A Key Role for CC Chemokine Receptor 4 in Lipopolysaccharide-induced Endotoxic Shock

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Abstract

CC chemokine receptor (CCR)4, a high affinity receptor for the CC chemokines thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC), is expressed in the thymus and spleen, and also by peripheral blood T cells, macrophages, platelets, and basophils. Recent studies have shown that CCR4 is the major chemokine receptor expressed by T helper type 2 (Th2) polarized cells. To study the in vivo role of CCR4, we have generated CCR4-deficient ($CCR4^{-/-}$) mice by gene targeting. $CCR4^{-/-}$ mice developed normally. Splenocytes and thymocytes isolated from the $CCR4^{-/-}$ mice failed to respond to the CCR4 ligands TARC and MDC, as expected, but also surprisingly did not undergo chemotaxis in vitro in response to macrophage inflammatory protein (MIP)-1α. The CCR4 deletion had no effect on Th2 differentiation in vitro or in a Th2-dependent model of allergic airway inflammation. However, $CCR4^{-/-}$ mice exhibited significantly decreased mortality on administration of high or low dose bacterial lipopolysaccharide (LPS) compared with $CCR4^{+/+}$ mice. After high dose LPS treatment, serum levels of tumor necrosis factor α, interleukin 1β, and MIP-1α were reduced in $CCR4^{-/-}$ mice, and decreased expression of MDC and MIP-2 mRNA was detected in peritoneal exudate cells. Analysis of peritoneal lavage cells from $CCR4^{-/-}$ mice by flow cytometry also revealed a significant decrease in the $F4/80^{+}$ cell population. This may reflect a defect in the ability of the $CCR4^{-/-}$ macrophages to be retained in the peritoneal cavity. Taken together, our data reveal an unexpected role for CCR4 in the inflammatory response leading to LPS-induced lethality.

Key words: CC chemokine receptor 4 • lipopolysaccharide • endotoxic shock • F4/80 antigen • T helper type 2 cells

Introduction

Chemokines are a superfamily of small proteins involved both in routine leukocyte trafficking and in the activation and recruitment of specific cell populations to sites of inflammation and infection. There are four classes of chemokines, named according to the spacing of the first two of a conserved four-cysteine motif: CC, CXC, C, and CX_3C. The majority of chemokines belong to the CC and CXC subfamilies. For the C and CX_3C subfamilies, only one member has been identified to date, lymphotactin and fractalkine (or neurotactin), respectively (1, 2). The biological activity of chemokines is mediated by their interaction with a family of seven-transmembrane G protein–coupled receptors. To date, there are published reports for five CXC chemokine–specific receptors, nine CC, a CX_3C, and a C chemokine receptor as well as the Duffy antigen and several virally encoded chemokine receptors (3, 4). In vitro studies have shown that most chemokines are able to bind and activate more than one chemokine receptor, and the majority of chemokine receptors bind multiple ligands although there are exceptions. Yet in spite of this apparent redundancy, there are now increasing numbers of reports in the literature associating specific chemokine receptor–ligand pairs with particular disease states or tissue-specific lymphocyte homing. Much of this information has been obtained from studies of chemokine or chemokine receptor knockout mice.

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We have previously reported the molecular cloning of both human and murine CC chemokine receptor (CCR)[1] 4 (5, 6). Although originally identified as a receptor for macrophage inflammatory protein (MIP)-1α and regulated upon activation, normal T cell expressed and secreted chemokine (RANTES), CCR4 is in fact a high affinity receptor for two recently described CC chemokines, thymus and activation-regulated chemokine (TARC) (7) and macrophage-derived chemokine (MDC) (8). CCR4 mRNA is expressed predominantly in the thymus, spleen, and in peripheral blood leukocytes, including T cells, basophils, monocytes (5), macrophages, and platelets (9). In addition, several reports have shown that CCR4 is highly expressed by human T cells polarized towards the Th2 phenotype (10–12). These Th2 cells are also responsive to the CCR4 ligands TARC and MDC, leading investigators to suggest a role for CCR4 in the development of Th2 responses. Finally, a recent report has shown that CCR4 is also expressed on all skin homing T cells expressing cutaneous lymphocyte antigen (CLA) (13).

To study the role of CCR4 in vivo, we have generated CCR4-deficient mice by gene targeting. Here, we show that in spite of the reported high level expression of CCR4 on human Th2-polarized cells, CCR4 deletion had no effect on the development of the response in a classical Th2-dependent murine model of airway inflammation. However, $CCR4^{-/-}$ mice showed an unexpected resistance to the lethal effects of LPS in two models of LPS-induced endotoxic shock.

Materials and Methods

Generation of CCR4-deficient Mice. The murine (m)CCR4 gene was isolated from an HM-1 embryonic stem (ES) cell library in λFIXII vector (Stratagene) by plaque hybridization using murine CCR4 cDNA as a probe (6). Two unique clones of 12.18 and 13.08 kb were shown to contain the mCCR4 coding sequence by PCR, using specific primers (6). An 8.5-kb fragment of genomic DNA confirmed to contain the CCR4 coding sequence by Southern blotting was subcloned into pBluescript II SK^- to generate pCCR4. The entire CCR4 coding sequence was then removed as an NheI-HpaI fragment and replaced with a neo cassette (derived from pMC1neoPolyA; CLONTECH Laboratories, Inc.). The resulting construct was digested with EcoRV and Eco47-3, and religated to generate a plasmid containing a long arm of homology of 4,904 bp and a short arm of homology of 1,318 bp. Finally, a thymidine kinase (tk) cassette was inserted into the HindIII-XhoI site of the plasmid to produce the targeting vector. The targeting vector was linearized with NotI and electroporated into HM-1 ES cells as described previously (14). Gancyclovir and G418-resistant clones were selected. DNA was isolated from resistant clones using DNAzol (GIBCO BRL), and the presence of the transgene was detected by PCR and verified by Southern hybridization on PstI-digested genomic DNA, using a 466-bp probe derived by AvrII-NsiI digestion of pCCR4. Seven independent transgene-containing ES cell clones were used to produce chimeric mice by blastocyst injection according to standard procedures (15). Two 100% chimeric males derived from two independent ES clones were mated with a 100% chimeric female to generate heterozygous $CCR4^{+/-}$ mice, and littermates from the matings of $CCR4^{+/-}$ mice were analyzed for the presence of homozygous $CCR4^{-/-}$ mice by Southern blot analysis or by PCR on tail DNA. The sequences of the PCR primers used are: (primer 1, neomycin gene) 5′-CGCTTCCTCGTGCTTTACGGTAT; (primer 2, CCR4 3′ untranslated region) 5′-ATAGCCTTGGCTGGTCTGGAACTA; (primer 3, mCCR4 coding region sense primer) 5′-CCAAAGATGAATGCCACAGAGGTCACAG; (primer 4, mCCR4 coding region antisense primer) 5′-TTACAAAGCGTCACGGAAGTCATG.

