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Role of CXCR3 in the Immune Response to Murine Gammaherpesvirus 68

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The chemokine IP-10 (CXCL10) and its cellular receptor CXCR3 are upregulated in the lung during murine gammaherpesvirus 68 (MHV-68) infection. In order to determine the role of the CXCR3 chemokine receptor in the immune response to MHV-68, CXCR3$^{-/-}$ mice were infected with the virus. CXCR3$^{-/-}$ mice showed delayed clearance of replicating MHV-68 from the lungs. This correlated with delayed T-cell recruitment to the lungs and reduced cytolytic activity prior to viral clearance. Splenomegaly and the numbers of latently infected cells per spleen were transiently increased. However, CXCR3$^{-/-}$ mice showed normal virus-specific antibody titers and effective long-term control of MHV-68 infection.

Intranasal administration of murine gammaherpesvirus 68 (MHV-68) results in acute productive infection of lung alveolar epithelial cells and a latent infection in several cell types (7, 17, 19, 24). Infectious virus is cleared from the lungs by a T-cell-mediated process (6, 20), whereas control of latent virus, once established, can be mediated by either T- or B-cell-dependent mechanisms (1, 9, 17). We previously showed that that both CXCR3 and its ligand IP-10 (CXCL10 or crg-2) were upregulated in the lungs of MHV-68-infected mice (15). Furthermore, our in vitro studies showed that neutralizing antibodies to IP-10 significantly reduced the chemotactic activity of bronchoalveolar lavage (BAL) fluid (15). Other groups have also reported that the CXCR3 ligands MIG (CXCL9) and IP-10 are upregulated in the lungs and spleens of MHV-68-infected mice (5, 25). CXCR3 and its ligands have been reported to play a role in the clearance and/or inflammatory responses to mouse hepatitis virus (10), lymphocytic choriomeningitis virus (3), herpes simplex type 1 (2), and vaccinia and ectromelia viruses (8, 11–13), whereas the neutralization of IP-10 in Theiler's virus infection had no effect on inflammation or viral persistence (21). Our previous studies (23) showed that the absence of CXCR3 had a modest effect on pulmonary inflammation during influenza virus infection and did not affect viral clearance. Thus, the role and influence of CXCR3 and its ligands varies during infection with different viruses. In the present study, we examined the importance of CXCR3 in the immune response to MHV-68.

Age- and sex-matched 6- to 12-week-old CXCR3$^{-/-}$ mice, which had been backcrossed for 10 generations to C57BL/6 (Jackson) mice, and wild-type CXCR3$^{+/+}$ C57BL/6 (Jackson) controls were used in all experiments. Mice were bred and housed under specific-pathogen-free conditions in the Animal Resource Center at the La Jolla Institute for Allergy and Immunology or Torrey Pines Institute for Molecular Studies. The genotypes of the CXCR3$^{+/+}$ and CXCR3$^{-/-}$ mice were verified by PCR on tail snips.

Our previous studies (15) suggested that upregulation of CXCR3 on activated T cells might be a key event in T-cell trafficking to the lung during MHV-68 infection and consequent viral clearance. In accordance with this hypothesis, mice homozygous for a targeted disruption of the CXCR3 gene showed a significant delay in viral clearance from the lungs (Fig. 1A). Thus, lung viral titers of CXCR3$^{-/-}$ mice were significantly higher than those of CXCR3$^{+/+}$ mice at days 7 to 10 postinfection (p.i.). By day 10 postinfection, all of the CXCR3$^{+/+}$ mice had cleared virus, whereas only one of six CXCR3$^{-/-}$ mice had done so. However, by day 13 postinfection, all of the CXCR3$^{-/-}$ mice had also cleared replicating virus from their lungs. Furthermore, CXCR3$^{-/-}$ mice were able to maintain effective long-term control of MHV-68, and no viral reactivation was observed in the lungs.

