{-/-}$ macrophages is similar to that of aaMφ.

Discussion

Coordinated interaction between both innate and acquired immune responses is crucial to the development of an effective response to foreign stimuli. In addition to regulating both arms of the immune system, chemokines and their receptors have also been recognized as key mediators of the cross-talk integrating nonspecific and specific defenses. Although CCR4 has been extensively studied in the context of acquired immunity, this investigation attempted to assess the contribution of CCR4 in models triggering innate immunity. Because previous studies have shown that CCR4$^{-/-}$ mice provide a protective phenotype to LPS challenge (23), we were interested in understanding the mechanisms of this resistance.

Our initial investigation confirmed that CCR4$^{-/-}$ mice were resistant to the in vivo effects of LPS, albeit at a reduced level than that previously reported (10). Contrary to these early studies using CCR4$^{-/-}$ mice backcrossed with C57BL/6 mice for four generations, our 10th generation mice were susceptible to a 100 mg/kg dose of LPS but were significantly resistant to a lower 10 mg/kg dose as compared with WT controls. Continued backcrossing may explain some of the phenotypic differences between these studies, as C57BL/6 mice are known to produce a more vigorous type 1 (IFN-γ-mediated) cytokine response (24) that can result in acute septic shock. In addition to LPS, we also demonstrated that CCR4$^{-/-}$ mice are more resistant to challenge with Pam$_3$Cys.

The innate immune system is responsible for the nonspecific recognition of foreign invaders. Pattern recognition receptors, including TLRs, interact with conserved structures found on invading microbes. Several TLRs are involved in the recognition of bacterial Ags such as LPS (TLR4), diacyl lipopeptides (TLR2/TLR6), flagellin (TLR5), and CpG DNA (TLR9). TLR binding elicits the production of inflammatory chemokines, as well as a multitude of other immune molecules, that attract and coordinate the activities of both innate and specific immune cells.

We demonstrated that CCR4$^{-/-}$ mice were protected against in vivo challenge with two different TLR agonists, LPS and Pam$_3$Cys, as well as a live bacterial infection. Alterations in TLR

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

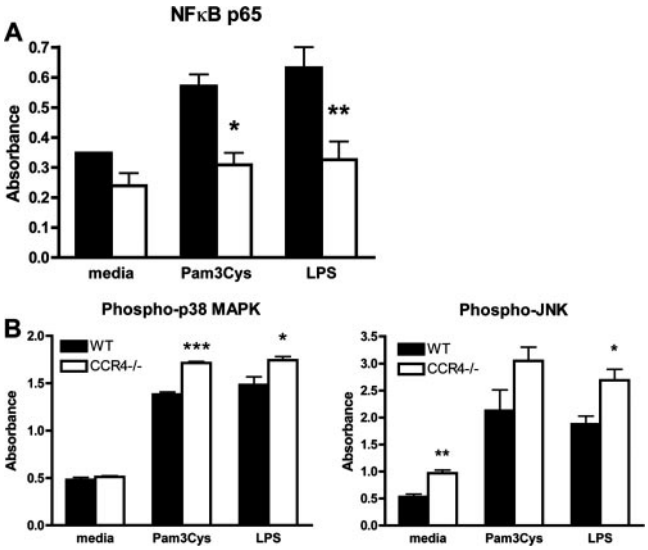

FIGURE 6. CCR4 modulates transcription factor activation. *A*, Macrophages from thioglycollate-recruited peritoneal cells (10^7/dish) were rested overnight and treated with medium, 2.5 μg/ml Pam_3Cys, or 1 μg/ml LPS. Nuclear extracts were prepared at 4 h after treatment (peak expression), and 20 μg of the extracts were analyzed using an NF-κB p65 ELISA. *B*, Naive resident peritoneal cells were plated (10^5 macrophages/well), rested overnight, and stimulated with medium, Pam_3Cys, or LPS. Phosphorylated p38 MAPK and JNK were measured at 30 min by ELISA. Absorbance was normalized to cells per well. All results shown represent data from two independent assays. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