Reverse Transcriptase PCR. Total RNA was isolated from thymocytes, splenocytes, or peritoneal lavage cells using Trizol™ (GIBCO BRL). 1 μg of total RNA from thymocytes and splenocytes or total RNA from 10^6 peritoneal exudate cells was reverse transcribed using Superscript™ (GIBCO BRL) and oligo $dT_{12–18}$ primer according to the manufacturer's instructions. One twentieth of the cDNA synthesis reaction was then subjected to 25 cycles of PCR using AmpliTaq™ (PerkinElmer) and PCR primers based on the EMBL/GenBank/DDBJ database entries for MIP-2 (X53798), mMDC (AF052505), and mCCR4 (X90862). PCR products were analyzed on 1% agarose gels stained with ethidium bromide, and bands migrating at the correct molecular weight were verified by direct sequencing. For semiquantitative reverse transcriptase (RT)-PCR, bands were quantitated using Kodak Digital Science v1.0 software and results are expressed as arbitrary units of mRNA.

In Vitro Differentiation of Th1 and Th2 Cells. $CD8^+$ and Ig^+ depleted cells from lymph nodes and spleens of naive $CCR4^{+/+}$ and $CCR4^{-/-}$ mice were cultured for 4 d on plates coated with an anti-CD3 antibody (145-2C11; BD PharMingen) as described previously (16) in the presence of either murine IL-12 (500 pg/ml; R&D Systems) plus anti–IL-4 mAb (10 μg/ml) or murine IL-4 (500 U/ml; ImmunoKontact) plus anti–IFN-γ mAb (10 μg/ml; BD PharMingen). After a 5-d culture period, cells were washed and restimulated for 24 h at a density of 2×10^5 cells per well on an anti-CD3–coated 96-well plate (Costar) in the presence of murine IL-2 (50 U/ml; R&D Systems).

Animals and Treatments. Homozygous $CCR4^{-/-}$ mice were backcrossed with C57BL/6 mice (Centre d'Elevage Janvier) for four generations. Age- and weight-matched $CCR4^{-/-}$ and $CCR4^{+/+}$ littermates from heterozygote ($CCR4^{+/-}$) matings from the fourth backcross were used in this study to control for strain background. Mice (20–25 g) of either sex were immunized intraperitoneally with 10 μg of OVA (A-5503; Sigma-Aldrich) in 0.2 ml of alum (Serva). Control mice received an injection of saline (0.9% wt/vol NaCl) alone. 14 d later, mice were anesthetized by inhaled 2% FORENE™ (Abbott) and 50 μg of OVA was administered to the lungs (in 50 μl of saline) intranasally as described previously (17). Control mice received 50 μl saline only. This procedure was repeated daily for 5 d. Animals were finally killed by lethal injection of 60 mg/kg pentobarbital. Student's *t* test was used for statistical analysis, except that for the analysis of the survival curves, we performed the log rank test (two-tailed).

[1]*Abbreviations used in this paper:* BAL, bronchoalveolar lavage; BALF, BAL fluid; BHR, bronchial hyperreactivity; CCR, CC chemokine receptor; D-gal, d-galactosamine; ES, embryonic stem; MCh, methacholine; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; Penh, enhanced pause; RANTES, regulated upon activation, normal T cell expressed and secreted chemokine; RT, reverse transcriptase; TARC, thymus and activation-regulated chemokine; Tlr4, Toll-like receptor 4.

Phenol-extracted bacterial LPS from *Escherichia coli* 055:B5 (List Biological Laboratories) was administered at 60, 90, and 120 mg/kg intraperitoneally for the high dose LPS shock model. For the low dose LPS shock model, mice received 1, 2, and 4 μg of LPS with 8 mg d-galactosamine (D-gal; Fluka) in 0.5 ml saline. Animals were killed by CO_2 asphyxiation at the time points indicated in the figures.

Evaluation of Bronchial Hyperresponsiveness. Bronchial hyperreactivity (BHR) was measured by recording respiratory pressure curves by whole body plethysmography (18) in response to inhaled methacholine (MCh; Sigma-Aldrich) using a Buxco® apparatus (EMKA Technologies). The airway reactivity was expressed in enhanced pause (Penh) as described previously (19).

Analysis of Blood and Lavage Fluids. Blood (20 μl) was collected from the retroorbital plexus of mice into a heparinized micropipette, then transferred to a Unopette microcollection system for platelet determination (Becton Dickinson). Three independent samples of platelets were counted in a hemocytometer. A minimum of 100 cells were counted, and the arithmetic mean of the three counts was calculated (20).

OVA-specific IgM, IgG1, IgG2a, and IgE titers were measured in serum samples obtained 3 d after the final intranasal treatment with OVA using a standard ELISA protocol (19). Quantification of murine TNF-α in serum and in culture supernatant and IL-1β, IL-6, and MIP-1α in serum was determined using cytokine-specific ELISAs as per the manufacturer's protocol (R&D Systems).

Bronchoalveolar lavage (BAL) and peritoneal lavage cells were harvested and differential cell counts were performed on cytospin preparations stained with Diff-Quik™ (Baxter Diagnostics). A minimum of 200 cells were counted per field, with 3 fields per sample for BAL, and 5 fields per sample for peritoneal lavage.

For phenotypic analysis of peritoneal cells by flow cytometry, lavaged cells were resuspended at 10^6 cells/ml in PBS containing 1% BSA and 0.01% azide (FACS buffer). Cells were incubated with Fc block (BD PharMingen) for 10 min at 4°C, washed twice with FACS buffer, then incubated for 20 min with FITC-labeled rat anti–mouse F4/80 antibody (Serotec). Cells were washed twice, resuspended in 200 μl FACS buffer, and analyzed on a Becton Dickinson FACScan™ flow cytometer with CELLQuest™ software.

