

Short communication

Study of the role of CCR5 in a mouse model of intranasal challenge with *Yersinia pestis*

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Abstract

CCR5 is a chemokine receptor used by HIV-1 to enter cells and has recently been found to act as a pathogen associated molecule pattern receptor. Current positive selection for the high frequency of a *CCR5-Δ32* allele in humans has been attributed to resistance to HIV, smallpox, and plague infections. Using an intranasal mouse model of *Y. pestis* infection, we have found that lack of CCR5 does not enhance host resistance to *Y. pestis* infection and that CCR5-mediated responses might have a protective role. *CCR5*^{-/-} mice exhibited higher levels of circulating RANTES and MIP-1 α than those exhibited by wild-type mice at the baseline and throughout the course of *Y. pestis* infection. High levels of RANTES and MIP-1 α , which are CCR5 ligands that mediate Natural Killer cell migration, may reflect compensation for the absence of CCR5 signaling.

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1. Introduction

CCR5 is a seven membrane-spanning chemokine receptor found primarily on the surface of immune cells where it appears to act as a pathogen associated molecule pattern receptor [1]. CCR5 was first identified as a key receptor for HIV-1 entry into T lymphocytes via a fusion-mediated event [2–5]. A *CCR5-Δ32* allele confers protection against HIV-1 infection, and is present in an average of 10% of humans of European origin. The *CCR5-Δ32* deletion in humans results in the deletion of the second extracellular loop of CCR5 which causes an earlier stop codon. Thus, the resulting truncated CCR5 molecule, lacking the final three transmembrane domains and the adjacent loops, is retained within the cell. Without CCR5 on the cell surface, the HIV envelope protein does not bind and the virus does

not enter the cell. There appears to be strong positive selection in humans, with no negative consequence to *CCR5* deletion homozygosity in either humans or mice [6–10].

A recent finding indicates that the frequency of the *CCR5-Δ32* allele in DNA samples from Bronze Age skeletons is comparable to that in modern German DNA samples [11], suggesting that during human evolution the deletion may have conferred resistance to microbial infections prevalent at different times. The evidence of continuous positive selection for the high frequency of the variant has been attributed to resistance to HIV, smallpox, and plague infections.

Tests for protection against infection of CCR5-deficient mice with *Yersinia pestis*, the agent for bubonic plague, yielded ambiguous results. When *Y. pestis* was intravenously inoculated, neither significant differences in survival nor differences in bacterial load in the caecum or Peyer's patches between CCR5^{+/+} and CCR5^{-/-} mice were observed [12]. On the other hand, peritoneal macrophages isolated from CCR5-deficient mice showed significantly reduced uptake of *Y. pestis*

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in vitro [13], suggesting the potential for an altered immune response, although subcutaneous infection of these mice yielded no significant protection against lethality in comparison to CCR5+/+ mice.

To further analyze whether the lack of CCR5 protects from *Y. pestis* infection, we have used a recently developed mouse mucosal model of intranasal challenge with *Y. pestis* [14] to assess survival in CCR5-deficient C57Bl/6 mice while monitoring the systemic serum cytokine response over the course of infection. *Y. pestis* infection induced substantial elevations in the serum concentration of multiple cytokines and chemokines in both CCR5-/- and wild-type mice. Serum concentrations of a CCR5 ligand, RANTES, were increased in CCR5-/- mice compared to wild-type mice both at baseline and after plague infection. Our work supports a non-protective role for the lack of CCR5 in the mouse model of intranasal challenge with *Y. pestis*.

2. Materials and methods

2.1. Bacterial strain

Y. pestis pgm- strain KIM5, was grown in BHI medium supplemented with 100 µM FeCl₂ (EMD Chemicals) at 150 rpm, 26 °C. Log phase culture was washed and resuspended in PBS at approximately 2 × 10⁷ bacteria/ml. Inoculum concentration was verified by counting colony-forming units (CFU) of plated dilutions.

2.2. Mouse infection model

Eight-week old CCR5+/+ and CCR5-/- mice (C57Bl/6, Jackson Labs, *n* = 15 and 13, respectively) were infected intranasally with 8 × 10⁵ CFU *Y. pestis* KIM5 and subsequently monitored for weight loss and other clinical signs of disease. Blood samples (50–100 µl) were obtained from all mice 5 days prior to and 7 days after infection. In addition, half the mice in each group were bled on alternate days through day 4 post infection. Serum was collected by centrifugation of mouse blood and stored frozen at -80 °C until analyzed. Mice were euthanized for humane reasons when moribund or sacrificed after 21 days.

2.3. Serum cytokine/chemokine analysis

Cytokine/chemokine analysis of 15 µl of serum was performed using a 22-plex premixed kit and protocol from Linco Research, supplemented with a final 15 min incubation in 4% paraformaldehyde for decontamination. Data were collected on a BioRad Bioplex System using Software 4.0.