expression and ultimately the recognition of bacterial Ags could be responsible for some of the different in vivo responses observed between WT and $CCR4^{-/-}$ mice. In fact, $CCR4^{-/-}$ mice exhibited increased transcript levels of all TLRs assayed after LPS treatment. A profile of the different leukocyte populations demonstrated that $CCR4^{-/-}$ mice had significantly higher numbers of $CD11b^+F4/80^+$, $CD11b^+TLR2^+$, and $CD11b^+TLR4^+$ leukocytes than WT animals at 4 h after LPS challenge. Thus, one potential mechanism for increased resistance in $CCR4^{-/-}$ mice may be enhanced pathogen recognition and leukocyte recruitment directed at the expeditious elimination of the bacteria and its Ags. Although increasing TLR expression facilitates a more rapid response to pathogens, we would expect the overall inflammatory response to be increased as well. Bacterial Ags such as LPS are well known for their ability to incite an overwhelming storm of inflammatory cytokines such as that observed during sepsis. Despite increases in TLR expression of $CCR4^{-/-}$ mice, few changes were seen in the overall chemokine profiles of infected WT and $CCR4^{-/-}$ mice. Therefore, other factors or mechanisms must be present that protect $CCR4^{-/-}$ mice from the tissue injury and mortality observed in WT mice after Pam_3Cys or LPS challenge, respectively.

The peritoneal cavity is normally a sterile environment, and macrophages are the principal resident leukocytes responsible for surveying this environment for pathogens and initiating the appropriate immune responses. The basal level of TLR expression in these cells provides a measure of their general ability to respond to bacterial stimuli. Analyses of naive resident peritoneal macrophages from WT and $CCR4^{-/-}$ mice revealed higher TLR2 and TLR4 expression in $CCR4^{-/-}$ macrophages, indicating an enhanced potential for pathogen recognition by knockout mice. Further analyses indicated different cytokine/chemokine profiles for WT vs $CCR4^{-/-}$ macrophages. Interestingly, no differences were seen in the expression of the inflammatory mediator CCL3, but naive and LPS-treated $CCR4^{-/-}$ macrophages produced significantly more anti-inflammatory/immunomodulatory mediators (IL-10, CCL2, CCL6, and CCL17) than similarly treated WT macrophages. This skewed response supports the decreased susceptibility of $CCR4^{-/-}$ mice to LPS/Pam_3Cys challenge in vivo. Although IL-10 has been shown to alter TLR4 expression, little information exists with regard to the ability of chemokines to alter the expression pattern of various TLRs. In preliminary investigations we did not observe significant alterations in TLR expression in WT mice treated with chemokines known to be associated with a polarized M2 response, including CCL2, CCL6, or CCL17.

Similarly as in T cell polarization, macrophages differentiate into classically activated macrophages (M1) or aaMφ (M2) (20, 22). Recently, IL-10, CCL2, and CCL17 expression has been associated with the development of aaMφ and the inhibition of classical activation (21, 25, 26). Our data suggested that $CCR4^{-/-}$ mice had a resident population of macrophages displaying a cytokine/chemokine phenotype characteristic of aaMφ. FIZZ1 is antagonistically regulated by IL-4 and IFN-γ and, therefore, is often used as a marker of aaMφ (25, 27). $CCR4^{-/-}$ macrophages expressed significantly higher levels of FIZZ1 than WT macrophages. Although alternative activation of WT macrophages using IL-4 and IL-13 treatment resulted in production of CCL2, CCL17, and FIZZ1, expression of these factors and IL-10 was always higher in $CCR4^{-/-}$ macrophages. These data provide further evidence illustrating that $CCR4^{-/-}$ mice have resident macrophages possessing an alternatively activated phenotype.

Although the focus of this investigation was on the peritoneal cells and leukocytes elicited into the peritoneum, we were interested in determining whether macrophages from other tissues have an altered phenotype in the $CCR4^{-/-}$ mice. In preliminary studies, arginase 1, a mediator associated with macrophage polarization to an M2 phenotype, was found to be overexpressed in bone marrow-derived macrophages recovered from $CCR4^{-/-}$ animals challenged in vivo and subsequently treated in vitro with either LPS or CpG. This finding suggests that the peritoneal macrophages are not unique in possessing the ability to express a polarized phenotype upon challenge.