Chemotaxis Assays. Spleens from $CCR4^{+/+}$ and $CCR4^{-/-}$ mice were dispersed through a 70-μm nylon cell strainer (Becton Dickinson). Erythrocytes were removed by hypotonic lysis. Cells were harvested by centrifugation and resuspended in RPMI medium containing 5% FCS and 2 mM glutamine. Chemotaxis assays were performed using the micro-Boyden chamber method with 5-μm filters (21). Recombinant human and mouse chemokines were purchased from R&D Systems or made in house.

Results and Discussion

The murine CCR4 gene was deleted through homologous recombination using the targeting vector shown in Fig. 1 a. Targeted ES cells were used to generate chimeric mice, which transmitted the transgene through the germline. Southern blot analysis confirmed that the CCR4 gene had been deleted using a DNA probe located in the 5′ noncoding region of the gene (Fig. 1 b). PCR was also used to confirm that the CCR4 coding sequence had indeed been deleted while confirming the presence of the neomycin transgene and 3′ untranslated sequence of CCR4. The CCR4 knockout mice were viable, appeared to develop normally, and showed no overt morphological or behavioral defects in the unstressed state. RT-PCR was used to demonstrate that the mRNA for CCR4 was not present in the thymus, spleen (Fig. 1 c), or peritoneal cells (Fig. 1 d) of targeted animals.

The ability of splenocytes and thymocytes, isolated from the targeted and wild-type mice, to migrate in response to the proposed CCR4 ligands was then assessed. Splenocytes (Fig. 1 e) and thymocytes (data not shown) from $CCR4^{-/-}$ mice had no chemotactic response to TARC or MDC, whereas splenocytes from $CCR4^{+/+}$ mice responded with the characteristic dose–response curves, confirming that the gene deleted in this study is an endogenous TARC and MDC receptor. Surprisingly, splenocytes isolated from the $CCR4^{-/-}$ mice did not respond to human MIP-1α (Fig. 1 e) or to murine MIP-1α (data not shown) in at least four independent experiments. However, the response to human RANTES was similar in both groups of mice (Fig. 1 e). Murine RANTES receptors described to date (CCR1, CCR5, and CCR3) have also been shown to bind and signal in response to MIP-1α in vitro. Yet RT-PCR analysis of the cells used in the study confirmed that deletion of the CCR4 gene did not interfere with expression of these other receptors (data not shown). This result suggested that in these cell types, CCR4 is a physiological receptor for MIP-1α. At present, we are unable to explain this astonishing result. However, most of the data on chemokine receptor ligand specificity has been obtained from in vitro studies. As such it is possible that in vivo, other factors affect chemokine receptor selectivity. Alternatively, our observations could reflect the need for cooperativity between distinct chemokine receptors to generate ligand specificity.

The role of CCR4 in the in vitro differentiation of T cells was addressed next. Naive $CD4^+$ T cells were cultured for 4 d on plates coated with an anti-CD3 antibody either in the presence of IL-12 plus anti–IL-4 antibody, which induces Th1 cell differentiation, or in the presence of IL-4 plus anti–IFN-γ antibody, which induces Th2 cell differentiation. Cells were then restimulated with the anti-CD3 antibody for 24 h. $CD4^+$ T cells initially cultured in the presence of IL-4 showed no significant difference in the production of IL-4 ($CCR4^{+/+}$ T cells, 105 ± 15 pg/ml; $CCR4^{-/-}$ T cells, 190 ± 10 pg/ml) after restimulation. Likewise, T cells cultured with IL-12 produced comparable levels of IFN-γ ($CCR4^{+/+}$, 76 ± 19 ng/ml; $CCR4^{-/-}$, 49 ± 8 ng/ml). These results indicate that in vitro, Th2 and Th1 cell differentiation was not impaired in the $CCR4^{-/-}$ mice. As in vitro–derived Th2 T cells have previously been shown to express CCR4, we tested whether the cells used in this study could respond to CCR4 ligands in chemotaxis assays. Cells derived from the $CCR4^{-/-}$ mice failed to migrate in response to MDC, whereas cells derived from the $CCR4^{+/+}$ mice had a robust chemotactic response to MDC (Fig. 2 a). A similar result was obtained using TARC (data not shown). In addition, in vitro–derived Th2 T cells from both wild-type and $CCR4^{-/-}$ mice responded to RANTES (Fig. 2 a).