The total number of cells infiltrating the lungs of CXCR3$^{+/+}$ mice was significantly higher than the number of cells infiltrating the lungs of CXCR3$^{-/-}$ mice at days 7 ($P < 0.001$) and 8 ($P < 0.01$) postinfection (Fig. 1B). There was no significant difference in the total cell counts in BALs at day 10 and at any subsequent time point after infection. The proportion of CD8$^+$ T cells in the BAL of CXCR3$^{-/-}$ mice were reduced compared to the proportion in the BAL of wild-type mice at days 7 to 10 postinfection ($P < 0.05$) (Fig. 2). There was also a significant reduction in the proportion of α/β T-cell receptor-positive (αβTCR$^+$) cells in the BAL of CXCR3$^{-/-}$ mice at days 8 and 10 postinfection (P was <0.05 at each time point) but not at day 7 (possibly due to a slight increase in the proportion of CD4 T cells offsetting the reduction in CD8 T cells at the latter time point). The proportions of CD8 cells in the BALs of

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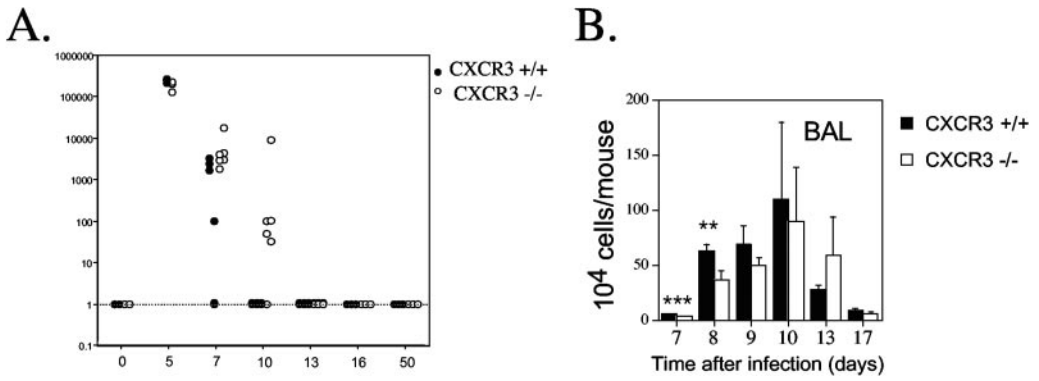

FIG. 1. Lung virus titers and numbers of cells in the bronchoalveolar lavages of $CXCR3^{+/+}$ and $CXCR3^{-/-}$ mice. (A) Delayed clearance of lytic MHV-68 from the lungs of $CXCR3^{-/-}$ mice. $CXCR3^{-/-}$ or $CXCR3^{+/+}$ mice were infected intranasally with 2×10^5 PFU of MHV-68 (clone G2.4). At various times after infection, lungs were harvested and virus titers determined in lung homogenates by plaque assay as described previously (1). Data are expressed as $\log_{10}$ PFU/0.1 g of lung tissue for individual mice. Lung virus titers of $CXCR3^{-/-}$ mice were significantly higher than those of $CXCR3^{+/+}$ mice at days 7 ($P = 0.026$) and 10 ($P = 0.015$) after infection (Mann-Whitney rank sum test). (B) Numbers of cells in the BALs. Numbers of cells recovered in the BALs were determined at intervals after intranasal infection of $CXCR3^{+/+}$ and $CXCR3^{-/-}$ mice with MHV-68. Data are mean cell counts (plus standard deviations) from individual mice, obtained in two to four independent experiments at each time point. Groups of three to five mice were used in each experiment. Viable cell counts were determined by trypan blue exclusion. Asterisks denote that the difference between cell numbers in the BALs of $CXCR3^{-/-}$ and $CXCR3^{+/+}$ mice was statistically significant at days 7 and 8 postinfection. ***, $P < 0.001$; **, $P < 0.01$ (Student's t test).

$CXCR3^{-/-}$ and wild-type mice by day 15 after infection were similar. Thus, there appeared to be a delay in CD8 T-cell trafficking to the lungs in $CXCR3^{-/-}$ mice. There was no significant difference in the proportions of $DX5^+$ lymphocytes (predominantly NK cells) or $CD19^+$ B cells in the BALs of $CXCR3^{+/+}$ and $CXCR3^{-/-}$ mice at any time point, suggesting that CXCR3 is not required for trafficking of these cell types to the lung.

