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

By Yolande Chvatchko, Arlene J. Hoogewerf, Alexandra Meyer, Sami Alouani, Pierre Juillard, Raphaele Buser, Francois Conquet, Amanda E.I. Proudfoot, Timothy N.C. Wells, and Christine A. Power

From the Serono Pharmaceutical Research Institute, 1228 Plan-les-Ouates/Geneva, Switzerland

Abstract

CC chemokine receptor (CCR4), 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<sup>−/−</sup>) mice by gene targeting. CCR4<sup>−/−</sup> mice developed normally. Splenocytes and thymocytes isolated from the CCR4<sup>−/−</sup> 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<sup>−/−</sup> mice exhibited significantly decreased mortality on administration of high or low dose bacterial lipopolysaccharide (LPS) compared with CCR4<sup>+/+</sup> mice. After high dose LPS treatment, serum levels of tumor necrosis factor α, interleukin 1β, and MIP-1α were reduced in CCR4<sup>−/−</sup> mice, and decreased expression of MDC and MIP-2 mRNA was detected in peritoneal exudate cells. Analysis of peritoneal lavage cells from CCR4<sup>−/−</sup> mice by flow cytometry also revealed a significant decrease in the F4/80<sup>+</sup> cell population. This may reflect a defect in the ability of the CCR4<sup>−/−</sup> 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<sub>3</sub>C. The majority of chemokines belong to the CC and CXC subfamilies. For the C and CX<sub>3</sub>C 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<sub>3</sub>C, 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.
We have previously reported the molecular cloning of both human and murine CC chemokine receptor (CCR) 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), CCR 4 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). CCR 4 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 CCR 4 is highly expressed by human T cells polarized towards the Th2 phenotype (10–12). These Th2 cells are also responsive to the CCR 4 ligands TARC and MDC, leading investigators to suggest a role for CCR 4 in the development of Th2 responses. Finally, a recent report has shown that CCR 4 is also expressed on all skin homing T cells expressing cutaneous lymphocyte antigen (CLA) (13).

To study the role of CCR 4 in vivo, we have generated CCR 4-deficient mice by gene targeting. Here, we show that in spite of the reported high level expression of CCR 4 on human Th2-polarized cells, CCR 4 deletion had no effect on the development of the response in a classical Th2-dependent murine model of airway inflammation. However, CCR 4−/− mice showed an unexpected resistance to the lethal effects of LPS in two models of LPS-induced endotoxic shock.

Materials and Methods

Generation of CCR 4-deficient Mice. The murine (m)CCR 4 gene was isolated from an HM-1 embryonic stem (ES) cell library in λFIXII vector (Stratagene) by plaque hybridization using murine CCR 4 cDNA as a probe (6). Two unique clones of 12.18 and 13.08 kb were shown to contain the CCR 4 coding sequence by PCR, using specific primers (6). An 8.5-kb fragment of genomic DNA confirmed to contain the CCR 4 coding sequence by Southern blotting was subcloned into plBuescript II SK− to generate pCCR 4 . The entire CCR 4 coding sequence was then removed as an Nhel-Hpal fragment and replaced with a neo cassette (derived from pMCINEOPlOA; 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-Xh site of the plasmid to produce the targeting vector. The targeting vector was linearized with NolI 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 PsI-digested genomic DNA, using a 466-bp probe derived by AvrII-Nsil digestion of pCCR 4 . 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 CCR 4+/− mice, and littermates from the matings of CCR 4+/− mice were analyzed for the presence of homozygous CCR 4−/− mice by Southern blot analysis or by PCR on tail DNA. The sequences of the PCR primers used are: (primer 1, neomycin gene) 5′-CGCT-TGCTGTGTTACGGAT; (primer 2, CCR 4 3′ untranslated region) 5′-ATAGCGTTGCTGTCGAGAAT; (primer 3, mCCR 4 coding region sense primer) 5′-CCAAA-GTGAATGCTGCAAGGCAG; (primer 4, mCCR 4 coding region antisense primer) 5′-TTACAAACGCTGACGAAATCATG.