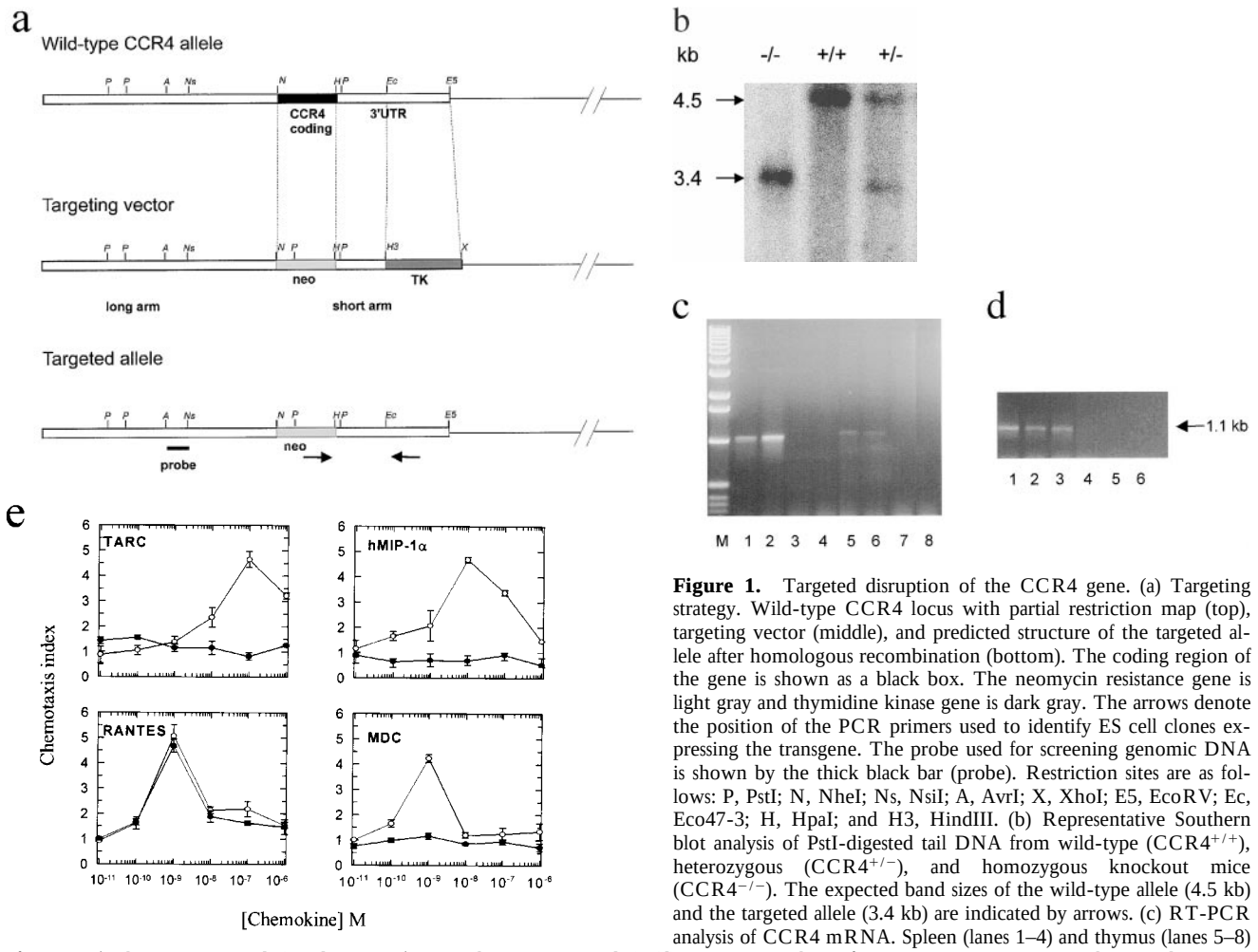

Figure 1. Targeted disruption of the CCR4 gene. (a) Targeting strategy. Wild-type CCR4 locus with partial restriction map (top), targeting vector (middle), and predicted structure of the targeted allele after homologous recombination (bottom). The coding region of the gene is shown as a black box. The neomycin resistance gene is light gray and thymidine kinase gene is dark gray. The arrows denote the position of the PCR primers used to identify ES cell clones expressing the transgene. The probe used for screening genomic DNA is shown by the thick black bar (probe). Restriction sites are as follows: P, PstI; N, NheI; Ns, NsiI; A, AvrI; X, XhoI; E5, EcoRV; Ec, Eco47-3; H, HpaI; and H3, HindIII. (b) Representative Southern blot analysis of PstI-digested tail DNA from wild-type ($CCR4^{+/+}$), heterozygous ($CCR4^{+/-}$), and homozygous knockout mice ($CCR4^{-/-}$). The expected band sizes of the wild-type allele (4.5 kb) and the targeted allele (3.4 kb) are indicated by arrows. (c) RT-PCR analysis of CCR4 mRNA. Spleen (lanes 1–4) and thymus (lanes 5–8) of $CCR4^{+/+}$ (lanes 1, 2, 5, and 6) and $CCR4^{-/-}$ mice (lanes 3, 4, 7, and 8). (d) RT-PCR analysis of CCR4 mRNA in peritoneal macrophages isolated from $CCR4^{+/+}$ (lanes 1–3) and $CCR4^{-/-}$ mice (lanes 4–6). The predicted band size of CCR4 PCR product indicated by the arrow is 1.1 kb. (e) Chemotaxis of splenocytes in response to CCR4 ligands. $CCR4^{+/+}$ (open circles) and $CCR4^{-/-}$ mice (filled circles). Results shown are the means of triplicate determinations for each concentration of chemokine, and are representative of at least four experiments.

The effect of the CCR4 deletion in vivo was studied using an OVA-induced murine model of airway inflammation, a predominantly Th2-associated response (22). Repeated intranasal OVA challenges of immunized $CCR4^{+/+}$ and $CCR4^{-/-}$ littermates resulted in a significant increase in BHR in response to inhaled MCh (3×10^{-2} M) compared with saline-challenged mice (Fig. 2 a). Penh values were 0.8 ± 0.1 in saline compared with 1.77 ± 0.2 in OVA-challenged $CCR4^{+/+}$ mice, and 0.64 ± 0.11 in saline compared with 1.88 ± 0.33 in OVA-challenged $CCR4^{-/-}$ mice. Comparable OVA-induced eosinophilia was observed both in BALF (Fig. 2 b) and in lung tissue (data not shown) of $CCR4^{+/+}$ and $CCR4^{-/-}$ littermates, a finding consistent with the induction of a Th2 response in the airways. In addition, no significant differences were observed in the BAL fluid (BALF) in either the total cell count or individual leukocyte populations (eosinophils, macrophages, lymphocytes, and neutrophils) between OVA-challenged $CCR4^{+/+}$ and $CCR4^{-/-}$ littermates (Fig. 2 b). To confirm that efficient antigen priming had occurred in the periphery, serum titers of OVA-specific IgM, IgG1, IgG2a, and IgE were measured. Again, all OVA-specific Ig titers were comparable in $CCR4^{+/+}$ and $CCR4^{-/-}$ littermates after OVA sensitization and challenge (Fig. 2 c). Taken together, our results suggest that deletion of the CCR4 gene does not impair the development of a Th2 response in vivo in this model. This is in contrast to the data recently reported by Gonzalo et al. (23), in which the administration of polyclonal antibodies against MDC, one of the ligands for CCR4, protected against eosinophilia and BHR. However, there are several explanations for the differences between the results. First, there may be an alternative receptor for MDC on activated cells. Second, the genetic background of mice used in each study may differentially influence susceptibility. Third, the polyclonal antibodies used may have depleted MDC binding cells. These hypotheses will be tested when specific mAbs against murine MDC become available.

