The Journal of Immunology

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J Immunol 1998; 160:4018-4025; ;
http://www.jimmunol.org/content/160/8/4018

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Impaired Macrophage Function and Enhanced T Cell-Dependent Immune Response in Mice Lacking CCR5, the Mouse Homologue of the Major HIV-1 Coreceptor

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The CC-chemokine receptor CCR5 has been shown to be the major coreceptor for HIV-1 entry into cells, and humans with homozygous mutation in the *ccr5* gene are highly resistant to HIV-1 infection, despite the existence of many other HIV-1 coreceptors. To investigate the physiologic function of CCR5 and to understand the cellular mechanisms of these clinical observations, we generated a CCR5-deficient mouse model (*ccr5*$^{-/-}$) by targeted deletion of the *ccr5* gene. We found that although developed normally in a pathogen-free environment, CCR5-deficient mice showed reduced efficiency in clearance of *Listeria* infection and exsert a protective effect aganist LPS-induced endotoxemia, reflecting a partial defect in macrophage function. In addition, CCR5-deficient mice had an enhanced delayed-type hypersensitivity reaction and increased humoral responses to T cell-dependent antigenic challenge, indicating a novel role of CCR5 in down-modulating T cell-dependent immune response. ***The Journal of Immunology*, 1998, 160: 4018–4025.**

Chemokines are small secreted proteins of 7 to 17 kDa (except the CX_3C chemokine (1, 2)) that induce chemotaxis of certain cell populations both in vivo and in vitro (3–5). Chemokines exert their functions through specific receptors that are expressed on different cell populations of hemopoietic origin. All chemokine receptors belong to the seven-transmembrane G protein-coupled receptor superfamily (3–5). In vitro, many chemokines bind several receptors and vice versa (4), making it difficult to understand the in vivo function of these molecules. CCR5 binds and responds in vitro to MIP-1α,[2] MIP-1β, and RANTES (6–9). However, MIP-1α and RANTES can also bind to CCR1 and CCR3 (murine) (10–14). To date, CCR5 is the only known receptor for MIP-1β (6–9). In vivo, MIP-1α has been shown to be involved in the pathogenesis of type II collagen-induced arthritis (15, 16), the T cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis (17), and virus-induced inflammation (18). The in vivo function of MIP-1β and RANTES has not yet been well documented.

All three CCR5 ligands can function as inhibitors of HIV-1 infection (19). Recently, five groups simultaneously identified CCR5 as the major coreceptor for macrophage-tropic HIV-1 isolates (20–24). Another chemokine receptor, CXCR4, was identified as a coreceptor for T cell-tropic HIV-1 isolates (25). Two other CC-chemokine receptors, CCR2b and CCR3, have also been shown to function as coreceptors for some, but not all HIV-1 strains (21, 23). Recently, several orphan chemokine receptors were shown to be able to function as HIV cofactors (26, 27). Moreover, a human CMV-encoded CC-chemokine receptor (US28) can also serve as a cofactor for HIV (28). Taken together, these findings suggest that HIV-1 may use a broad range of coreceptors for infection.

Nevertheless, CCR5 has been shown to be the essential receptor in the establishment of HIV-1 infection. A 32-bp deletion allele of the *ccr5* gene (Δ*ccr5*) was detected at a frequency of 20% heterozygosity and 1% homozygosity in the Caucasian population (29, 30). Homozygosity for the Δ*ccr5* allele is strongly associated with the resistance to HIV-1 infection (29–32). Heterozygosity for Δ*ccr5* leads to prolonged AIDS-free survival time, diminished viral load, and slower decrease in their $CD4^+$ T cell count, suggesting that Δ*ccr5* allele exerts a protective effect on these infected individuals (33, 34). However, once AIDS is diagnosed, those individuals tend to have an accelerated decrease in $CD4^+$ cell counts and reduced survival time, suggesting a dual effect of the Δ*ccr5* allele (35).

Whereas the intensive study of the relation between CCR5 and HIV-1 has shed light on the mechanisms of HIV-1 entry into cells, the normal in vivo role of this receptor is still unknown. In the present study, we report a partial defect in macrophage function and an enhanced T cell-dependent (TD) immune response in the CCR5-deficient mice. Our results suggest that, in addition to the lack of HIV-1 docking molecule, the overactive immune system may also contribute to the protection effect in the CCR5 null and CCR5 heterozygous humans.

Materials and Methods

Targeted disruption of the mouse ccr5 *gene*

A genomic clone containing the *ccr5* locus was isolated from a mouse library (cloned in λDASHII from Stratagene, La Jolla, CA) prepared from D3 (129/sv) ES cell DNA. The targeting vector was constructed in the pPNT vector for positive/negative selection with G418 (the *n*E*o* gene) and

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Received for publication September 5, 1997. Accepted for publication December 11, 1997.

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[2] Abbreviations used in this paper: MIP, macrophage-inflammatory protein; DTH, delayed-type hypersensitivity; ^{125}I-MIP-1α, ^{125}I-labeled macrophage-inflammatory protein-1α; KLH, keyhole limpet hemocyanin; NP, (4-hydroxy-3-nitro-phenyl) acetic acid; TD, T cell-dependent; TI, T cell-independent.

