Cutting Edge: Protective Effect of CX3CR1⁺ Dendritic Cells in a Vaccinia Virus Pulmonary Infection Model

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The protective host immune response to viral infections requires both effective innate and adaptive immune responses. Cross-talk between the two responses is coordinated by the chemokine network and professional APCs such as dendritic cells (DCs). In mice, subpopulations of myeloid DCs in peripheral tissues such as lungs and in blood express CX3CR1 depending on the inflammation state. We thus examined the host response of mice deficient in the chemokine receptor CX3CR1 to an intranasal vaccinia virus infection. CX3CR1-deficient mice displayed significantly more severe morbidity and mortality compared with control wild-type mice within 10 d following vaccinia virus infection. CX3CR1+/− mice had increased viral loads and a reduced T cell response compared with wild-type mice. Finally, an adoptive transfer of CX3CR1+/+ DCs completely protected CX3CR1−/− mice to a previously lethal infection. This study therefore opens up the possibility of novel antiviral therapeutics targeting lung DC recruitment.

The protective host immune response to viral infections requires coordination between the innate and adaptive responses. The speed at which the host innate cells respond is crucial in determining the subsequent outcome of infection (1). This response is controlled by the inflammatory process including chemical signals such as chemokines (2). Dendritic cells (DCs) are professional APC and as such key instigators of protective immunity (3). Understanding how DCs sense an inflammation state. We thus examined the host response of mice deficient in the chemokine receptor CX3CR1 to an intranasal vaccinia virus infection. CX3CR1-deficient mice displayed significantly more severe morbidity and mortality compared with control wild-type mice within 10 d following vaccinia virus infection. CX3CR1+/− mice had increased viral loads and a reduced T cell response compared with wild-type mice. Finally, an adoptive transfer of CX3CR1+/+ DCs completely protected CX3CR1−/− mice to a previously lethal infection. This study therefore opens up the possibility of novel antiviral therapeutics targeting lung DC recruitment.

The chemokine receptor CX3CR1 and its ligand, fractalkine (CX3CL1), are important for directing leukocyte migration from the blood into tissues (4–6). Expressed by DCs, NK cells, circulating monocytes, and CD8 T cells in both humans and mice, this receptor enables capture and migration of these cells into infected tissues particularly in states of inflammation (4). Expression of CX3CL1 is enhanced by inflammatory cytokines such as TNF-α and IFN-γ (7), and it has been implicated in many chronic inflammatory lung diseases (6). The respiratory syncytial virus G protein has a CX3C chemokine motif that binds CX3CR1 modifying its response (8). In mouse studies, this resulted in a reduced antiviral T cell response (9). Its effect on DCs was not examined in this study.

In the current study, we show that CX3CR1+/− mice are far more susceptible to vaccinia virus (VV) infection compared with wild-type (wt) mice on two genetic backgrounds. Adoptive transfer of wt DCs to CX3CR1−/− mice resulted in complete protection against death to a VV infection.

Materials and Methods

Mice

Two genetic backgrounds were used in this study. C57BL/6 CX3CR1−/− and CX3CR1+/− mice were backcrossed six generations onto the BALB/c background (F6 BALB/c). For some experiments, CX3CR1−/−, CCR5−/−, CCR5−/−, CX3CR1−/−, and CX3CR1+/− C57BL/6 mice were also used as indicated. All mice were housed at the Centre d’Expérimentation Fonctionnelle of the Université Pierre et Marie Curie under specified pathogen-free conditions and used for experiments at 6–10 wk old; mice in infection experiments were age matched with controls. Female BALB/c and C57BL/6 mice were purchased from Charles River Laboratories. All experiments complied with French legislation and guidelines for animal research.

Virus

VV Copenhagen strain (Dr. M.-P. Kieny, Transgene Laboratories, Strasbourg, France) was produced in our laboratory on the BHK21 cell line. Modified virus Ankara (MVA) (Dr. B. Verrier, Transgene Laboratories) was used for supplemental figures. Mice were infected intranasally (i.n.) with 5 × 10^6 PFU (40 μl) live VV or MVA after anesthesia with ketamine/xylazine by i.p. injection.

The online version of this article contains supplemental material.

Abbreviations used in this article: Ct, threshold cycle; DC, dendritic cell; DLN, draining lymph node; i.n., intranasal; KO, knockout; MVA, modified virus Ankara; VV, vaccinia virus; wt, wild-type.

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Measurement of viral load (mRNA) by semiquantitative real-time PCR

Total lung RNA was extracted with the Absolutely RNA RT-PCR Miniprep kit (Stratagene), and cDNA was generated with 1 μg RNA, Primer Oligo dT12-18 (0.5 mg/ml), 2’-deoxynucleoside 5’-triphosphate (10 mM each), RNase out (40 U), 0.1 M DTT, and 5× First Strand Buffer and SuperScript II RNaseH Reverse Transcriptase according to the manufacturer’s instructions (Invitrogen).