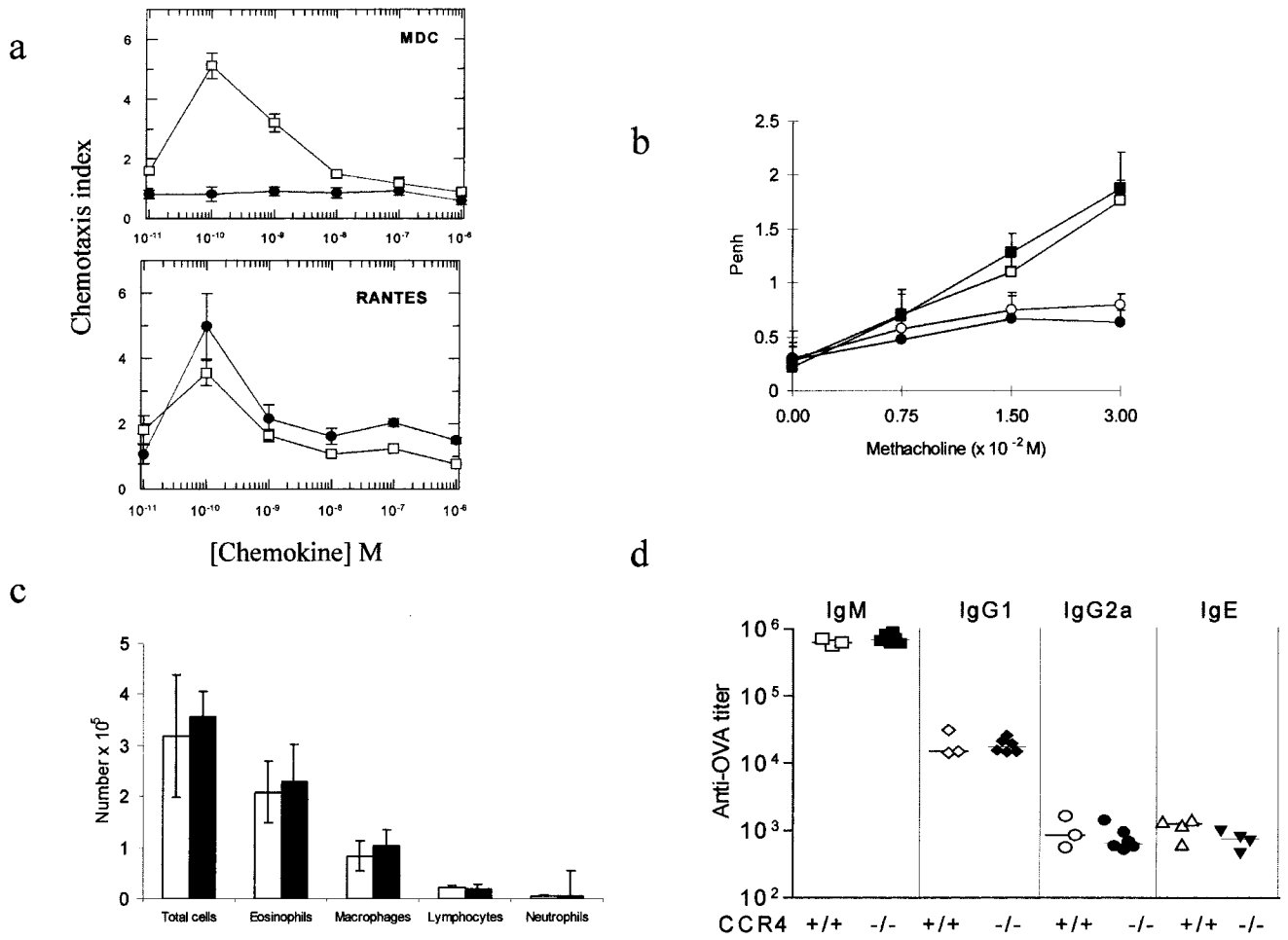

Figure 2. Effect of CCR4 deletion on Th2 responses in vitro and in vivo. (a) Chemotactic response of in vitro–derived Th2 T cells to MDC (top) and RANTES (bottom). $CCR4^{+/+}$ (open symbols) and $CCR4^{-/-}$ (filled symbols). CCR4-deficient mice develop allergic airway inflammation. (b) Airway reactivity in response to MCh in $CCR4^{+/+}$ (open symbols) and $CCR4^{-/-}$ (filled symbols) mice after OVA priming followed by intranasal challenge with saline (circles, $n = 10$) or OVA (squares, $n = 13$). (c) Total cell count and individual leukocyte populations in BALF. $CCR4^{+/+}$ (white bars, $n = 13$) and $CCR4^{-/-}$ (black bars, $n = 13$). (d) OVA-specific Ig serum titers of OVA-primed and challenged mice were measured by ELISA. Data are shown for one experiment representative of three different experiments, for each parameter measured.

CCR4 is also expressed on other cell types, such as platelets (9), monocytes (5, 24), and macrophages (Parums, D., and Power, C.A., unpublished data; see also Fig. 1 d). Therefore, we assessed the effect of the CCR4 deletion during LPS-induced endotoxic shock, an inflammatory model in which these cells types have been implicated (25, 26). LPS (60–120 mg/kg) was injected intraperitoneally into $CCR4^{+/+}$ and $CCR4^{-/-}$ littermates and survival was assessed daily for 6 d (Fig. 3 a). All $CCR4^{+/+}$ mice died between 2 and 4 d after LPS injection. In contrast, 14 out of 15 $CCR4^{-/-}$ mice were alive on day 6, demonstrating significant resistance to 60 mg/kg LPS ($P < 0.001$). Indeed, the $CCR4^{-/-}$ mice were also strikingly resistant to LPS doses of up to 120 mg/kg. Control mice (saline-injected $CCR4^{-/-}$ mice, $n = 4$, and $CCR4^{+/+}$ mice, $n = 4$) remained alive and healthy throughout the 6-d study (data not shown). Interestingly, during the first few hours after LPS administration, $CCR4^{-/-}$ mice still showed signs of endotoxemia such as shivering and lethargy. However, these effects were visually milder than in the $CCR4^{+/+}$ mice.

Intraperitoneal injection of a high dose of LPS is followed by a marked thrombocytopenia and accumulation of platelets in the liver and spleen (27). Blood samples from $CCR4^{-/-}$ and $CCR4^{+/+}$ mice contained similar numbers of platelets. Furthermore, in an independent experiment, a superimposable decrease in blood platelet count occurred in both $CCR4^{+/+}$ ($n = 3$) and $CCR4^{-/-}$ mice ($n = 3$) in the first 20 h after injection of high dose LPS (Fig. 3 b), indicating that there was no obvious difference in platelet mobilization between the two groups. However the platelet count returned to normal in the $CCR4^{-/-}$ mice by 5 d after treatment.