0022-1767/98/$02.00

gancyclovir (the *tk* gene). A 7.5-kb *Cla*I-*Sal*I fragment of the *ccr5* clone was subcloned into the 3′ flanking region of the *neo* cassette, and a 3.8-kb *Pst*I-*Pst*I fragment was subcloned into the 5′ flanking region of the *neo* cassette. As a result, a 0.77-kb *Cla*I-*Pst*I fragment encoding all of the seven-transmembrane domains of the *ccr5* gene was replaced by the *neo* gene. The targeting vector was electroporated into both CJ7 and R1 ES cells, and 19 targeted ES clones were obtained from 120 double-resistant colonies picked. Homologous recombination was confirmed by Southern blot analysis of tail DNA digested with *Eco*RI; the wild-type allele generates a 4-kb fragment, while the targeted allele gives an approximately 14-kb fragment. Two independent clones were selected for blastocyst injection into ICR mice. Resulting male chimeras were mated with ICR females to yield germline transmission of the targeted allele, and heterozygous offsprings were then interbred.

Cell culture, Northern blot analysis, and cytokine analysis

A quantity amounting to 1×10^6 peritoneal macrophages was cultured in medium alone (RPMI 1640 plus 10% filtered low endotoxin FBS) or in the presence of LPS (1 μg/ml) (*Escherichia coli* 0111:B4; Sigma Chemical, St. Louis, MO) and IFN-γ (100 U/ml) (Sigma Chemical) for 24 h. Splenic T cells were isolated with murine T cell enrichment columns (R&D Systems, Minneapolis, MN). Cell purity was confirmed by FACS analysis, and more than 80% of the cells were Thy-1.2$^+$. Purified T cells (1×10^6) were cultured in the presence of PMA (20 ng/ml) and PHA (1 μg/ml), or in dishes coated with anti-CD3 and anti-CD28 Abs (1 μg/ml) for the indicated periods of time. Total RNA was isolated using RNAzol (Cinna/Biotecx Laboratories, Friendswood, TX), according to the manufacturer's instructions. The *Pst*I-*Apa*I fragment of the *ccr5* gene and the *Xba*I-*Bam*HI fragment of the *ccr2* gene that correspond to the 3′ untranslated regions of these genes were used as probes. The *ccr1*, *ccr3*, and *ccr4* probes were generated by reverse-transcriptase PCR and contained the entire open reading frame of these genes. All of the probes were labeled using RadPrime DNA labeling system (Life Technologies, Rockville, MD) to similar specific activities. Cytokine production of the culture supernatant was measured by ELISA (Life Technologies).

Binding assays

A quantity amounting to 5×10^5 cells was washed with PBS and incubated for 15 min at 37°C in 200 μl assay buffer (HBSS, 25 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% NaN_3, and 0.2% BSA) containing 0.05 to 2.5 nM ^{125}I-MIP-1α in the presence or absence of 100-fold excess of unlabeled MIP-1α. After incubation, cells were washed three times with 500 μl assay buffer, and cell-bound radioactivity was counted in a scintillation counter. Mouse rMIP-1α was purchased from R&D Systems. ^{125}I-MIP-1α was labeled by Bolton-Hunter to a sp. act. of 2200 mCi/mmol (DuPont NEN Custom Iodination Laboratory, Boston, MA).

Thioglycolate-induced peritonitis

Six- to eight-week-old mice were injected i.v. with 3 ml sterile Brewer's thioglycolate broth (3%) and sacrificed 72 h later. Total leukocytes elicited into the peritoneal cavity were harvested by peritoneal lavarges and counted with a hemacytometer. Differential cell counts were determined by Diff-Quick staining of cytospin slide preparations and counting cells of 10 high power views. The number of different cell populations was calculated by multiplying percentage by total cell counts.

Listeria monocytogenes *infection*

Six- to eight-week-old mice were injected i.v. with 2500 CFU of *Listeria* and sacrificed on day 5 after infection. Numbers of viable *Listeria* in lung, liver, and spleen of infected animals were determined by plating serial dilution of organ homogenates in PBS on sheep blood agar. Statistical analysis of the samples was performed using the Mann-Whitney two-sample test (unpaired nonparametric analysis), and the two-tailed *p* value was calculated.

Delayed-type hypersensitivity (DTH) reaction and histopathologic analysis

The mouse abdominal skin was shaved, and 400 μl FITC solution (0.5% in 1:1 acetone/dibutyl phathalate) or solvent, only for the nonsensitized group, was applied epicutaneously. After 6 days, the baseline thickness of the animal's right ear was measured using a precision thickness gauge (0.01–12.5 mm; Swiss Precision Instruments, Los Angeles, CA). Each side of the right ear was then treated epicutaneously with 10 μl FITC solution, and the ear thickness was measured 24 h later. For histopathologic analysis, mice were sacrificed and one-half of the ears were fixed by immersion in 10% neutral buffered Formalin. Tissues were processed by standard methods, embedded in paraffin blocks, sectioned at 4 to 6 μm, stained with hematoxylin and eosin, and examined by light microscopy. Severity was graded without knowledge of treatment group, as follows: 0, none; 1, minimal; 2, mild; 3, moderate; 4, marked. Statistical analysis was performed using the unpaired Student's *t* test to calculate the two-tailed *p* values. For immunohistochemical analysis, frozen tissue sections of the other half of the ears were stained with anti-CD4 Ab (Life Technologies; 1/100 dilution), or anti-Mac1 Ab (Boehringer Mannheim, Indianapolis, IN; 1/100 dilution).