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 1010 peritoneal exudates was reverse transcribed using Superscript™ (GIBCO BRL) and oligo dT12–18 primer according to the manufacturer’s instructions. Oligonucleotide primers for the CCR 4 cDNA synthesis reaction was then subjected to 25 cycles of PCR using AmpliTaq™ (PerkinElmer) and PCR primers based on the EMBL/GenBank/DDB database entries for MIP-2 (X53798), MDC (AF052505), and MCCR 4 (X90862). PCR products were analyzed on 1% agarose gels stained with ethidium bromide, and bands migrating at the correct molecular weight were visualized by direct sequencing. Semiquantitative reverse transcriptase (RT)-PCR, bands were quantitated using Kodak Digital Science 1.0 software and results are expressed as arbitrary units of mrNA.

In Vitro Differentiation of Th1 and Th2 Cells. CD8− and IgG-depleted cells from lymph nodes and spleens of naïve CCR 4−/− and CCR 4+/− 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 2×106 cells per well on an anti-CD3-coated 96-well plate (Costar) in the presence of murine IL-2 (50 U/ml) and R&D Systems.

Animals and Treatments. Homozygous CCR 4−/− mice were backcrossed with C57BL/6 mice (Centre d’Elevage Janvier) for four generations. Age- and weight-matched CCR 4+/− and CCR 4+/− littermates from C57BL/6 litters were used to produce chimeric mice by blastocyst injection 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% FOR ENE™ (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).
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 CO₂ 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).

A analysis of Blood and Lavage Fluids. Blood (20 μl) was collected from the retroorbital plexus of mice into a heparinized microcuvette, then transferred to a Uno petri dish collection system for platelet determination (Becton Dickinson). Three independent samples of platelet samples 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 standardized 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 by cytosine 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 10⁶ 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 asayed were performed using the micro-Boyden chamber method with 5-μm filters (21). R 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. 1a. Targeted ES cells were used to generate chimeric mice, in 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. 1b). 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. 1c), or peritoneal cells (Fig. 1d) 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. 1e) 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. 1e) 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. 1e). M urine 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 in vitro. 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. 2a). 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. 2a).
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).

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; ES, EcoRV; Ec, Eco47-3; H, Hpal; 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 in peritoneal macrophages isolated from CCR4+/+ (lanes 1–3) and CCR4−/− (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.
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$^{1/1}$ and CCR4$^{2/2}$ littermates and survival was assessed daily for 6 d (Fig. 3 a). All CCR4$^{1/1}$ mice died between 2 and 4 d after LPS injection. In contrast, 14 out of 15 CCR4$^{2/2}$ mice were alive on day 6, demonstrating significant resistance to 60 mg/kg LPS ($P$, 0.001). Indeed, the CCR4$^{1/1}$ mice were also strikingly resistant to LPS doses of up to 120 mg/kg. Control mice (saline-injected CCR4$^{2/2}$ mice, $n = 4$, and CCR4$^{2/2}$ 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$^{2/2}$ mice still showed signs of endotoxemia such as shivering and lethargy. However, these effects were visually milder than in the CCR4$^{1/1}$ 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$^{1-/-}$ and CCR4$^{2+/-}$ mice contained similar numbers of platelets. Furthermore, in an independent experiment, a superimposable decrease in blood platelet count occurred in both CCR4$^{1-/-}$ ($n = 3$) and CCR4$^{2+/-}$ 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$^{2+/-}$ 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 $\mu$g) is enhanced by coinjection of 8 mg of D-gal (28). Within 24 h of intraperitoneal injection of the LPS
1760 CCR4 Knockout Mice Are Resistant to Endotoxic Shock

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 $\mu$g of LPS plus D-gal ($P < 0.02$; Fig. 3 c), also demonstrating increased resistance to low dose LPS. However, when administered at 4 $\mu$g 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 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 $\mu$g (squares, n = 12), 2 $\mu$g (circles, n = 4), and 4 $\mu$g (triangles, n = 4) of LPS plus D-gal (8 mg). The data shown are from three different experiments.