For quantitative RT-PCR, we used the DUAL Labeled Probes and primers from MWG-Biotech and Applied Biosystems TagMan Master Mix with uracil-N-glycosylase. We thank Vincent Calvez (Pitié-Salpêtrière Hospital, Paris, France) for the RT-PCR assay. Real-time detection and analysis were performed on an ABI 7700 thermocycler (PerkinElmer). The data were analyzed quantitatively by measuring the threshold cycles (Ct) in a Microsoft Excel program (Microsoft) and graphically by an amplification plot. The Ct is the cycle at which a significant increase in fluorescence occurs; hence, a Ct value <45 indicates a positive result.

ELISPOT assays

ELISPOT assays were performed with the mouse-specific IFN-γ kit (Diaclone Research), following the manufacturer’s specifications and as described elsewhere (10). Cells (100,000 cells/well) were incubated on polyvinylidene difluoride plates (Millipore) during 42 h at 37°C in a 5% CO2 atmosphere with and without virus at 0.1 PFU/cell. Spot-forming units were counted onto an Axioplan 2 microscope using the KS-ELISPOT software (Zeiss).

Adoptive transfer

For adoptive cell transfers, spleens and lymph nodes of mice were prepared by Collagenase type IV treatment (Sigma-Aldrich) and positively selected using CD11c (N418) or NK (DX5)-coupled MicroBeads (Miltenyi Biotec). Cells were purified following the manufacturer’s instructions. The cells were washed twice in PBS, and 1.5 × 106 cells were injected i.v. through the retro-orbital vein of the recipient mice 1 h before VV infection by the i.n. route.

Statistics

Statistical significance was examined by a Mann–Whitney or unpaired Student t test as indicated. Survival curves were compared using a log-rank test. Statistical significance was set at p < 0.05. Prism 4 software (GraphPad) was used for data handling, analysis, and graphic representation.

Results

CX3CR1−/− mice have increased susceptibility to vaccinia virus

The role of CX3CR1 was assessed in two genetic backgrounds to VV infection. Firstly, using CX3CR1−/− mice on a C57BL/6 background, the role of CX3CR1 in controlling a VV infection was assessed (Fig. 1A). Following an i.n. infection with VV, CX3CR1−/− mice exhibited significantly more severe weight loss by days 7, 8, and 11 compared with control wt mice (p < 0.05). No difference in the kinetics of weight loss was observed between the two groups. We also tested two other chemokine receptors that have been implicated in the recruitment of innate cells: CCR1 and CCR5 (11, 12). CCR1−/− and CCR5−/− mice were infected but did not exhibit any differences in weight loss compared with control mice (Fig. 1B). These results showed that the absence of CX3CR1, but not CCR1 or CCR5, in C57BL/6 mice increased the morbidity of VV infection.

To further investigate the role of CX3CR1 in controlling VV, we also investigated CX3CR1−/− mice backcrossed for six generations onto a BALB/c background (F6 BALB/c mice). Following an i.n. infection with VV, both wt and knockout (KO) mice lost weight to a similar degree during the first few days (Fig. 1C). However, 100% wt BALB/c mice (n = 21) survived the infection (Fig. 1D), in sharp contrast to littermate CX3CR1−/− BALB/c mice (n = 14), which began to die by day 7, with <50% surviving at day 15 after challenge. The 42% of mice that survived regained normal weight loss after day 15. The large numbers in this experiment and

FIGURE 1. CX3CR1−/− mice are more susceptible than wt mice to i.n. vaccinia infection. A, Percentage weight loss of wt (○; n = 14) and CX3CR1−/− (●; n = 16) mice on a C57BL/6 background following i.n. VV infection with 5 × 106 PFU. Data represent three pooled experiments. **p < 0.01, ***p < 0.001, Mann–Whitney U test. B, Percentage weight loss of CCR1−/− (○; n = 7), CCR5−/− (△; n = 6), and wt (○; n = 5) mice on a C57BL/6 background after VV infection. C, Percentage weight loss before death time point of wt (○; n = 6) and CX3CR1−/− (●; n = 6) mice on an F6 BALB/c background following VV infection. D, Survival of wt (○; n = 21) and CX3CR1−/− (●; n = 14) mice on an F6 BALB/c background following VV infection. Data represent three pooled experiments. ****p < 0.0001, log-rank (Mantel–Cox) test.
the highly significant difference (p = 0.0007) in survival rates implicated an important role for CX3CR1 in protection to VV.