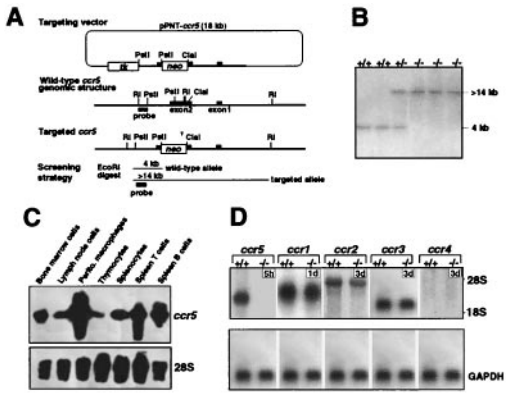

FIGURE 1. Targeted disruption of the mouse *ccr5* gene. *A*, From *top*, the targeting vector pPNT-*ccr5*, wild-type *ccr5* genomic structure, and targeted *ccr5* structure. The thick lines represent *ccr5* genomic DNA. Closed boxes indicate the two exons of the *ccr5* gene. The *PGK-n*E*o* and the *PGK-tk* cassettes are shown as open boxes. Homologous recombination of the targeting vector with wild-type *ccr5* genomic DNA replaced a 0.77-kb fragment of the exon 2 encoding all of the seven-transmembrane domains by the *PGK-neo* cassette. The targeted allele eliminated the two *Eco*RI (RI) sites in the open reading frame of *ccr5*. Using a probe outside the targeting vector (hatched box), the wild-type allele was detected as a 4-kb *Eco*RI fragment, while the targeted allele was detected as a fragment of more than 14 kb. *B*, Representative Southern blot analysis of wild-type (+/+), heterozygous (+/−), and homozygous (−/−) offsprings. Tail DNA was digested with *Eco*RI and hybridized with the flanking probe shown in *A*. *C*, Northern blot analysis of *ccr5* expression in different cell populations of hemopoietic origin. Total RNA was isolated from different cells and analyzed with a *ccr5*-specific probe. The same blot was stripped and tested with a probe specific for 28S rRNA. *D*, Northern blot analysis of *ccr5* and other CC-chemokine receptor genes in wild-type and homozygous mutant mice. Total RNA was prepared from resident peritoneal macrophages of each genotype, and tested with a *ccr5*-specific probe. Identical blots were probed with mouse *ccr1*, *ccr2*, *ccr3*, and *ccr4* cDNA fragments. All of the blots were stripped and analyzed with a probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Exposure time are 5 h for *ccr5*, 1 day for *ccr1*, 3 days for *ccr2*, *ccr3*, and *ccr4*, and 5 h for GAPDH (hybridization efficiency could be influenced by the gene sequence).

Ig production analysis

The TD Ag, keyhole limpet hemocyanin coupled to (4-hydroxy-3-nitrophenyl) acetic acid (NP-KLH), was precipitated in alum. Mice were injected i.p. with either 100 μg of alum-precipitated NP-KLH or 10 μg of the T cell-independent (TI) Ag, NP-LPS, in a 200 μl vol and bled at 0, 7, 14, and 21 days postimmunization. Basal levels of serum Ig were determined by isotype-specific ELISA. The levels of NP-specific Ig were determined by the use of ELISA plates coated with NP17-BSA for capture, and goat anti-mouse isotype-specific Abs conjugated to horseradish peroxidase (Southern Biotechnology, Birmingham, AL), as previously described (36). The level of each Ag-specific isotype was determined by comparison with a standard curve. Statistical analysis was performed using the unpaired Student's *t* test to calculate the two-tailed *p* values.

Results

Targeted disruption of the mouse ccr5 *gene*

The *ccr5* gene was deleted by replacing all of the seven-transmembrane domains with the *n*E*o* gene (Fig. 1*A*). Germline transmission