Our final set of studies focused on analyzing the signaling cascades initiated by TLR binding in WT and $CCR4^{-/-}$ macrophages. Considering our data illustrating the expression profiles of these two different strains of cells, we predicted that we would also discover differences in the signaling pathways activated by TLR challenge. NF-κB is the primary transcription factor responsible for initiating transcription of most inflammatory cytokines/chemokines. When inactive, it is held in the cytoplasm by the inhibitor protein IκBα. Activation of this pathway results in phosphorylation of IκBα, releasing NF-κB into the nucleus where it is active. Therefore, nuclear NF-κB was measured to indirectly assess the overall activity of this pathway. Untreated $CCR4^{-/-}$ cells had reduced levels of nuclear p65 compared with basal WT levels. LPS and Pam_3Cys were potent triggers of this pathway in WT cells; however, neither had an effect on $CCR4^{-/-}$ macrophages. This finding is extremely important, as suppression of this pathway would provide a mechanism explaining the reduced storm of cytokines initiated during an in vivo LPS challenge, improving tissue and host survival.

Despite the absence of NF-κB activation, macrophages from $CCR4^{-/-}$ mice produce high levels of anti-inflammatory/regulatory cytokines and chemokines, suggesting that other transcriptional pathways are up-regulated. In fact, we observed significantly higher induction of both p38 MAPK and JNK pathways in $CCR4^{-/-}$ macrophages in comparison to WT cells. Both p38