There was a significant reduction in the cytotoxic T lymphocyte (CTL) response in the BAL of $CXCR3^{-/-}$ mice at day 8 after infection ($P < 0.01$) (Fig. 3A), consistent with the subsequent delay in viral clearance in these mice (Fig. 1). Cytotoxicity was measured by using a redirected chromium release assay (1), which measures both virus-specific and nonspecific cytotoxicity. A previous publication (1) and our unpublished data (F. Giannoni and S. Sarawar, unpublished data) suggest that the majority of the redirected cytotoxic activity is mediated by $CD3^+CD8^+$ cells in this model of MHV-68 infection. The reduction in CTL activity did not seem to be explicable on a per-cell basis by the relative numbers of CD8 T cells in the BAL of $CXCR3^{+/+}$ or $CXCR3^{-/-}$ mice. This suggests that there might have been a selective effect either on the trafficking

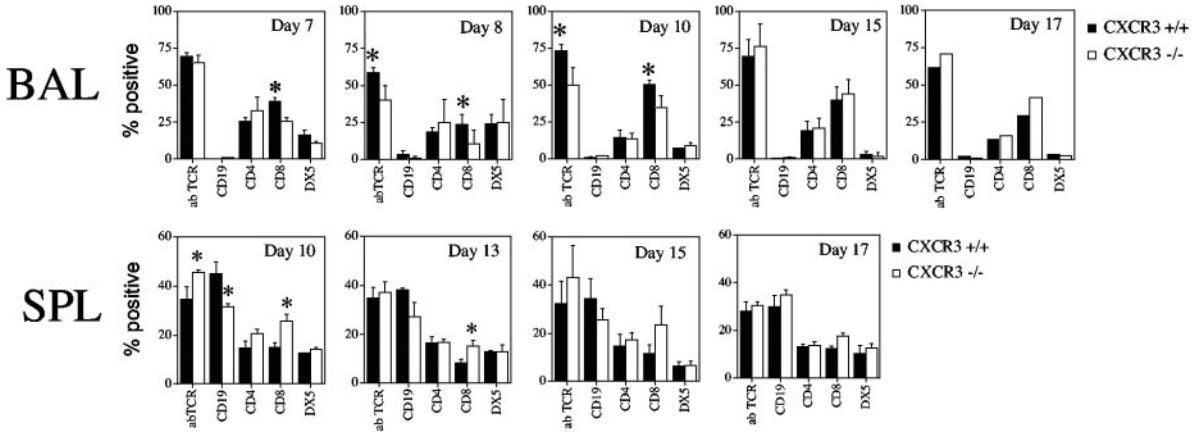

FIG. 2. Lymphocyte subsets in the bronchoalveolar lavages and spleens (SPL) of $CXCR3^{+/+}$ and $CXCR3^{-/-}$ mice following infection with MHV-68. $CXCR3^{-/-}$ and $CXCR3^{+/+}$ mice were infected intranasally with MHV-68. At various times after infection, mice were killed by Avertin overdose, and BALs and splenocytes were harvested. Single-cell suspensions were prepared, and leukocytes were stained with phycoerythrin or fluorescein isothiocyanate-conjugated monoclonal antibodies as described previously (14). The resulting populations were analyzed by flow cytometry. Combined data from two independent experiments are shown. Groups of three mice were used in each experiment. Asterisks denote significant differences in the percentage of CD8 cells in the BALs of $CXCR3^{+/+}$ and $CXCR3^{-/-}$ mice at days 7 through 10 after infection (an asterisk indicates that P is <0.05) and in the percentage of αβTCR (abTCR)-positive cells in the BALs of $CXCR3^{-/-}$ mice at days 8 and 10 postinfection (P was <0.05 at each time point). There was a significant difference between the percentages of $\alpha\beta TCR^+$ cells and CD8 T cells in the spleens of $CXCR3^{+/+}$ and $CXCR3^{-/-}$ mice at days 10 and 13 after infection (an asterisk indicates that P is <0.05 for each cell subset; Student's t test).