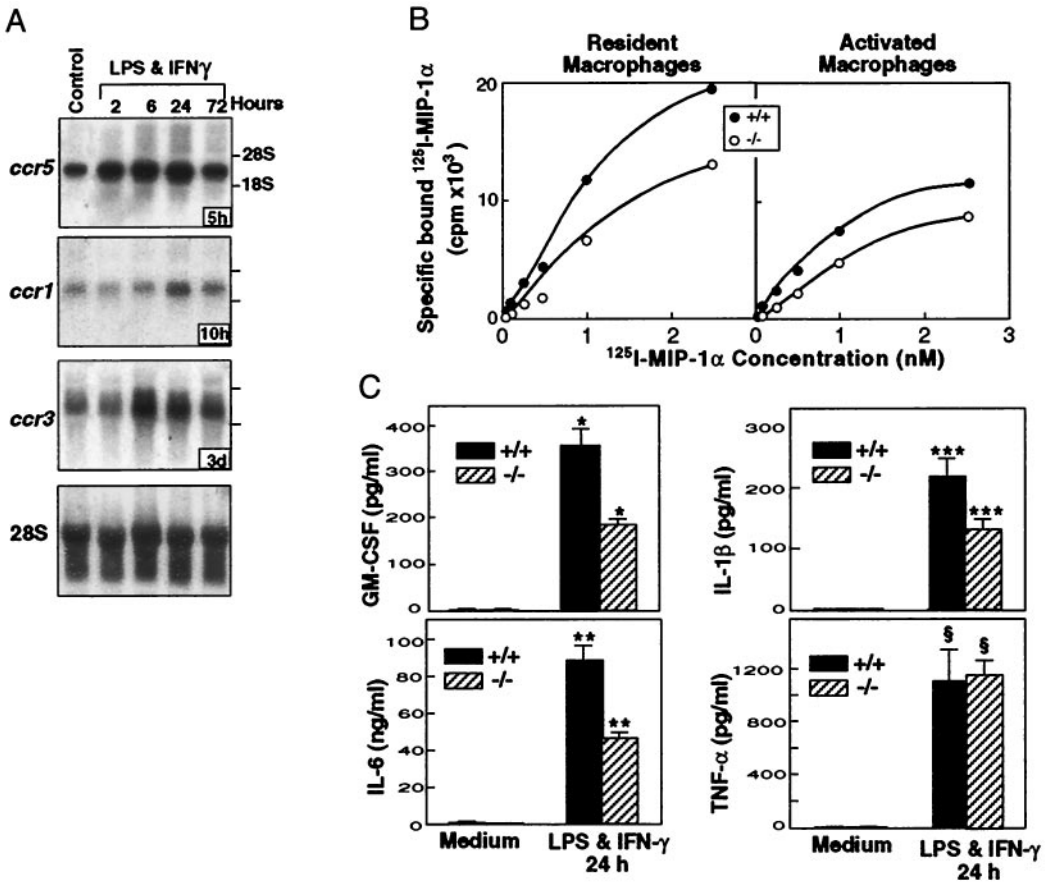

FIGURE 2. MIP-1α binding and cytokine production by peritoneal macrophages. *A*, Northern blot analysis of *ccr5* and other MIP-1/RANTES receptor gene expression in resident macrophages and in macrophages upon in vitro activation. The same blot was stripped and reprobed with a probe specific for 28S rRNA. Exposure time is 5 h for *ccr5*, 10 h for *ccr1*, 3 days for *ccr3*, and 30 min for 28S (hybridization efficiency could be influenced by the gene sequence). *B*, Binding of MIP-1α to peritoneal macrophages. Residential peritoneal macrophages were cultured in the presence or absence of LPS (1 μg/ml) and IFN-γ (100 U/ml) for 24 h before binding reaction. Values of specific ^{125}I-MIP-1α binding correspond to total ^{125}I-MIP-1α binding minus nonspecific ^{125}I-MIP-1α binding ($n = 3$ for each genotype). *C*, Cytokine production by peritoneal macrophages. Values are mean $\pm$ SEM ($n = 4$ for each genotype). *, **, ***$p < 0.003$, $^{\S}p = 0.99$ (Student's *t* test).

was obtained with two independently isolated homologous recombinant ES clones. Heterozygous matings generated offspring homozygous for the disrupted *ccr5* allele (*ccr5*$^{-/-}$) in Mendelian proportions. Deletion of the *ccr5* gene was confirmed by Southern blot analysis (Fig. 1*B*). Since the highest levels of *ccr5* mRNA can be detected in peritoneal macrophages of wild-type animals (Fig. 1*C*), its absence was confirmed by Northern blot analysis of peritoneal macrophage RNA hybridized with a *ccr5*-specific probe (Fig. 1*D*). The expression of other CC-chemokine receptor genes, *ccr1*, *ccr2*, *ccr3*, and *ccr4*, was not affected in the *ccr5* knockout mice (Fig. 1*D*).

CCR5-deficient mice were indistinguishable from their wild-type and heterozygous littermates when raised in a pathogen-free environment. Histopathologic analysis of major organs did not reveal alterations in their gross morphology. Flow-cytometric analysis of cells from thymus, spleen, lymph nodes, and bone marrow using a large number of T, B, granulocyte, and monocyte/macrophage cell markers showed no alterations in the different cell populations, indicating that CCR5 does not play an essential role in the development of the hemopoietic system (data not shown). Even though all of the three ligands of CCR5, MIP-1α, MIP-1β, and RANTES can induce chemotaxis of certain cell populations in vitro (37, 38), macrophage recruitment by thioglycolate elicitation in CCR5-deficient mice was comparable with wild-type animals (data not shown). Macrophage infiltration into glucan-induced granulomas in lung or liver also revealed no significant difference between CCR5-deficient mice and wild-type controls (data not shown), indicating that CCR5 does not play an essential role in macrophage recruitment in mice.

Decreased cytokine production in CCR5-deficient macrophages

In addition to CCR5, CCR1 and CCR3 also bind and respond to MIP-1α or RANTES in vitro (10–14). Northern blot analysis revealed that *ccr1* and *ccr3* were expressed at relatively lower levels compared with that of *ccr5*, in both resident and in vitro activated macrophages (Fig. 2*A*). To investigate the MIP-1α-binding capacity of macrophages in the absence of CCR5, ^{125}I-MIP-1α binding was measured. ^{125}I-MIP-1α bound to HEK293 cells expressing CCR5 with a K_d of 0.1 nM. Equilibrium binding could be reached within 15 min at 37°C and was saturable at 1 to 2 nM (Zhou et al., unpublished observations). Under identical conditions, equilibrium binding of increasing concentrations of ^{125}I-MIP-1α to resident wild-type macrophages approached, but did not reach, saturation binding at 2.5 nM. Equilibrium binding of increasing concentrations of ^{125}I-MIP-1α to resident CCR5-deficient macrophages showed a similar binding pattern to that of wild-type controls; however, the overall binding was reduced compared with that of wild type (Fig. 2*B*). Consistently, there was decreased MIP-1α

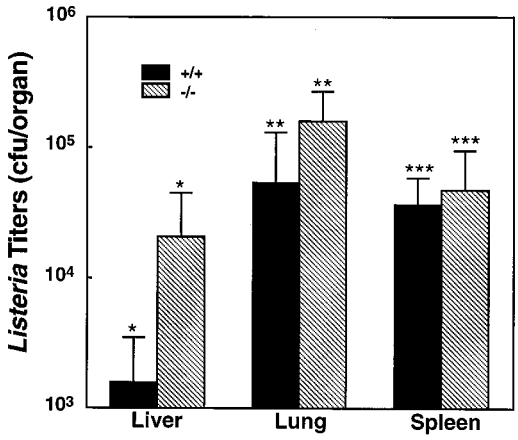

FIGURE 3. *L. monocytogenes* infection. Wild-type (+/+) and CCR5-deficient (−/−) mice were infected i.v. with 2500 CFU of *Listeria*. Values are mean ± SEM ($n = 5$ for each genotype). *$p < 0.03$, **$p = 0.5$, ***$p = 0.1$ (unpaired nonparametric Mann-Whitney test). The result is a representative of two independent experiments.

binding to in vitro activated CCR5-deficient macrophages compared with that of wild-type controls (Fig. 2*B*). These results indicate that CCR5 contributes to the MIP-1α-binding activity in wild-type macrophages. Cytokine production of the corresponding cell culture supernatants was measured by ELISA. The production of granulocyte-macrophage CSF, IL-1β, and IL-6 by CCR5-deficient macrophages decreased to 50% compared with that of wild-type controls (Fig. 2*C*, $p < 0.003$, Student's *t* test), while TNF-α (Fig. 2*C*, $p = 0.99$, Student's *t* test) and IL-10 (data not shown) production remained at similar level. These results suggested a partial defective macrophage function in CCR5-deficient mice.