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

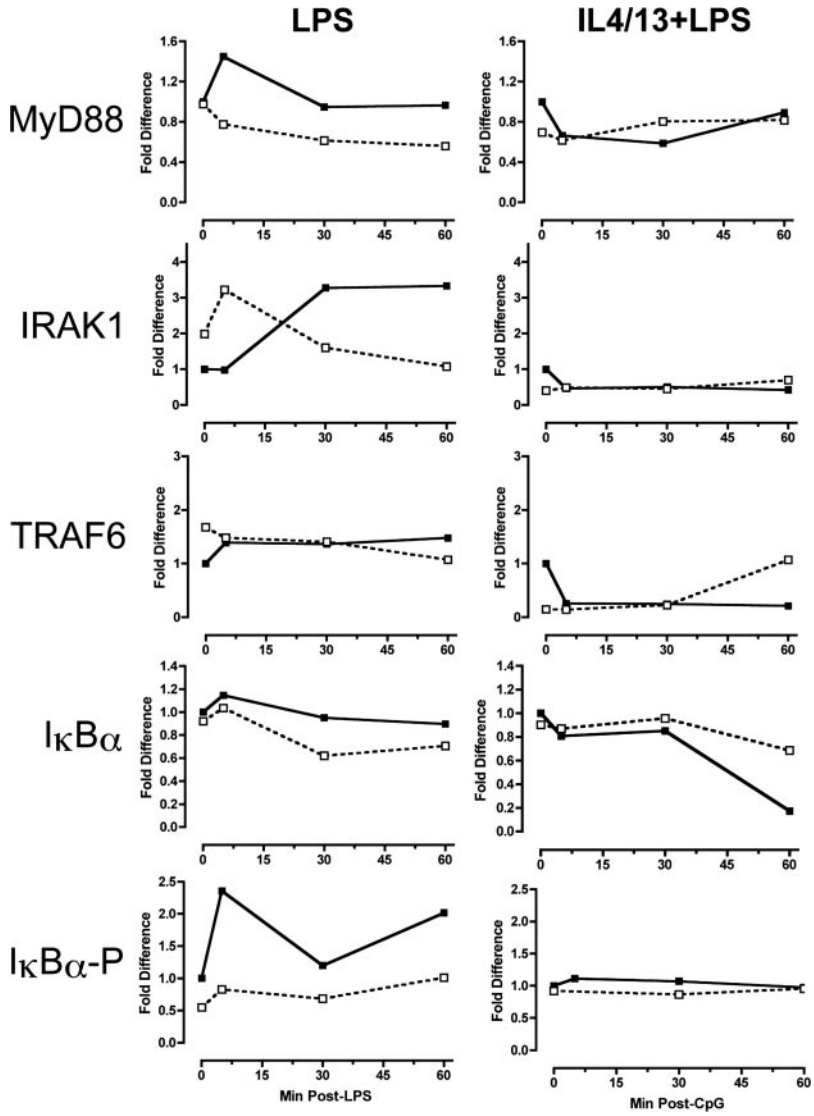

FIGURE 7. MyD88-dependent signaling is suppressed in CCR4$^{-/-}$ macrophages. Resident peritoneal macrophages were plated (5 × 106 per plate), rested overnight, and treated for 0, 5, 30, or 60 min with 1 μg of LPS per milliliter. Total cell lysates were prepared and subjected to SDS-PAGE (25 μg/each) and immunostaining for signaling proteins in the MyD88-dependent pathway. Protein loading was controlled to β-actin expression. Targets were expressed as fold differences relative to untreated WT cells. In a separate set of experiments, macrophages were pretreated with 100 ng/ml IL-4 plus IL-13 for 48 h followed by 1 μg/ml LPS. Representative data from two experiments are shown. IκBα-P denotes phosphorylated IκBα.

MAPK and JNK are members of the serine/threonine MAPK family and are involved in inflammatory and stress responses. p38 MAPK targets several transcription factors including STAT1, Myc/Max, Elk-1, CHOP, MEF2, ATF-2, and CREB, whereas JNK activates c-Jun, ATF-2, Elk-1, JunD, and AP-1. Therefore, altered signaling pathways are able to compensate for NF-κB deficiency in CCR4$^{-/-}$ mice but skew the cytokine/chemokine responses following challenge with TLR agonists. A similar phenomenon was noted for tumor-associated macrophages (TAMs) (28). TAMs were associated with an alternatively activated phenotype, demonstrated high levels of IL-10 and CCL2, exhibited defective NF-κB signaling, and up-regulated IRF-3/STAT1 signaling. These studies with TAMs appear to parallel our studies with CCR4$^{-/-}$ macrophages and accentuate their similarities to aaMφ.

All TLRs, with the exception of TLR3, are able to stimulate the MyD88-dependent signaling pathway, increasing NF-κB translocation (29). Upon ligand binding, the adaptor protein MyD88 interacts with the cytoplasmic tail of the TLR and initiates the activation of a cascade of intracellular signaling mediators, including IRAK1 and TRAF6. In CCR4$^{-/-}$ macrophages, activation of this entire pathway, with the exception of IRAK1, is suppressed. IRAK1 has been shown to initiate STAT3-dependent IL-10 expression (30), potentially contributing to the increased IL-10 production seen in CCR4$^{-/-}$ mice and macrophages. IL-4/IL-13 treatment of WT macrophages shut down activation of the NF-κB pathway, indicating that alternative activation inhibits classical activation of NF-κB while driving the use of other pathways.

In summary, our data provide a novel mechanistic insight into the role of CCR4 in the innate immune response. Using CCR4$^{-/-}$ mice, we have demonstrated that these animals are resistant to the deleterious effects of TLR agonist challenge, as well as bacterial infection. The response of these mice to CpG challenge/TLR9 activation is the subject of ongoing studies in our laboratory. Although CCR4$^{-/-}$ mice have increased early cellular recruitment in response to LPS, CCR4$^{-/-}$ macrophages possess an altered phenotype, more closely resembling aaMφ. These cells rapidly induce TLR expression, allowing the recognition and binding of pathogens. Finally, the signal transduction pathways in these macrophages are altered, diminishing the NF-κB response and thus preventing the classic cytokine storm that is induced in inflammatory diseases such as sepsis.

Disclosures

The authors have no financial conflict of interest.