As a comparison, the effect of the CCR4 deletion in a low dose LPS endotoxic shock model was studied. In this model, the susceptibility of mice to a low doses of LPS (1, 2, and 4 μg) is enhanced by coinjection of 8 mg of D-gal (28). Within 24 h of intraperitoneal injection of the LPS

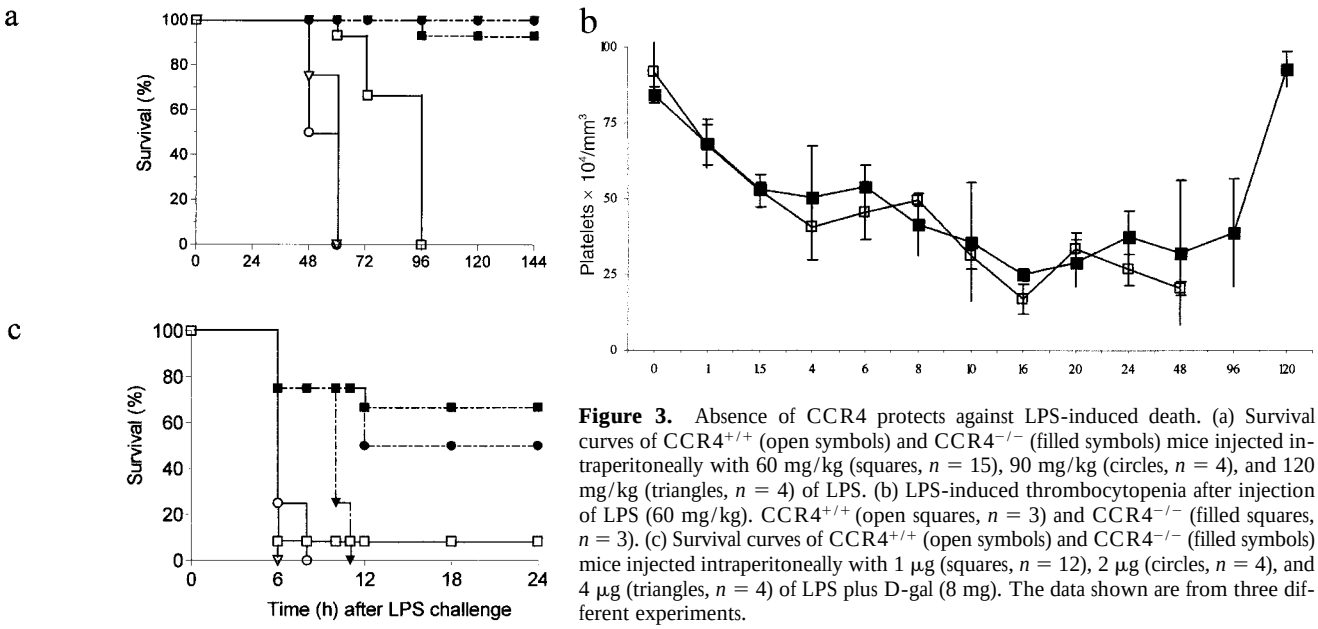

Figure 3. Absence of CCR4 protects against LPS-induced death. (a) Survival curves of $CCR4^{+/+}$ (open symbols) and $CCR4^{-/-}$ (filled symbols) mice injected intraperitoneally with 60 mg/kg (squares, $n = 15$), 90 mg/kg (circles, $n = 4$), and 120 mg/kg (triangles, $n = 4$) of LPS. (b) LPS-induced thrombocytopenia after injection of LPS (60 mg/kg). $CCR4^{+/+}$ (open squares, $n = 3$) and $CCR4^{-/-}$ (filled squares, $n = 3$). (c) Survival curves of $CCR4^{+/+}$ (open symbols) and $CCR4^{-/-}$ (filled symbols) mice injected intraperitoneally with 1 μg (squares, $n = 12$), 2 μg (circles, $n = 4$), and 4 μg (triangles, $n = 4$) of LPS plus D-gal (8 mg). The data shown are from three different experiments.

and D-gal combination, only 1 in 12 of the $CCR4^{+/+}$ mice survived, whereas 9 out of 12 of the $CCR4^{-/-}$ littermates survived when treated with 1 μg of LPS plus D-gal ($P < 0.02$; Fig. 3 c), also demonstrating increased resistance to low dose LPS. However, when administered at 4 μg of LPS with D-gal, all $CCR4^{-/-}$ mice tested died but with a 6-h delay compared with the wild-type mice.