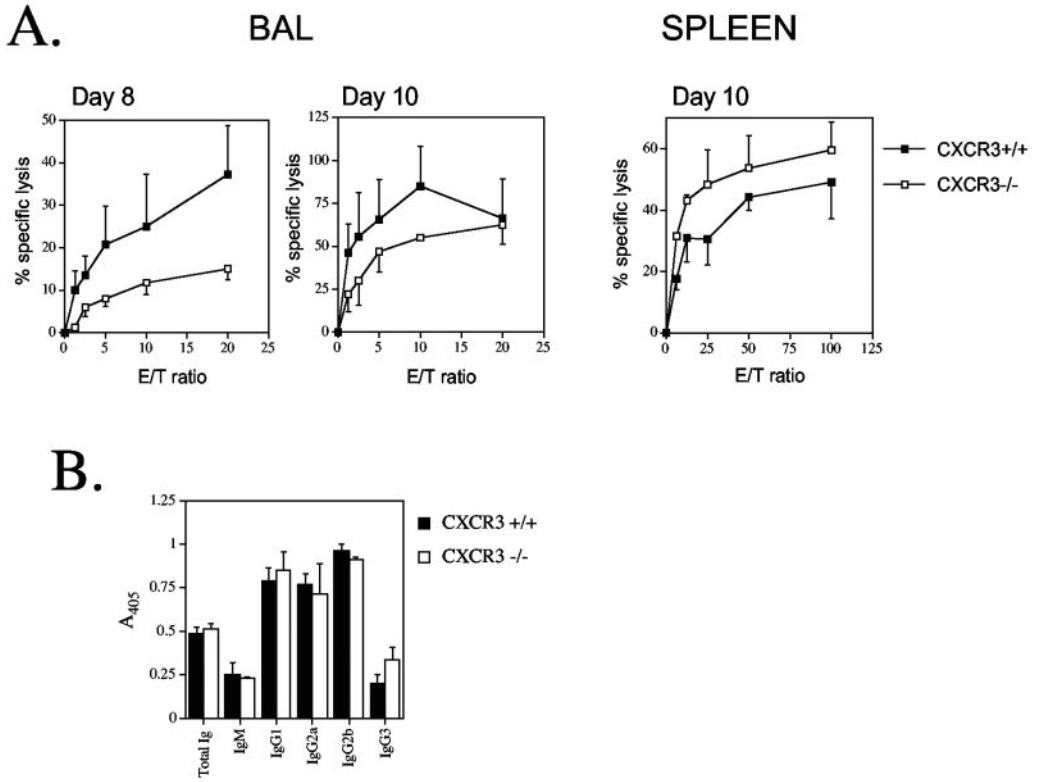

FIG. 3. CTL activity and antiviral antibody responses in CXCR3$^{+/+}$ and CXCR3$^{-/-}$ mice. (A) Reduced CTL activity in the lungs of CXCR3$^{-/-}$ mice. BAL or spleen was harvested from CXCR3$^{+/+}$ or CXCR3$^{-/-}$ mice at days 8 and 10 after infection with MHV-68. CTL activity was determined by a 6-h redirected ^{51}Cr release assay using FcR^{+} P815 cells, in the presence of 2 μg/ml 2C11 anti-CD3 antibody as targets. Mean percentages of specific lysis ± standard deviations for BAL CTL are shown. Data are combined from four independent experiments at day 8 and three experiments at day 10, each experiment using groups of three to five mice. There was a significant reduction in the BAL of CXCR3$^{-/-}$ mice at day 8 postinfection ($P < 0.01$; paired t test). E/T ratio, effector/target ratio. (B) MHV-68-specific antibody responses. Serum was collected from CXCR3$^{+/+}$ and CXCR3$^{-/-}$ mice 50 days after infection with MHV-68. Virus-specific antibody responses were determined as described previously (16). Data are expressed as mean serum antibody titers plus standard deviations for three individual mice. Ig, immunoglobulin.