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

References

1. Bonecchi, R. G. Bianchi, P. P. Bordignon, D. D'Ambrosio, R. Lang, A. Borsati, S. Sozzani, P. Allavena, P. A. Grey, A. Mantovani, and F. Sinigaglia. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med.* 187: 129–134.
2. Inoue, T., S. Fujishima, E. Ikeda, O. Yoshie, N. Tsukamoto, S. Aiso, N. Aikawa, A. Kubo, K. Matsushima, and K. Yamaguchi. 2004. CCL22 and CCL17 in rat radiation pneumonitis and in human idiopathic pulmonary fibrosis. *Eur. Respir. J.* 24: 49–56.
3. Belperio, J. A., M. Dy, L. Murray, M. D. Burdick, Y. Y. Xue, R. M. Strieter, and M. P. Keane. 2004. The role of the Th2 CC chemokine ligand CCL17 in pulmonary fibrosis. *J. Immunol.* 173: 4692–4698.
4. Katoh, S., K. Fukushima, N. Matsumoto, K. Matsumoto, K. Abe, N. Onai, K. Matsushima, and S. Matsukura. 2003. Accumulation of CCR4-expressing $CD4^+$ T cells and high concentration of its ligands (TARC and MDC) in bronchoalveolar lavage fluid of patients with eosinophilic pneumonia. *Allergy* 58: 518–523.
5. Yoneyama, H., A. Harada, T. Imai, M. Baba, O. Yoshie, Y. Zhang, H. Higashi, M. Murai, H. Asakura, and K. Matsushima. 1998. Pivotal role of TARC, a CC chemokine, in bacteria-induced fulminant hepatic failure in mice. *J. Clin. Invest.* 102: 1933–1941.
6. Jakubzick, C., H. Wen, A. Matsukawa, M. Keller, S. L. Kunkel, and C. M. Hogaboam. 2004. Role of CCR4 ligands, CCL17 and CCL22, during *Schistosoma mansoni* egg-induced pulmonary granuloma formation in mice. *Am. J. Pathol.* 165: 1211–1221.
7. Kim, S. H., M. M. Cleary, H. S. Fox, D. Chantry, and N. Sarvetnick. 2002. CCR4-bearing T cells participate in autoimmune diabetes. *J. Clin. Invest.* 110: 1675–1686.
8. Kawasaki, S., H. Takizawa, H. Yoneyama, T. Nakayama, R. Fujisawa, M. Izumizaki, T. Imai, O. Yoshie, I. Homma, K. Yamamoto, and K. Matsushima. 2001. Intervention of thymus and activation-regulated chemokine attenuates the development of allergic airway inflammation and hyperresponsiveness in mice. *J. Immunol.* 166: 2055–2062.
9. Gonzalo, J. A., Y. Pan, C. M. Lloyd, G. Q. Jia, G. Yu, B. Dussault, C. A. Powers, A. E. Proudfoot, A. J. Coyle, D. Gearing, and J. C. Gutierrez-Ramos. 1999. Mouse monocyte-derived chemokine is involved in airway hyperreactivity and lung inflammation. *J. Immunol.* 163: 403–411.
10. Chvatchko, Y., A. J. Hoogewerf, A. Meyer, S. Alouani, P. Juillard, R. Buser, F. Conquet, A. E. Proudfoot, T. N. Wells, and C. A. Power. 2000. A key role for CC chemokine receptor 4 in lipopolysaccharide-induced endotoxic shock. *J. Exp. Med.* 191: 1755–1764.
11. Schuh, J. M., C. A. Power, A. E. Proudfoot, S. L. Kunkel, N. W. Lukacs, and C. M. Hogaboam. 2002. Airway hyperresponsiveness, but not airway remodeling, is attenuated during chronic pulmonary allergic responses to *Aspergillus* in $CCR4^{-/-}$ mice. *FASEB J.* 16: 1313–1315.
12. Huser, N., C. Tertilt, K. Gerauer, S. Maier, T. Traeger, V. Assfalg, R. Reiter, C. D. Heidecke, and K. Pfeffer. 2005. CCR4-deficient mice show prolonged graft survival in a chronic cardiac transplant rejection model. *Eur. J. Immunol.* 35: 128–138.
13. Ness, T. L., K. J. Carpenter, J. L. Ewing, C. J. Gerard, C. M. Hogaboam, and S. L. Kunkel. 2004. CCR1 and CC chemokine ligand 5 interactions exacerbate innate immune responses during sepsis. *J. Immunol.* 173: 6938–6948.
14. Ness, T. L., C. M. Hogaboam, R. M. Strieter, and S. L. Kunkel. 2003. Immunomodulatory role of CXCR2 during experimental septic peritonitis. *J. Immunol.* 171: 3775–3784.