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 $\mu$g/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 <sup>−/−</sup> mice 1.5 h after LPS injection, which returned to baseline by 4 h after treatment. Interestingly, CCR4 <sup>−/−</sup> mice failed to induce significant levels of serum TNF-α after LPS injection (<i>P</i> < 0.002 at 1.5 h; Fig. 4 a). Cells isolated by peritoneal lavage from naïve CCR4 <sup>−/−</sup> mice produced significantly lower levels of TNF-α when cultured for 18 h in the presence of LPS, compared with cells isolated from naïve CCR4 <sup>+/+</sup> mice (107 ± 11 compared with 201 ± 18 pg/ml, <i>P</i> < 0.0079; Fig. 4 b). In addition, a sixfold decrease in serum IL-1β in CCR4 <sup>−/−</sup> mice was observed 3 h after LPS injection (<i>P</i> < 0.002) compared with CCR4 <sup>+/+</sup> 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 <sup>−/−</sup> mice the regulation of IL-6 can be independent from that of TNF-α and IL-1β. Further analysis of the CCR4 <sup>−/−</sup> samples also revealed a parallel decrease in serum MIP-1α levels (<i>P</i> < 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 <sup>−/−</sup> 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 <sup>+/+</sup> mice (white bars, n = 3) and CCR4 <sup>−/−</sup> 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 <sup>+/+</sup> (thick black line) and CCR4 <sup>−/−</sup> (thin black line). The results shown represent the results of one out of two independent experiments.
lished data). However, CCR 4−/− mice had significantly fewer macrophages than CCR 4+/+ 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 cytopsins 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 CCR 4−/− mice compared with the CCR 4+/+ 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 CCR 4−/− mice (data not shown). These results may imply a defect in the ability of CCR 4−/− mice to retain a particular macrophage population expressing F4/80, in the peritoneum, which in turn may confer LPS resistance in CCR 4−/− 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 CCR 4−/− mice (results not shown). However, the identity of the affected lymphocyte population and its contribution, if any, to the mechanism of LPS resistance in CCR 4−/− 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-R1 (36), although CCR 4-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 role of CCR 4 in immunity, inflammation, and other biological functions. The authors wish to thank Dr. Marie Kosco-Vilbois for critical reading of the manuscript, and Prof. Thomas Martin, Dr. Jerome Pugin, Dr. Manfred Kopf, and Dr. Irene Garcia for helpful discussions.

Submitted: 20 September 1999
Revised: 6 March 2000
Accepted: 8 March 2000

References
1. Luster, A.D. 1998. Chemokines—chemotactic cytokines that mediate inflammation. N. Engl. J. Med. 338:436–445.
2. Ward, S.G., and J. Westwick. 1998. Chemokines—understanding their role in T-lymphocyte biology. Biomed. J. 333:457–470.
3. Proudfoot, A.E., T.N. Wells and P.R. Clapham. 1999. Chemokine receptors—future therapeutic targets for HIV? Biomed. Pharmacol. 57:451–463.
4. Wells, T.N., and T.W. Schwartz. 1997. Plagiarism of the host immune system: lessons about chemokine immunology from viruses. Curr. Opin. Biotechnol. 8:741–748.
5. Power, C.A., A. Meyer, K. Nemeth, K.B. Bacon, A.J. Hoogewerf, A.E. Proudfoot, and T.N. Wells. 1995. Molecular cloning and functional expression of a novel CC chemokine receptor cDNA from a human basophilic cell line. J. Biol. Chem. 270:19495–19500.
6. Hoogewerf, A., D. Black, A.E. Proudfoot, T.N. Wells, and C.A. Power. 1996. Molecular cloning of murine CC CKR-4 and high affinity binding of chemokines to murine and human CC CKR-4. Biomed. Biophys. Res. Commun. 218:337–343.
7. Imai, T., M. Baba, M. Nishimura, M.T. Kikazaki, and O. Yoshie. 1999. The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. J. Biol. Chem. 274:15036–15042.
8. Imai, T., D. Chantry, C.J. R aport, C.L. Wood, M. Nishimura, R. Godiska, O. Yoshie, and P.W. Gray. 1998. Macrophage-derived chemokine is a functional ligand for the CC chemokine receptor 4. J. Biol. Chem. 273:1764–1768.
9. Power, C.A., J.M. Clemetson, K.J. Clemetson, and T.N. Wells. 1995. Chemokine and chemokine receptor mRNA expression in human platelets. Cytokine. 7:479–482.
10. Sallusto, F., D. Lenig, C.R. Mackay, and A. Lanzavecchia.
1998. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. J. Exp. Med. 187:875–883.