of a subset of CD8 T cells in CXCR3$^{-/-}$ mice or, as has been shown in other models (4), that CXCR3 may play a role in T-cell activation. However, by day 10 after infection, there was less difference between cytotoxic T cell responses in BALs from wild-type and CXCR3$^{-/-}$ mice, (Fig. 3A), which is also consistent with the ensuing viral clearance in the CXCR3$^{-/-}$ mice. The CTL response in both CXCR3$^{+/+}$ and CXCR3$^{-/-}$ mice was too low to measure at day 7 postinfection.

Serum antibody titers were determined by enzyme-linked immunosorbent assay 50 days after infection with MHV-68 as described previously (16). There was no significant difference in the total virus-specific serum antibody titers or antibody subclass distributions in CXCR3$^{+/+}$ and CXCR3$^{-/-}$ mice. (Fig. 3B).

Following intranasal infection with MHV-68, latency is established in the spleen and splenomegaly is induced (18, 22). Splenomegaly in CXCR3$^{-/-}$ mice was significantly increased (Fig. 4A and B) whether measured by increased spleen weight (P was <0.01 at days 13 and 15 p.i., and P was <0.001 at day 17 p.i.) or cellularity (P was <0.01 at day 17 p.i.). The frequencies of latently infected cells enumerated in the spleens of wild-type and CXCR3$^{-/-}$ mice by using an infectious centers assay were similar (Fig. 4C). However, the total number of latently infected cells in CXCR3$^{-/-}$ mice was significantly increased (P was <0.05 at day 17 p.i.) (Fig. 4D) due to the overall increase in splenic cellularity. Little or no replicating virus (<3 PFU/10^7 splenocytes) was detected in the spleens of either CXCR3$^{-/-}$ or CXCR3$^{+/+}$ mice. The percentage of CD8 T cells in the spleens of CXCR3$^{-/-}$ mice was higher than that for wild-type mice at days 10 to 13 after infection (Fig. 2), although CTL activity was not significantly affected (Fig. 3A). The increase in CD8 T-cell numbers may have been too small to increase CTL activity significantly. Alternatively, the increase may have predominantly involved CD8 T cells lacking cytolytic activity. These data suggest that the increase in splenomegaly and latently infected cells was not due to a decrease in CD8 T cells or CTL activity in the spleens of CXCR3$^{-/-}$ mice. Furthermore, there was no significant change in the number of splenic NK cells in CXCR3$^{-/-}$ mice. It is possible that the increased splenomegaly in CXCR3$^{-/-}$ mice reflects the delayed viral clearance in the lung, which leads to increased or prolonged seeding of latency in the spleen. Splenomegaly was eventually resolved in CXCR3$^{-/-}$ mice and effective long-term control of latent MHV-68 was established.

Taken together, our data suggest that the delay in viral clearance in CXCR3$^{-/-}$ mice results from an early reduction in T-cell trafficking to the lung and delayed development of the CTL response. However, the mice do eventually mount an