15. Chensue, S. W., D. G. Remick, C. Shmyr-Forsch, T. F. Beals, and S. L. Kunkel. 1988. Immunohistochemical demonstration of cytoplasmic and membrane-associated tumor necrosis factor in murine macrophages. *Am. J. Pathol.* 133: 564–572.
16. Waage, A., P. Brandtzaeg, T. Espevik, and A. Halstensen. 1991. Current understanding of the pathogenesis of Gram-negative shock. *Infect Dis. Clin. North Am.* 5: 781–791.
17. Schmitz, J., A. Owyang, E. Oldham, Y. Song, E. Murphy, T. K. McClanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, et al. 2005. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 23: 479–490.
18. Brint, E. K., D. Xu, H. Liu, A. Dunne, A. N. McKenzie, L. A. O'Neill, and F. Y. Liew. 2004. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nat. Immunol.* 5: 373–379.
19. Xu, D., W. L. Chan, B. P. Leung, F. Huang, R. Wheeler, D. Piedrafita, J. H. Robinson, and F. Y. Liew. 1998. Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J. Exp. Med.* 187: 787–794.
20. Mantovani, A., A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M. Locati. 2004. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 25: 677–686.
21. Katakura, T., M. Miyazaki, M. Kobayashi, D. N. Herndon, and F. Suzuki. 2004. CCL17 and IL-10 as effectors that enable alternatively activated macrophages to inhibit the generation of classically activated macrophages. *J. Immunol.* 172: 1407–1413.
22. Gordon, S. 2003. Alternative activation of macrophages. *Nat. Rev. Immunol.* 3: 23–35.
23. Chvatchko, Y., A. E. Proudfoot, R. Buser, P. Juillard, S. Alouani, M. Kosco-Vilbois, A. J. Coyle, R. J. Nibbs, G. Graham, R. E. Offord, and T. N. Wells. 2003. Inhibition of airway inflammation by amino-terminally modified RANTES/CC chemokine ligand 5 analogues is not mediated through CCR3. *J. Immunol.* 171: 5498–5506.
24. Darville, T., C. W. Andrews, Jr., J. D. Sikes, P. L. Fraley, L. Braswell, and R. G. Rank. 2001. Mouse strain-dependent chemokine regulation of the genital tract T helper cell type 1 immune response. *Infect. Immun.* 69: 7419–7424.
25. Raes, G., W. Noel, A. Beschin, L. Brys, P. de Baetselier, and G. H. Hassanzadeh. 2002. FIZZ1 and Ym as tools to discriminate between differentially activated macrophages. *Dev. Immunol.* 9: 151–159.
26. Tsuda, Y., H. Takahashi, M. Kobayashi, T. Hanafusa, D. N. Herndon, and F. Suzuki. 2004. CCL2, a product of mice early after systemic inflammatory response syndrome (SIRS), induces alternatively activated macrophages capable of impairing antibacterial resistance of SIRS mice. *J. Leukocyte Biol.* 76: 368–673.
27. Raes, G., P. De Baetselier, W. Noel, A. Beschin, F. Brombacher, and G. Hassanzadeh Gh. 2002. Differential expression of FIZZ1 and Ym1 in alternatively versus classically activated macrophages. *J. Leukocyte Biol.* 71: 597–602.
28. Biswas, S. K., L. Gangi, S. Paul, T. Schioppa, A. Saccani, M. Sironi, B. Bottazzi, A. Doni, B. Vincenzo, F. Pasqualini, et al. 2006. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-κB and enhanced IRF-3/STAT1 activation). *Blood* 107: 2112–2122.
29. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu. Rev. Immunol.* 21: 335–376.
30. Huang, Y., T. Li, D. C. Sane, and L. Li. 2004. IRAK1 serves as a novel regulator essential for lipopolysaccharide-induced interleukin-10 gene expression. *J. Biol. Chem.* 279: 51697–51703.

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022