To examine whether the increased mortality of CX3CR1<sup>−/−</sup> mice was associated with an increased viral load, we extracted lung mRNA and performed semiquantitative RT-PCR on total lung tissues from KO and wt BALB/c mice at day 5 postinfection (prior to death) (Fig. 2). Significantly more (p = 0.0019) viral mRNA was detected in the lungs of infected CX3CR1<sup>−/−</sup> mice compared with wt controls, suggesting that a failure to control viral replication in this organ led to the increased mortality.

These results demonstrate that CX3CR1 is a key molecule in protection against VV pulmonary infection in mice.

<sub>CX3CR1<sup>−/−</sup></sub> mice showed a reduced specific T cell response to VV infection

As we observed a difference in the response to VV between KO and wt mice at early time points, we speculated that the effect was T cell rather than B cell mediated. Therefore, to examine the early T cell response to VV infection, the lungs, spleen, and draining lymph nodes (DLNs) of wt and CX3CR1<sup>−/−</sup> mice were removed and tested by ELISPOT assay for VV-specific IFN-γ production at day 5 (Fig. 3A) on BALB/c background and day 14 (Fig. 3B) on a C57BL/6 background postinfection. It has to be noted that 30% BALB/c mice died after 6 to 7 d postinfection (Fig. 1D). Thus, it is difficult to compare the T cell response between groups prior to death. Immune responses observed in the lungs are low; however, notable differences in VV-specific T cell immune responses can be observed in the spleen (Fig. 3A). In the C57BL/6 mouse model, all mice are surviving. Thus, we performed the IFN-γ ELISPOT assay at day 14 and observed a lower VV-specific T cell response in CX3CR1<sup>−/−</sup> compared with wt mice (Fig. 3B).

Altogether, these results demonstrated that CX3CR1<sup>−/−</sup> mice had a reduced VV-specific IFN-γ response following VV infection in the lungs and spleen.

**FIGURE 2.** CX3CR1<sup>−/−</sup> mice contain higher virus replication levels postinfection with VV. BALB/c mice were infected with 5 × 10<sup>6</sup> PFU, and lung tissues were harvested at day 5. VV viral load (mRNA measure) (mean Ct ± SEM) detection in lung tissues of wt infected (●; n = 3), wt noninfected (NI) (●; n = 3), and CX3CR1<sup>−/−</sup> infected (●; n = 3) lung cells on day 5 following VV infection as measured by semi-quantitative RT-PCR. **p < 0.01, unpaired t test.

**FIGURE 3.** Specific effector T cell response to VV infection in CX3CR1<sup>−/−</sup> and wt mice. Mice (n = 6–8) were infected with 5 × 10<sup>6</sup> PFU of VV, and lungs, spleen, and lung DLNs were harvested at indicated time points. IFN-γ-producing VV-specific effector T cells (mean ± SEM) quantified by ELISPOT assay on the lungs, spleen, and DLNs of wt (white) and CX3CR1<sup>−/−</sup> (black) mice at day 5 (prior death time point) (BALB/c background) (A) and on day 14 (C57BL/6 background) (B). Data represent two pooled experiments. ∗p < 0.05; Mann–Whitney U test.

Adaptive transfer of wt DCs to CX3CR1<sup>−/−</sup> mice restored protection against VV infection

As DCs and NK cells express CX3CR1 and also link the innate and adaptive responses, we wanted to test which cell type might be responsible for the protective response to VV infection. We investigated the expression of CX3CR1 in myeloid cell subpopulations in the lungs of CX3CR1<sup>+/GFP</sup> mice (Supplemental Fig. 1A–E). This showed that CX3CR1 is expressed by both CD11c<sup>+</sup>CD11<sup>bhi</sup> (myeloid DC) and at a lower expression level in ~63% of CD11c<sup>−</sup>CD11<sup>bhi</sup> non-DCs (Supplemental Fig. 1B, 1D). Significant modifications of percentage of CD11c<sup>−</sup>CD11<sup>bhi</sup> DC population were observed in the lungs of CX3CR1<sup>−/−</sup> compared with wt mice 24 h following MVA infection (Supplemental Fig. 1F). In contrast, CD11c<sup>−</sup>CD11<sup>bhi</sup> (monocytes/NK/myeloid cells) did not differ in CX3CR1<sup>−/−</sup> mice compared with wt mice postinfection (Supplemental Fig. 1H).