Reduced efficiency in the clearance of L. monocytogenes *infection and protection of LPS-induced endotoxemia in CCR5-deficient mice*

To assess the in vivo impact of the partial defective macrophage function in CCR5-deficient mice, groups of mutant and wild-type animals were analyzed for their responses in two infection and inflammation models. First, the susceptibility to *L. monocytogenes* infection was investigated. The intracellular bacterium *Listeria* can survive in nonactivated macrophages, and effective clearance of infection requires activation of macrophages (39). Mice of 6 to 8 wk old were infected i.v. with 2500 CFU of *Listeria* and sacrificed 5 days after infection. *Listeria* titers of liver from CCR5-deficient mice were 10-fold higher than that of wild-type controls, while *Listeria* titers of lung and spleen were at similar levels ($^{*}p < 0.03$, $^{**}p = 0.5$, $^{***}p = 0.1$, Student's *t* test) (Fig. 3), indicating a mildly reduced efficiency in *Listeria* clearance in the liver of CCR5-deficient mice.

In the second model, the contribution of CCR5 in LPS-induced endotoxemia was determined. Both MIP-1α and RANTES have been shown to mediate macrophage influx and early mortality in endotoxemia (40, 41). Pretreatment with either anti-MIP-1α or anti-RANTES reduced the mortality of lethal doses of LPS (40, 41). CCR5-deficient mice and wild-type controls were administered i.p. with five doses of LPS, and survival was monitored up to 4 days. As shown in Table I, treatment with the highest dose, 2 mg/animal, resulted in a 20% survival of CCR5-deficient mice vs 0% survival of wild-type mice; the second highest dose, 0.8 mg/animal, resulted in a 50% survival of mutant mice vs 17% of wild-type mice; and treatment with the lower doses, 0.4, 0.2, or 0.1 mg/animal, resulted in 78, 100, or 100% survival of mutant mice vs 50, 80, or

Table I. *Effect of LPS injection*[a]

Genotype	LPS (mg/mouse)	Survival (survival/total)	LD_{50} (mg/kg)
Wild-type	2	0/10	18.15*
	0.8	6/33	
	0.4	8/15	
	0.2	8/10	
	0.1	5/5	
$ccr5^{-/-}$	2	2/10	34.06*
	0.8	16/32	
	0.4	10/13	
	0.2	10/10	
	0.1	5/5	

[a] LPS was injected i.p. into 8- to 12-week-old mice in 0.2 ml PBS solution. Mortality was recorded over 4 days after challenge. Surviving mice were monitored for 30 days (no mice died once they survived for 3 days).
* $p < 0.03$, unpaired nonparametric Mann-Whitney test.

100% survival of wild-type mice. Spearman Karben Estemate calculated LD_{50} values were 34.05 (mg LPS/kg animal body weight) for mutant mice vs 18.15 (mg LPS/kg animal body weight) for wild-type mice (*$p < 0.03$, unpaired nonparametric Mann-Whitney test). These results indicated a significant protection in LPS-induced endotoxemia in CCR5-deficient mice.

Increased cytokine production by CCR5-deficient T cells

CCR5 is expressed at relatively low levels on the surface of resting T cells. Upon in vitro activation by anti-CD3 Ab and IL-2, CCR5 protein levels increase dramatically, suggesting a role for CCR5 in T cell function (42). To investigate the possible redundancy among receptors, we first checked the expression of all known MIP-1/RANTES receptor genes in T cells by Northern blot analysis. *ccr5* was the only receptor gene expressed in T cells, and the only gene whose expression can be induced in vitro with either PMA plus PHA or anti-CD3 plus anti-CD28 Abs. There was no detectable expression of *ccr1* or *ccr3* (Fig. 4*A*). We then measured the MIP-1α-binding activity in T cells. Equilibrium binding of increasing concentrations of ^{125}I-MIP-1α to resting wild-type T cells was saturable and reached maximal binding at 0.5 to 1 nM. In contrast, there was no detectable equilibrium binding of ^{125}I-MIP-1α to resting CCR5-deficient T cells (Fig. 4*B*). Consistently, there was a dramatically decreased MIP-1α binding to in vitro activated CCR5-deficient T cells compared with that of wild-type controls at low concentrations of ^{125}I-MIP-1α (Fig. 4*B*). However, with increasing concentrations of ^{125}I-MIP-1α, a low affinity MIP-1α binding was detected in both wild-type and CCR5-deficient T cells (Fig. 4*B*), probably due to the induced expression of CCR3 on a subset of activated T cells (Th2 cells) (43) and/or to the induction of an unknown low affinity CCR5-binding receptor. Cytokine production was also measured in the corresponding culture supernatants. Surprisingly, there was a marked increase in the production of IFN-γ (fivefold), granulocyte-macrophage CSF (2.5-fold), and IL-4 (twofold) by CCR5-deficient T cells compared with wild-type controls (*, **$p < 0.0003$, ***$p < 0.02$, Student's *t* test), while no significant difference in the production of IL-2 or TNF-α ($^{\S}p = 0.13$, $^{\S\S}p = 0.22$, Student's *t* test) (Fig. 4*C*). There was no difference in the production of chemokine ligands of CCR5 (MIP-1α, MIP-1β, or RANTES, data not shown).