2.4. Statistical analysis

Survival was compared by the Fisher exact test. Survival times were compared by the Mann–Whitney rank sum test. Weights were compared by the *t*-test on each day after infection. The primary weight outcome was defined by the last day

on which at least 80% of both groups were alive. Concentrations of cytokines and chemokines were compared at multiple time points by the Wilcoxon rank sum test. All tests were two-tailed.

3. Results

3.1. CCR5-/- animals are not more resistant to intranasal infection of *Y. pestis* than wild-type

To study the role of CCR5 in immunity to *Y. pestis* infection, we used an intranasal mouse model of infection with *Y. pestis* strain KIM5 previously described [14]. Using the intranasal model, we previously demonstrated that after intranasal inoculation of mice with *Y. pestis* strain KIM5, the initial lung infection is followed by a systemic spread of the disease into liver and spleen. Also, the intranasal model has been used to demonstrate the reduced virulence of *Y. pestis* mutants in new and known virulence-related genes [14].

CCR5-/- and CCR5+/+ C57Bl/6 mice were infected with 8 × 10⁵ CFU of *Y. pestis*. As shown in Fig. 1A, overall survival of CCR5-/- mice is not significantly different from that of CCR5+/+ control mice, indicating that lack of CCR5 does not protect from the intranasal challenge with *Y. pestis*. Survival in CCR5 KO mice did not differ significantly from wild-type B6 mice (46% versus 73%, *P* = 0.246; difference = -27%, 95% confidence interval -62% to 8%). The confidence interval excludes a major resistance phenotype in the CCR5-/- mice, but does not exclude the possibility the CCR5-/- mice are more susceptible than wild-type to this intranasal plague challenge. There was a trend toward decreased survival time in the CCR5-/- mice (*P* = 0.108).

In order to more closely analyze the effects of infection on the health of the mice, morbidity was determined quantitatively by tracking weight change which is commonly used as an objective measure of illness. CCR5-/- and CCR5+/+ mice were weighed daily for 14 days after infection and the average percentage of weight loss for all animals in each group was calculated (Fig. 1B). Infected mice began to lose weight soon after the infection, on average losing 15–20% of their weight by 6 days post infection. By 14 days after infection, surviving CCR5-/- and CCR5+/+ mice had regained most of their initial weight. There was no significant difference in weight loss on any day from day 1 to day 6 (*P* = 0.619 on day 6, the primary weight endpoint day). After day 6, weight comparisons were not done due to survivor bias.

3.2. The levels of chemoattractants that mediate natural killer cell migration are higher in CCR5-/- animals than in wild-type

Chemokines and cytokines play a key role in the control of bacterial infections by activating immune cells and organizing their migration. Thus, we measured the levels of RANTES and MIP-1α, which are known to signal through CCR5, as well as the levels of additional chemokines and cytokines in serum of wild-type and CCR5-/- animals at various times during

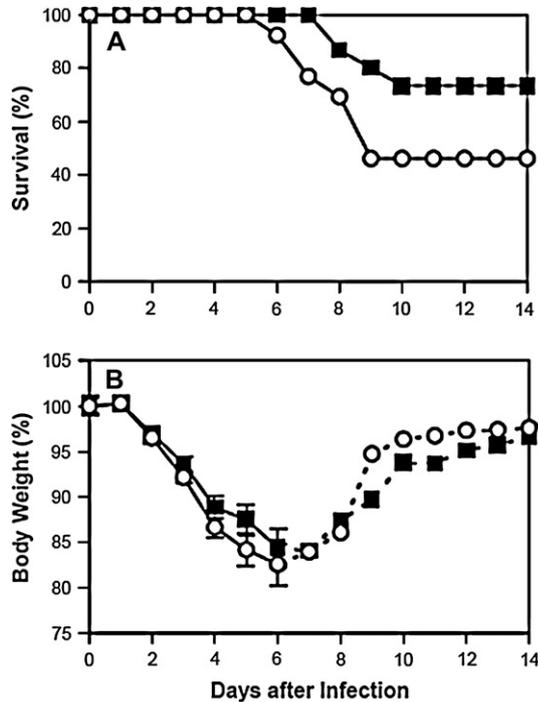


Fig. 1. CCR5^{-/-} and CCR5^{+/+} mice have similar mortality and morbidity following intranasal exposure to *Y. pestis*. Seven to eight week-old female CCR5^{-/-} C57Bl/6 ($n = 13$, open circles) and CCR5^{+/+} C57Bl/6 ($n = 15$, closed squares) mice were inoculated intranasally with *Y. pestis* KIM5 (8×10^5 CFU/mouse). (A) Mortality and (B) morbidity, in terms of average relative body weight, were monitored for 14 days. No significant differences were observed either in overall survival ($P = 0.246$, Fisher's exact test) or in time of survival ($P = 0.108$, Mann-Whitney rank sum test) between the two groups. Weight difference at day 6, prior to onset of survivor bias, was also not significant ($P = 0.619$, t -test).

infection with *Y. pestis*. Chemokine and cytokine profiles in serum were studied as a readout of systemic inflammation because *Y. pestis* KIM5 is present in the lungs at relatively low numbers and the lung infection is often cleared and followed by a systemic spread of the bacteria into liver and spleen [14].