11. Bonecchi, R., G. Bianchi, P.P. Bordignon, D. D’Ambrosio, R. Lang, A. Borsatti, S. Sozzani, P.P. Allavena, P.A. Gray, A. M antovani, 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.

12. D’Ambrosio, D., A. Iellem, R. Bonecchi, D. Mazzeo, S. Sozzani, A. Mantovani, and F. Sinigaglia. 1998. Selective up-regulation of chemokine receptors CCR 4 and CCR 8 upon activation of polarized human type 2 T cells. J. Immunol. 161:5111–5115.

13. Campbell, J.J., G. Haraldsen, J. Pan, J. R ottman, S. Qin, P. Ponath, D.P. Andrew, R. Wamke, N. R ufing, N. Kassam, et al. 1999. The chemokine receptor CCR 4 in vascular recognition by cutaneous but not intestinal memory T cells. Nature. 400:776–780.

14. Conquet, F., Z.I. Bashir, C.H. Davies, H. Daniel, F. Ferra guti, F. Bordi, K. Franz-Bacon, A. Reggiani, V. M atarese, and F. Conde. 1994. Motor deficit and impairment of synaptic plasticity in mice lacking mGlur 1. Nature. 372:237–243.

15. Kopf, M., G. Le Gros, M. Bachmann, M.C. Lamers, H. Bluethmann, and G. Kohler. 1993. Disruption of the murine IL-6 gene blocks Th2 cytokine responses. Nature. 362:245–248.

16. Tsuyuki, S., J. Tsuyuki, K. Einsle, M. Kopf, and A.J. Coyle. 1999. The chemokine receptor CCR4 in vascular recruitment and altered airway responsiveness. J. Exp. Med. 185:1671–1679.

17. Hamelmann, E., J. Schwarze, K. Takeda, A. O shiba, G.L. Larsen, C.G. Irvin, and E.W. Gelfand. 1997. Non-invasive measurement of airway responsiveness in allergic mice using barometric plethysmography. Am. J. Respir. Crit. Care Med. 156:766–775.

18. Chvatchko, Y., M.H. Kosco-Vilbois, S. Herren, J. Lefort, and J.-Y. Bonnefoy. 1996. Germinal center formation and local immunoglobulin E (IgE) production in the lung after an airway antigenic challenge. J. Exp. Med. 184:2353–2360.

19. Tacchini-Cottier, F., C. Vesin, M. Redard, W. Buurman, and P.F. Piguet. 1998. Role of T NF R 1 and T NF R 2 in TNF-induced platelet consumption in mice. J. Immunol. 160:6182–6186.

20. Lusti-Narayanan, M., A. Chollet, C.A. Power, B. Allet, A.E. Proudfoot, and T.N.C. Wells. 1999. Amino terminally modified RANTES analogues demonstrate differential effects on RANTES receptors. J. Biol. Chem. 274:32478–32485.

21. Pajkrt, D. and S.J. van Deventer. 1996. The cellular response in sepsis. Curr. Top. Microbiol. Immunol. 216:119–132.

22. Freudenberg, M.A., D. Keppler, and C. Galanos. 1986. Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin. Infect. Immun. 51:891–895.