Enhanced DTH reaction in CCR5-deficient mice

The elevated cytokine production by CCR5-deficient T cells suggested an enhanced TD immune response in CCR5-deficient mice. To test this hypothesis, we first compared the DTH reaction in CCR5-deficient and control mice. It has been shown that patients

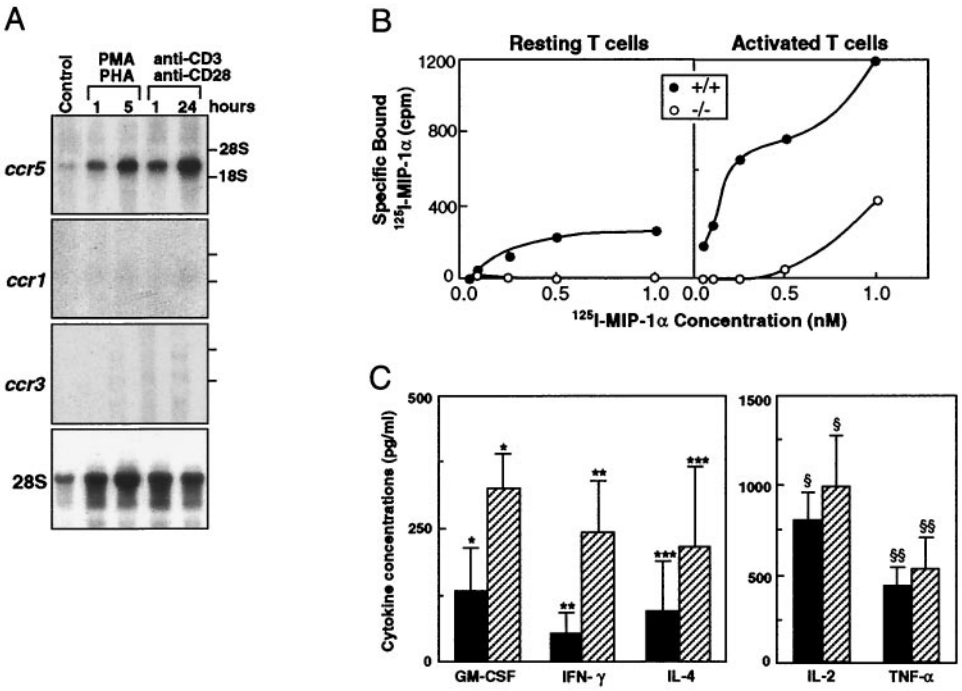

FIGURE 4. MIP-1α binding and cytokine production by T cells. *A*, Northern blot analysis of *ccr5* and other MIP-1/RANTES receptor genes in resting and in vitro activated T cells. The same blot was stripped and reprobed with a cDNA fragment specific for 28S rRNA. Exposure time was 3 days for *ccr5*, *ccr1*, and *ccr3*, and 30 min for 28S (hybridization efficiency could be influenced by the gene sequence). *B*, Binding of MIP-1α to T cells. Cells were incubated for 24 h in the absence (control) or presence of anti-CD3 plus anti-CD28 before binding reaction. Values of specific ^{125}I-MIP-1α binding correspond to total ^{125}I-MIP-1α binding minus nonspecific ^{125}I-MIP-1α binding ($n = 3$ for each genotype). *C*, Cytokine production by in vitro activated T cells. Cells were incubated for 36 h with anti-CD3 plus anti-CD28 before cytokine determination. Values are mean ± SEM ($n = 8$ for wild type and $n = 6$ for $ccr5^{-/-}$). *, **$p < 0.0003$, ***$p < 0.02$, $^{\S}p = 0.13$, $^{\S\S}p = 0.22$ (Student's *t* test).

with a subnormal DTH reaction had a significantly more rapid progression to AIDS than did patients with a normal DTH reaction (44). The abdominal skin of both CCR5-deficient and wild-type animals was exposed to FITC. After the afferent phase, the ears were treated epicutaneously with the hapten solution, and the ear thickness was measured 24 h later. While nonsensitized mice of both genotypes ($ccr5^{-/-}$ and wild type) had no significant changes in ear thickness, sensitized mice had a strong reaction, and the response was twofold higher in CCR5-deficient mice than in wild-type controls (*$p < 0.02$, Student's *t* test) (Fig. 5*A*). Histopathologic analysis of ear sections showed that nonsensitized mice of both genotypes were nonremarkable or had minimal inflammation (Fig. 5, *B–D*). In contrast, the sensitized wild-type mice showed mild to moderate dermal edema and inflammatory infiltrates with multifocal microabscesses consisting predominantly of neutrophils (Fig. 5, *B* and *E*). Sensitized CCR5-deficient mice had histologic changes similar to the sensitized wild-type mice, but with increased severity (**$p < 0.03$, Student's *t* test) (Fig. 5*B*). The ears of these mice were moderately thickened, with dense infiltrates of neutrophils, lymphocytes, and macrophages, and contained intraepithelial pustules and microabscesses (Fig. 5, *B* and *F*). Immunohistochemical staining with anti-CD4 and anti-Mac1 Abs showed more $CD4^-$-positive and Mac1-positive cells in the sensitized CCR5-deficient mice than in wild-type controls (data not shown). Since the DTH reaction is mediated by $CD4^+$ T cells, these results indicate an enhanced cell-mediated immune response in CCR5-deficient mice.