To further confirm that the protective role of CX3CR1 is only mediated by DCs, we purified DCs and NK cells from wt mice and adoptively transferred them to CX3CR1<sup>−/−</sup> mice before a VV challenge. Strikingly, CX3CR1<sup>−/−</sup> recipients of wt DCs survived 100% the VV challenge (Fig. 4A). In contrast, control VV-infected CX3CR1<sup>−/−</sup> mice began to die on day 7 with a 42% mortality rate by day 12 (p = 0.0229) (Fig. 4A). In contrast, the adoptive transfer of NK cells from wt
CX3CR1+/+ mice (wt NK cells (background) received either wt DCs (C (Mantel–Cox) test. In this model, the adoptive transfer of wt CD11c+ cells (Whitney U) showed no significant differences between CX3CR1+/+ mice against mortality after VV infection. CX3CR1+/+ DCs was confirmed in the C57BL/6 mouse model (Fig. 4B). In contrast, recipients of CX3CR1+/+ NK cells showed no protective response. As professional APCs, DCs are the key instigators of the adaptive immune response (16, 17). Our evidence presented in this study shows that expression of CX3CR1 is crucial for DC-mediated protection from a VV lung infection. It has previously been shown that blood monocytes are recruited to inflamed tissues and that this migration is dependent upon CX3CR1 (18). The short-lived CX3CR1loGr1+ subpopulation differentiated into DCs and induced naive T cell proliferation in vivo. In our model, we observed a reduced VV-specific T cell response in infected CX3CR1−/− mice compared with control mice, suggesting that this was at least partly how the DCs exerted their protective influence. In fact, very recently, the lung-migrating CD103+ DC population was implicated as the major contributor to CD8 T cell activation following poxvirus infection (16). However, without the adaptive immune response, the innate cells could merely delay morbidity compared with nonvaccinated controls (19). Therefore, it is likely that a combination of both innate and adaptive immune responses are necessary for full protection against a lethal respiratory infection such as VV.

Interestingly, in a mouse model of tuberculosis lung infection, the absence of CX3CR1 had no discernible effect on virus replication levels. This result together with the short mortality kinetics postinfection suggested that a component of the innate immune response was lacking in CX3CR1−/− mice.

To assess which cell type might be important for CX3CR1-mediated protection, we purified DCs and NK cells from wt mice and adoptively transferred them to CX3CR1−/− mice before challenging with VV. Conclusively, 100% of CX3CR1+/+ DC recipient mice survived compared with a 42% mortality rate in the control nontransferred CX3CR1−/− mice. In contrast, recipients of CX3CR1+/+ NK cells showed no protective response. As professional APCs, DCs are the key instigators of the adaptive immune response (16, 17). Our evidence presented in this study shows that expression of CX3CR1 is crucial for DC-mediated protection from a VV lung infection. It has previously been shown that blood monocytes are recruited to inflamed tissues and that this migration is dependent upon CX3CR1 (18). The short-lived CX3CR1loGr1+ subpopulation differentiated into DCs and induced naive T cell proliferation in vivo. In our model, we observed a reduced VV-specific T cell response in infected CX3CR1−/− mice compared with control mice, suggesting that this was at least partly how the DCs exerted their protective influence. In fact, very recently, the lung-migrating CD103+ DC population was implicated as the major contributor to CD8 T cell activation following poxvirus infection (16). RAG-1−/− mice that lack T and B cells but retain innate immune cells previously showed a delayed onset of severe VV respiratory disease when vaccinated with MVA 2 d earlier (19). However, without the adaptive immune response, the innate cells could merely delay morbidity compared with nonvaccinated controls (19). Therefore, it is likely that a combination of both innate and adaptive immune responses are necessary for full protection against a lethal respiratory infection such as VV.
disease outcome (20). However, for mycobacteria infections, resident lung macrophages instigate the immune response, and these cells do not express CX3CR1, unlike lung DCs (21) (data not shown). Another study using CX3CR1<sup>−/−</sup> mice supported this, showing that alveolar CX3CR1<sup>−/−</sup> DCs did not take part in the early antimicrobial response despite their increased numbers in bacillus Calmette-Guérin–infected lungs (21). Previous studies have implicated the chemokine CCR2 for DC accumulation in the lungs in response to a particulate Ag challenge (22). However, this immune response is largely of a Th2 type involving very different effector pathways compared with a viral infection (Th1 type). As DCs are responsible for skewing the immune response to either a Th1 or Th2 pathway, the chemokines produced in the lungs to the different types of Ag challenge may influence this (17). In fact, it has been demonstrated that CD103<sup>+</sup> DCs in the lung derive from Ly6c<sup>hi</sup> blood monocytes dependent on CCR2 (23). In contrast, CD11b<sup>hi</sup> DCs derive from Ly6c<sup>lo</sup> monocytes, which are mediated by CX3CR1 (23). CX3CR1 and its ligand fractalkine have also been implicated in the pathogenesis of atherosclerosis and coronary heart disease (24), and targeting this chemokine has been proposed as a potential therapy. However, the results in this study indicate that it may increase susceptibility to infectious diseases. We have demonstrated in this paper a clear and nonredundant role for fractalkine-mediated protection to VV infection. This opens up the possibility of novel therapeutics targeting increased DC/monocyte lung recruitment during pulmonary viral infections.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


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