LPS is known to stimulate the release of proinflamma-

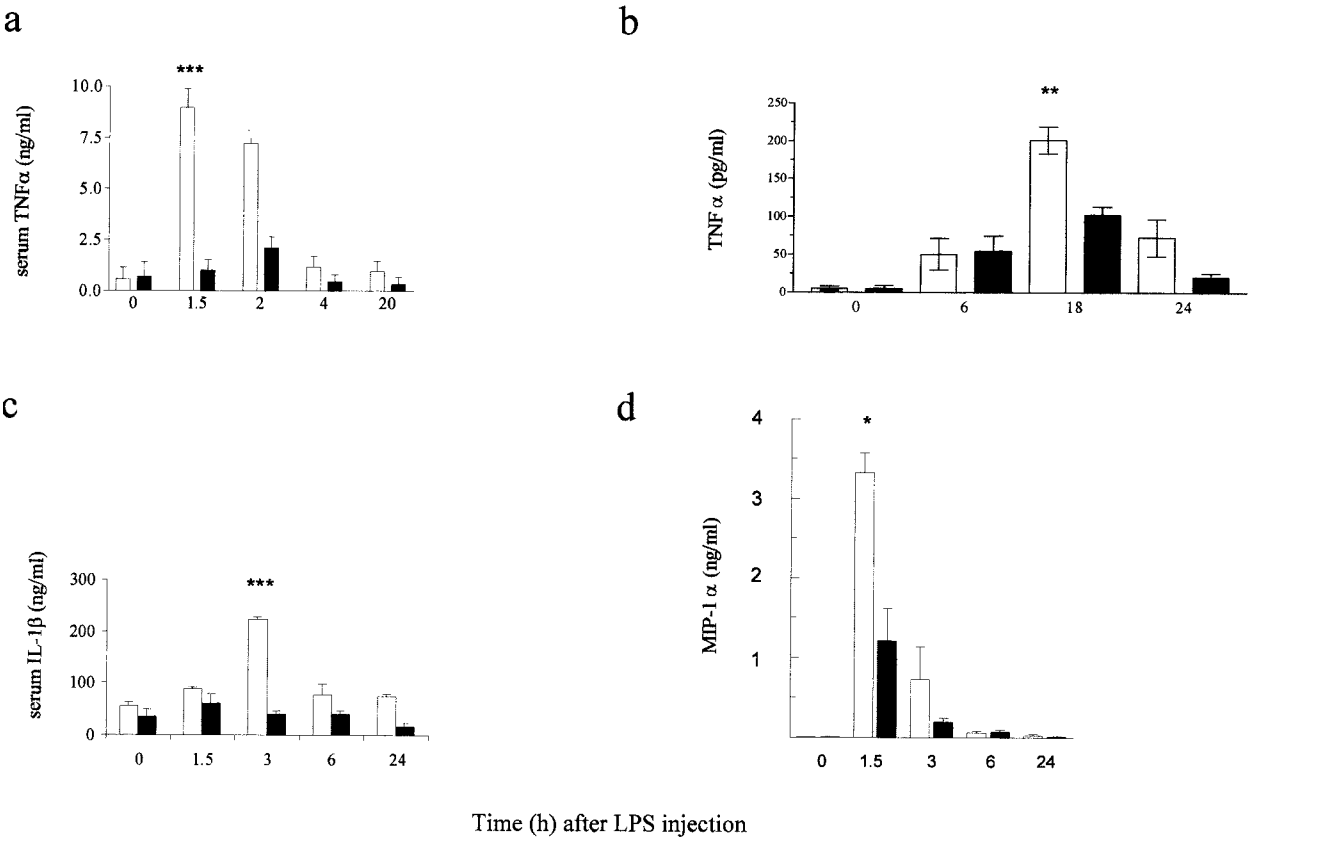

Figure 4. Time course of LPS-induced cytokines. Serum levels of TNF-α (a), IL-1β (c), and MIP-1α (d) after 60 mg/kg LPS treatment; and in vitro production of TNF-α by peritoneal lavage cells stimulated with 1 μg/ml LPS (b). $CCR4^{+/+}$ mice (white bars, $n = 3$) and $CCR4^{-/-}$ mice (black bars, $n = 3$). Results shown are representative of at least two different experiments.

tory cytokines such as TNF-α and IL-1β from monocytes, macrophages, and neutrophils (29). Therefore, the effect of the high dose LPS response was studied in more detail. A sharp increase in serum TNF-α levels was observed in $CCR4^{+/+}$ mice 1.5 h after LPS injection, which returned to baseline by 4 h after treatment. Interestingly, $CCR4^{-/-}$ mice failed to induce significant levels of serum TNF-α after LPS injection ($P < 0.002$ at 1.5 h; Fig. 4 a). Cells isolated by peritoneal lavage from naive $CCR4^{-/-}$ mice produced significantly lower levels of TNF-α when cultured for 18 h in the presence of LPS, compared with cells isolated from naive $CCR4^{+/+}$ mice (107 ± 11 compared with 201 ± 18 pg/ml, $P < 0.0079$; Fig. 4 b). In addition, a sixfold decrease in serum IL-1β in $CCR4^{-/-}$ mice was observed 3 h after LPS injection ($P < 0.002$) compared with $CCR4^{+/+}$ mice (Fig. 4 c). This suggests that the observed resistance to LPS may in part be due to decreased TNF-α and IL-1β production, implying that CCR4 is indirectly involved in the production of these cytokines. In contrast, IL-6 production was unaltered (data not shown), suggesting that in $CCR4^{-/-}$ mice the regulation of IL-6 can be independent from that of TNF-α and IL-1β. Further analysis of the $CCR4^{-/-}$ samples also revealed a parallel decrease in serum MIP-1α levels ($P < 0.0249$ at 1.5 h; Fig. 4 d), pointing to a possible macrophage defect.

The cellular composition of the peritoneal lavage was therefore assessed at various times after injection of high dose LPS. No significant differences were seen in the total number of cells at early time points. At 24 h after high dose LPS treatment, the number of neutrophils detected in the lavage of both groups of mice was comparable (Fig. 5 a). This is as expected, since to date there is no evidence for the expression of CCR4 on neutrophils. Furthermore, we have shown that neutrophils isolated from the peritoneal cavity of wild-type mice after thioglycollate treatment do not respond to CCR4 ligands in chemotaxis assays. In addition, thioglycollate-elicited neutrophils from $CCR4^{-/-}$ mice respond normally to MIP-1α and MIP-2 (our unpub-