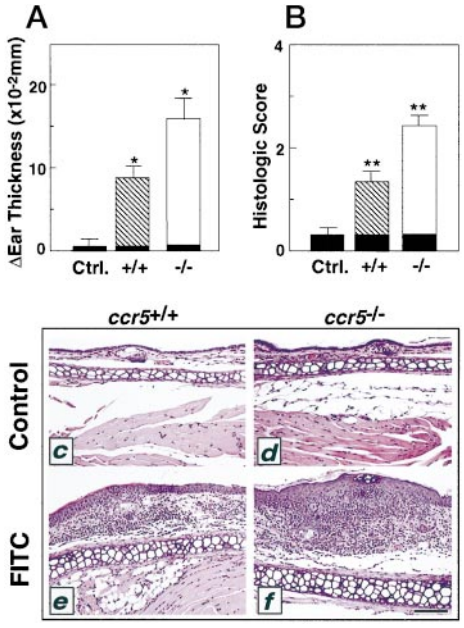

FIGURE 5. DTH reaction in CCR5-deficient mice. *A*, Depiction of changes in ear thickness. Baseline ear thickness was recorded 6 days after sensitization; the ears were treated epicutaneously with hapten solution and measured 24 h later. *B*, Histopathologic grading of inflammation. Closed bars indicate the changes of ear thickness (*A*) and histopathologic grading (*B*) of naive animals. Values are reported as mean ± SEM ($n = 5$ for each genotype). *$p < 0.02$, **$p < 0.03$ (Student's *t* test). *C–F*, Histopathology of DTH reaction in the ears. Nonsensitized wild-type (*C*) and $ccr5^{-/-}$ (*D*) mice. DTH reaction in wild-type (*E*) and $ccr5^{-/-}$ (*F*) mice. Size bar equals 200 μm. Severity was graded without knowledge of treatment group, as follows: 0, none; 1, minimal; 2, mild; 3, moderate; 4, marked.

Enhanced humoral responses to TD antigenic challenge in CCR5-deficient mice

To further analyze this issue, we compared the humoral response to a specific antigenic challenge in CCR5-deficient and wild-type

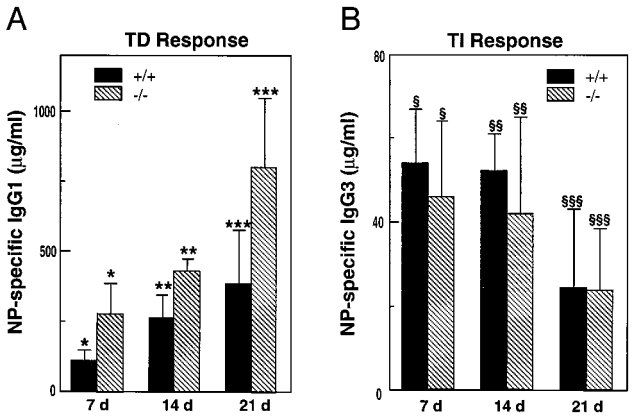

FIGURE 6. Ig production in CCR5-deficient mice. *A*, Immune response to the TD Ag NP-KLH. Mice were immunized with NP-KLH, and anti-NP IgG1 levels in sera were determined 7, 14, and 21 days postimmunization. *B*, Immune response to the TI Ag NP-LPS. Mice were immunized with NP-LPS, and anti-NP IgG3 levels in sera were determined 7, 14, and 21 days postimmunization. Values are mean ± SEM ($n = 8$ for wild type and $n = 6$ for $ccr5^{-/-}$). *, **, ***$p < 0.02$, §, §§, §§§$p > 0.05$ (Student's *t* test).

animals. Mice were injected with either the TD Ag NP-KLH or the TI Ag NP-LPS, and the Ig levels at 0, 7, 14, and 21 days postimmunization were measured. Although basal levels of total Ig and different isotypes remain similar between CCR5-deficient mice and wild-type controls (data not shown), the production of NP-specific IgG1, the predominant isotype produced during a normal TD response to soluble protein Ags, was increased approximately twofold at days 7, 14, and 21 postimmunization in CCR5-deficient mice ($p < 0.02$, Student's *t* test) (Fig. 6*A*). In contrast, the production of NP-specific IgG3, the main isotype produced during a normal TI response to carbohydrate Ag, was similar in mutant and control mice ($p > 0.05$, Student's *t* test) (Fig. 6*B*). These results confirm the enhanced immune reaction in CCR5-deficient mice, specifically in TD responses.

Discussion

To investigate the physiologic function of CCR5 and to understand the cellular mechanisms of the hyperresistance to HIV-1 infection in the CCR5 null humans, we generated a CCR5-deficient mouse model ($ccr5^{-/-}$) by targeted deletion of the *ccr5* gene. We found that although developed normally in a pathogen-free environment, CCR5-deficient mice showed reduced efficiency in the clearance of *Listeria* infection and exsert a protection effect against LPS-induced endotoxemia. In addition, CCR5-deficient mice had an enhanced DTH reaction and increased humoral responses to TD antigenic challenge.

The lack of the developmental defect correlates with the observation that CCR5 null humans have no obvious health problems (29–32). Similarly, no developmental defects are observed in mice deficient for either MIP-1α or CCR1 (18, 45). In contrast, mice deficient in the CXC chemokine SDF-1 (46) or the CXC chemokine receptor CXCR2 (47) do present developmental alterations, suggesting that the absence of one of the CC chemokines or their receptors is better tolerated, possibly due to the higher degree of redundancy in this chemokine subfamily (4).