Analysis of serum by Luminex assay revealed higher serum concentrations of chemokines and cytokines in wild-type C57Bl/6 animals in response to infection when compared to the uninfected baseline levels in the same animals (Fig. 2 and data not shown). Overall, the levels of chemokines and cytokines begin increasing 48 h postinfection and they significantly increased 72 h postinfection. The analysis of serum chemokines and cytokines also indicates that infected CCR5^{-/-} mice exhibit higher levels of circulating RANTES and MIP-1 α than those exhibited by wild-type mice at the baseline and throughout the course of *Y. pestis* infection (Fig. 2). Since RANTES and MIP-1 α are CCR5 ligands, their higher levels in CCR5^{-/-} animals than in wild-type mice may be due to an attempt to compensate for the absence of CCR5 signaling. No significant differences between wild-type and CCR5^{-/-} animals were observed in response to infection for chemokines IP10 or KC or various cytokines tested, including IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, TNF- α , and IFN- γ (data not shown).

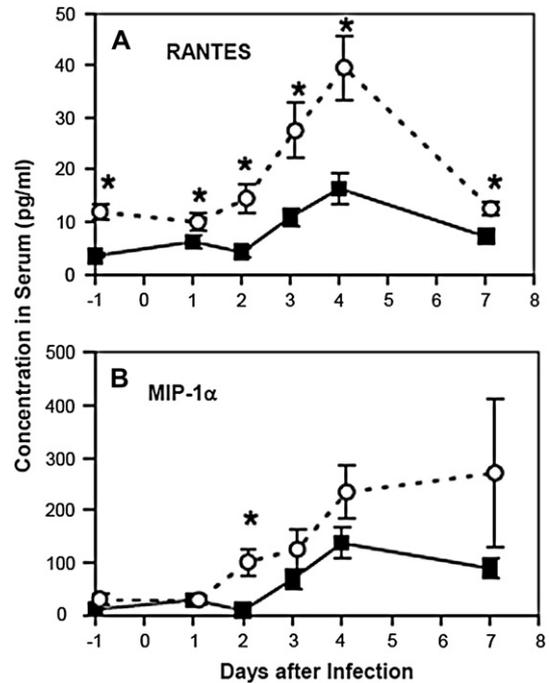


Fig. 2. CCR5-deficient mice exhibit an altered serum chemokine response when exposed to *Y. pestis*. Serum was drawn from all mice 5 days prior to and 7 days after infection with *Y. pestis*. Additional serum was obtained from half the mice on alternate days during the first 4 days after infection. Chemokine and cytokine levels were analyzed by Luminex assay with Lindco reagents. Shown are the average chemokine concentrations in picograms per milliliter over time for RANTES and MIP-1 α in the serum of infected CCR5^{-/-} (open circles) and CCR5^{+/+} (closed squares) C57Bl/6 mice. Error bars, SEM. Statistically significant differences between the strains, indicated by asterisks, were observed in RANTES and MIP-1 α ($P < 0.05$, Wilcoxon rank sum test).

4. Discussion

These experiments provide the first evidence indicating that the CCR5 deletion does not provide protection against *Y. pestis* using intranasal inoculation. The confidence interval around the survival rates observed in this study (46% for CCR5^{-/-} mice versus 73% for wild-type, $P = 0.246$; difference = -27%, 95% confidence interval -62% to 8%) excludes any significant survival advantage. Although we cannot rule out the possibility that CCR5 has a beneficial effect in the host response to *Y. pestis*, the survival analysis rules out the possibility of a protective role for the lack of CCR5 in this model. These results support previous plague studies using different routes that have also found no benefit to the lack of CCR5. Taken together, these results suggest that plague was not a selective factor in the emergence of the non-functional CCR5- $\Delta 32$ allele in humans.

Interestingly, the chemokines with elevated levels in the CCR5 deletion strain in response to infection, RANTES and MIP-1 α , are chemoattractants that mediate Natural Killer (NK) cell migration. The migration of immune cells, including NK cells, is a key process to control bacterial infections that requires finely tuned mechanisms of control. The higher levels of these chemokines observed during *Y. pestis* infection in

CCR5 animals compared to those of wild-type animals open the possibility that the lack of CCR5-mediated responses may be compensated by the elevated levels of chemokines which can eventually activate additional CCR receptors to maintain an appropriate NK cell response to *Y. pestis* infection. Since no significant differences were observed in response to infection for chemokines IP10 and KC (GRO- α) or the various cytokines tested, including IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, TNF- α , and IFN- γ , we do not believe that inflammation plays a detrimental role in CCR5 $-/-$ animals. Further experiments will be required to address the role of NK cells in immunity to *Y. pestis* and the mechanisms that elicit their activation and migration.

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