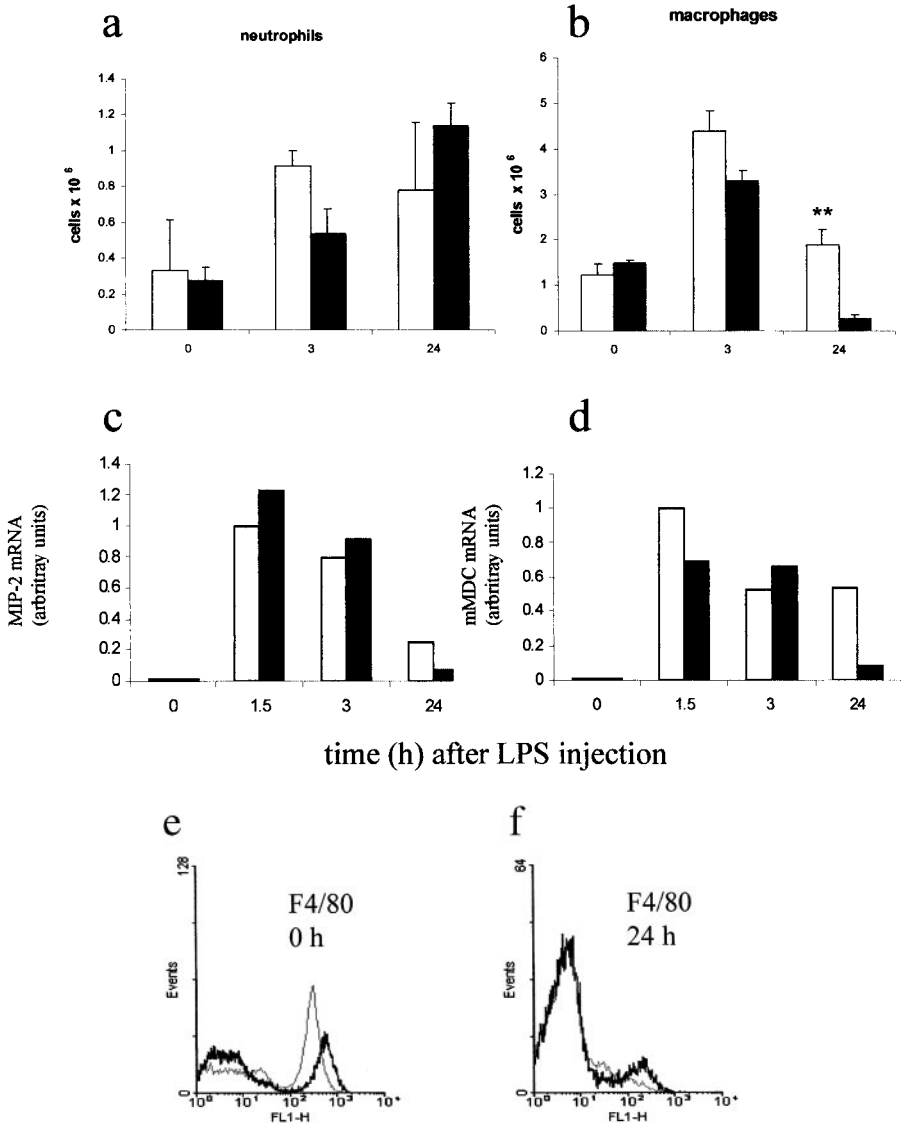

Figure 5. Analysis of peritoneal lavage cells after high dose LPS treatment. Quantitation of (a) neutrophils and (b) macrophages on cytospin slides prepared from the peritoneal lavage at times indicated. Effect of LPS treatment on macrophage chemokine expression by peritoneal cells as measured by semiquantitative RT-PCR: (c) MIP-2 and (d) MDC. $CCR4^{+/+}$ mice (white bars, $n = 3$) and $CCR4^{-/-}$ mice (black bars, $n = 3$). Analysis of F4/80 expression by peritoneal cells using flow cytometry (e) before LPS treatment, and (f) 24 h after LPS treatment. $CCR4^{+/+}$ (thick black line) and $CCR4^{-/-}$ (thin black line). The results shown represent the results of one out of two independent experiments.

lished data). However, $CCR4^{-/-}$ mice had significantly fewer macrophages than $CCR4^{+/+}$ mice ($P < 0.0099$; Fig. 5 b). These findings were consistent with the observed decrease in the mRNA expression of the macrophage-associated chemokines MIP-2 (30) and MDC (31) (Fig. 5, c and d). We confirmed the apparent differences in macrophage numbers seen on the cytospins by FACS® analysis for macrophage markers. We found decreased numbers of $CD11b^+$ and $CD14^+$ cells (data not shown) and an absence or severely reduced number of F4/80-expressing cells in the $CCR4^{-/-}$ mice compared with the $CCR4^{+/+}$ mice 24 h after LPS treatment (Fig. 5 f). However, $F4/80^+$ cells were detected in naive mice (Fig. 5 e) as well as at earlier time points after LPS injection in the $CCR4^{-/-}$ mice (data not shown). These results may imply a defect in the ability of $CCR4^{-/-}$ mice to retain a particular macrophage population expressing F4/80, in the peritoneum, which in turn may confer LPS resistance in $CCR4^{-/-}$ mice.

The F4/80 antigen is an unusual seven-transmembrane receptor in that its extracellular domain is composed of EGF domain repeats. Its ligand(s) and precise function remain unknown (32), but while it is well established that activated macrophages express F4/80, a population of $CD5^+$ peritoneal B cells has also recently been shown to express this marker (33). Interestingly, we also noted a decrease in the number of lymphocytes in the peritoneal lavage 24 h after high dose LPS treatment in $CCR4^{-/-}$ mice (results not shown). However, the identity of the affected lymphocyte population and its contribution, if any, to the mechanism of LPS resistance in $CCR4^{-/-}$ mice awaits further investigation.

Resistance to LPS-induced lethality has now been demonstrated in several gene-deleted mice, including macrophage migration inhibition factor (MIF [34]), intercellular adhesion molecule 1 (ICAM-1 [35]), and TNF-RI (36), although CCR4-deficient mice are unique in that they can be resistant in both the high and low dose models of LPS-induced endotoxic shock. A great deal is known about the proinflammatory cytokine production after LPS treatment, yet relatively little about the signal transduction pathway by which LPS induces host cell activation. In the currently accepted model, LPS monomers are catalytically transferred by a lipid exchange molecule, LBP (37), to CD14 (38). The LPS transmembrane coreceptor, Toll-like receptor 4 (Tlr4), interacts with the LPS–CD14 complex and initiates LPS signaling (39). The mechanism by which CCR4-deficient mice are resistant to LPS is unclear, but may be due to a difference in downstream signaling events. The use of gene microarrays may help to elucidate the differences between the $CCR4^{+/+}$ and $CCR4^{-/-}$ mice in this respect.

In conclusion, we have shown that 24 h after high dose LPS treatment there is a significant reduction in the numbers of macrophages found in the peritoneum of $CCR4^{-/-}$ mice. At the same time, we noted a decrease in macrophage-associated serum cytokines TNF-α, IL-1β, and MIP-1α. In addition, peritoneal lavage cells had decreased levels of mRNA for the chemokines MDC and MIP-2, which are thought to be produced mainly by activated macrophages. Taken together, these results are consistent with either a defect in macrophage function or the absence of a specific population, which is supported by the disappearance of the $F4/80^+$ population at 24 h after LPS treatment. Identification of the precise mechanisms of LPS-induced cell stimulation is important for our understanding of bacterial pathogenesis and for the development of strategies to protect against gram-negative bacterial infection. Targeted deletion of the CCR4 gene has revealed an unexpected role for this receptor in LPS-induced endotoxic shock. Thus, although there is only limited relevance of the endotoxic shock models used here in mice, to the condition of septic shock in humans, it remains to be seen whether neutralization of CCR4 either by antibodies or chemokine receptor antagonists will have any therapeutic effect in vivo. Further studies with this animal model should also help elucidate the role of CCR4 in immunity, inflammation, and other biological functions.

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