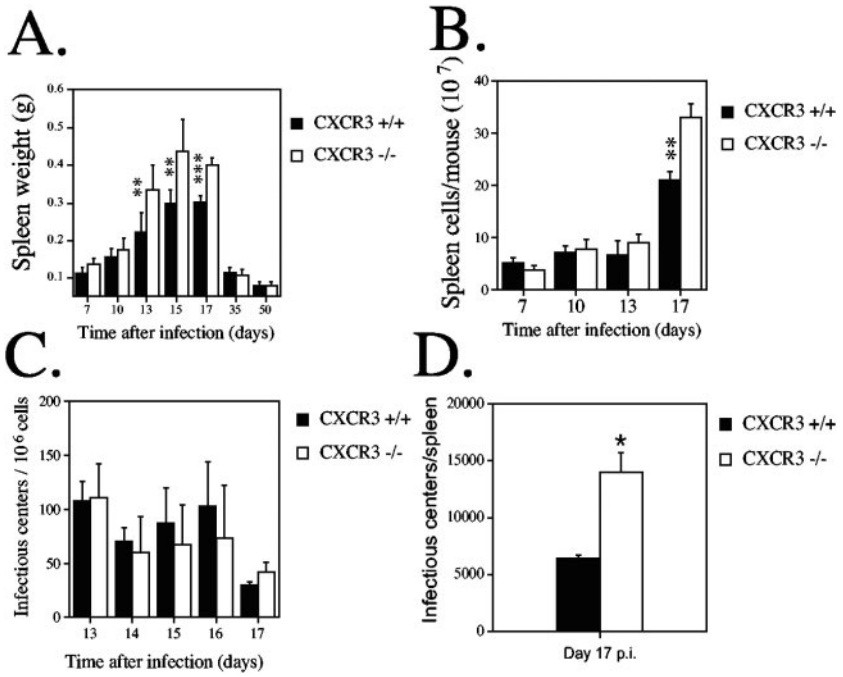

FIG. 4. CXCR3$^{-/-}$ mice show increased splenomegaly and an increase in the number of latently infected cells in the spleen. Spleens were harvested at the specified times after infection with MHV-68. (A) Increased weights of spleens of CXCR3$^{-/-}$ mice. Data are mean spleen weights plus standard deviations for two independent experiments at each time point. Groups of three mice were used in each experiment. Asterisks denote that the difference in spleen weights between CXCR3$^{-/-}$ and CXCR3$^{+/+}$ mice was statistically significant at days 13, 15, and 17 postinfection. **, $P < 0.01$; ***, $P < 0.001$ (Student's t test). (B) Increased splenic cellularity in CXCR3$^{-/-}$ mice. Single-cell suspensions were prepared from individual mouse spleens, and viable cell counts were determined by trypan blue exclusion. Data are mean cell counts plus standard deviations for two independent experiments at each time point. Groups of three mice were used in each experiment. Spleen cell counts for CXCR3$^{-/-}$ mice were significantly higher than those for CXCR3$^{+/+}$ mice at day 17 after infection ($P < 0.01$; Student's t test). (C) The frequencies of infectious centers in the spleens of CXCR3$^{+/+}$ and CXCR3$^{-/-}$ mice were determined by plaque assay after overnight incubation of splenocytes on NIH 3T3-cell monolayers. Data are expressed as mean numbers of infectious centers/10^7 splenocytes for duplicate determinations. Groups of three mice at were used at each time point. (D) Increased total number of infectious centers in the spleens of CXCR3$^{-/-}$ mice. Data are expressed as mean numbers of infectious centers ± standard errors of the means for duplicate determinations on splenocytes from three individual mice at day 17 p.i. An asterisk denotes that the difference in total numbers of infectious centers in the spleens of CXCR3$^{-/-}$ and CXCR3$^{+/+}$ mice was statistically significant. *, $P < 0.05$.

effective immune response to MHV-68, and it is likely that both CTL and antiviral antibody responses contribute to the long-term control of MHV-68 in CXCR3$^{-/-}$ mice. A wide variety of chemokine receptors are upregulated on T cells during activation, and it is not unexpected that there would be some degree of functional overlap between them. The fact that T cells eventually migrated to the lungs and cleared replicating MHV-68 in the absence of CXCR3 suggests that they were responding via alternative chemokine receptors. We have previously shown that, in addition to CXCR3, chemokine receptors CCR1, CCR2, CCR3, and CCR5 are upregulated in the lungs during MHV-68 infection (15). The increased expression of these receptors coincides with the development of the inflammatory infiltrate, suggesting that they may also be involved in T-cell trafficking to the lungs (15). Therefore, it is likely that in the absence of CXCR3, T cells are able to respond via one or more of these receptors to chemokines produced in the lung. However, the initial impairment in viral control in mice lacking CXCR3 or its ligand observed in this study and previous studies of other systems (2–4, 8, 10–12) suggests that this chemokine-receptor interaction plays a unique role in the immune response.

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