23. Shibazaki, M., M. Nakamura, and Y. Endo. 1996. Biphasic, organ-specific, and strain-specific accumulation of platelets induced in mice by a lipopolysaccharide from Escherichia coli and its possible involvement in shock. Infect. Immun. 64:5290–5294.

24. Galanos, C., M.A. Freudenberg, and W. Reutter. 1979. Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc. Natl. Acad. Sci. USA. 76:5939–5943.

25. Gutierrez-Ramos, J.C. and H. Bluethmann. 1997. Molecules and mechanisms operating in septic shock: lessons from knockout mice. Immunol. Today. 18:329–334.

26. Wallen, K.R., N.W. Lukacs, T.J. Sandford, R.M. Strieter, and S.L. Kunkel. 1997. Elevated levels of macrophage inflammatory protein 2 in severe murine peritonitis increase neutrophil recruitment and mortality. Infect. Immun. 65:3847–3851.

27. Godiska, R., D. Chantry, C.J. Raport, S. Sozzani, P. Allavena, D. Leviten, A. Mantovani, and P.W. Gray. 1997. Human macrophage-derived chemokine (MDC), a novel chemotacticant for monocytes, monocyte-derived dendritic cells, and natural killer cells. J. Exp. Med. 185:1595–1604.

28. McKnight, A.J., A.J. Macfarlane, P. Dri, L. Turley, A.C. Willis, and S. Gordon. 1996. Molecular cloning of F4/80, a murine macrophage-restricted cell surface glycoprotein with homology to the G-protein-linked transmembrane 7 hormone receptor family. J. Biol. Chem. 271:486–489.

29. Borrello, M.A., and R.P. Phipps. 1996. The B/macrophage cell: an elusive link between CD5+ B lymphocytes and macrophages. Immunol. Today. 17:471–475.

30. Bozza, M., A.R. Satoskar, G. Lin, B. Lu, A.A. Humbles, C. Gerard, and J.R. David. 1999. Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis. J. Exp. Med. 189:341–346.

31. Xu, H., J.A. Gonzalez, Y. St. Pierre, I.R. Williams, T.S. Kuper, R.S. Cotran, T.A. Springer, and J.C. Gutierrez-Ramos. 1994. Leukocytosis and resistance to septic shock in intracellular adhesion molecule 1-deficient mice. J. Exp. Med. 180:95–109.

32. Pfeffer, K., S.D. Wright, R.A. Ramos, P.S. Tobias, R.J. Ulevitch, and J. Innis. 1989. Lipopolysaccharide (LPS) binding protein on mouse macrophages. J. Exp. Med. 180:1231–1241.

33. Bozza, M., A.R. Satoskar, G. Lin, B. Lu, A.A. Humbles, C. Gerard, and J.R. David. 1999. Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis. J. Exp. Med. 189:341–346.

34. Wright, S.D., R.S. Cotran, T.A. Springer, and J.C. Gutierrez-Ramos. 1994. Leukocytosis and resistance to septic shock in intracellular adhesion molecule 1-deficient mice. J. Exp. Med. 180:95–109.

35. Pfeffer, K., R.A. Ramos, and J.C. Gutierrez-Ramos. 1993. Mice deficient for the 55 kd TNF receptor are resistant to endotoxic shock. J. Exp. Med. 170:1231–1241.

36. Bozza, M., A.R. Satoskar, G. Lin, B. Lu, A.A. Humbles, C. Gerard, and J.R. David. 1999. Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis. J. Exp. Med. 189:341–346.

37. Xu, H., J.A. Gonzalez, Y. St. Pierre, I.R. Williams, T.S. Kuper, R.S. Cotran, T.A. Springer, and J.C. Gutierrez-Ramos. 1994. Leukocytosis and resistance to septic shock in intracellular adhesion molecule 1-deficient mice. J. Exp. Med. 180:95–109.