Even though all three ligands of CCR5 induce chemotaxis of certain cell populations in vitro (37, 38), no recruitment defect was observed in either macrophage or T cells in CCR5-deficient mice. This suggests that CCR5 function is compensated by other CC chemokine receptors or that CCR5 is not involved in chemotaxis in vivo. Severe defect in neutrophil infiltration has been reported in the absence of either CCR1 or CXCR2 (45, 47), whereas CCR2 is essential for macrophage recruitment (48). To date, there is no strong evidence for a chemokine receptor being responsible for T cell migration in vivo. In the DTH model, more $CD4^+$ and $Mac1^+$ cells were infiltrated in the CCR5-deficient mouse ears than in wild-type ears (data not shown). These results suggested that, in the wild-type animal, the abundance of CCR5 on the surface of macrophages or T cells might dilute the functional concentration of ligands (MIP-1α/RANTES) to their receptors (CCR1, CCR3, or others) and indirectly reduce the cell infiltration to the inflammation site.

Peritoneal macrophages from CCR5-deficient mice after LPS challenge were much smaller in size and less foaming compared with those from wild-type mice (data not shown), indicating they are less active than wild-type macrophages. Reduced production of several other cytokines by macrophages in the absence of CCR5 also suggested a partially defective macrophage function. However, a minor reduced efficiency in clearance of *Listeria* infection indicated that CCR5 did not play a major role in this macrophage-mediated host defense model. Nevertheless, this minor defect might still be enough to take account for the partial protection of LPS-induced endotoxemia in CCR5-deficient mice. Lethal dose LPS-induced septicemia includes shock, multiorgan dysfunction, and death (49). Mice died of acute suppurative hepatitis, lymphopolysis in multiple lymphoid organs, and acute adrenocortical necrosis (data not shown). It has been shown, in addition to neutrophils, lymphocytes and macrophages (either newly recruited blood monocytes or resident tissue macrophages) also contribute to the endotoxin-induced organ injury (50). Thus, partial impaired macrophages in CCR5-deficient mice might be less virulent in damaging tissues; as a result, mice might have better chance to recover. TNF-α is one of the important cytokines involved in LPS pathway. However, we did not observe reduced TNF-α production. TNF-α receptor-deficient mice were only resistant to low doses of LPS after sensitized by galactosamine, but not to high doses of LPS (51–53), indicating that the TNF-α pathway is not involved in the high dose LPS-induced endotoxemia, which was the experiment performed in our studies. At the later stage of AIDS development, when patients' lymphoid tissues are quantitatively and qualitatively impaired, and $CD4^+$ cells are steadily declining, tissue macrophages play a critical role in fighting opportunistic infections (54). AIDS patients heterozygous for the Δccr5 allele have an accelerated decrease in $CD4^+$ cell counts and reduced survival time (35). We speculate that macrophages in these patients might also be partially defective and have reduced capacity in clearance of opportunistic infection.

The increased production of both type 1 (IFN-1γ) and type 2 (IL-4) cytokines by CCR5-deficient T cells is different from that observed in CCR1-deficient mice, in which an impaired type 1-type 2 cytokine balance was observed (45). These results indicate a novel role for CCR5 in down-modulating T cell function. There are at least two explanations for the overproduction of cytokines in the absence of CCR5. First, signaling through CCR5 might function as part of a negative regulatory cycle of T cell activation. To date, CCR5 is the only known receptor for MIP-1β (6–9). MIP-1β inhibits MIP-1α-induced macrophage activation (55). However, the activities of MIP-1α and MIP-1β on T cell activation have not been well studied. Second, the overproduction of cytokines in the absence of CCR5 might be the result of enhanced signaling through other receptors having overlapped ligand-binding profile with CCR5. Even though the production of chemokines did not increase in the absence of CCR5 (data not

shown), the ligand-receptor ratio might still increase in the absence of the abundant CCR5.

CD4 facilitated the direct interactions between CCR5 and HIV-1 envelope protein gp120, suggesting that $CD4^-$-dependent CCR5-gp120 binding is important to HIV-1-membrane fusion and virus entry (56, 57). Therefore, it was hypothesized that the resistance to HIV-1 infection in CCR5 null individuals was due to the lack of HIV-1 docking molecules (29, 30). However, identification of increased number of HIV-1 coreceptors argues that this hypothesis is the sole explanation for the hyperresistance to HIV-1 infection in CCR5 null individuals (26–28). Our results of enhanced TD immune responses in the CCR5-deficient mouse model suggest that there might be an additional level of defense in the CCR5 null humans: the overreactive immune system in these individuals might trigger a stronger immune response at the early phase of viral infection and clear viral particles more efficiently. Therefore, our data favor the efforts to look for reagents that block both CCR5-ligand and CCR5-gp120 interactions as preventive therapeutics against HIV-1 infection.

The levels of CCR5 expression on T cells vary dramatically from individual to individual (42). Our results further suggest that investigation of the correlation between the expression levels of CCR5 and the intensity of immune responses might be very informative to understand the pathology of allergic or autoimmune diseases. The CCR5-deficient mice will provide a useful tool to study the potential role of ligand-CCR5 interactions in these and other inflammatory or infectious models.

Acknowledgments

We thank Dr. Sergio Lira, Mavis Swerdel, and Alice Lee in the Transgenic Unit and all of the staff in Veterinary Sciences at Bristol-Myers Squibb for generating and maintaining the animals; Cheryl Rizzo and Anne Lewin for excellent technical assistance; and Willy Kratil for computer graphics assistance. We also thank Drs. Daniel Carrasco, Tin-Chen Hsu, and Jorge H. Caamaño for suggestions and comments on this manuscript.

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