Original Article

CXCL14 deficiency does not impact the outcome of influenza or *Escherichia coli* infections in mice

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Abstract

Introduction: Chemokines are small proteins that regulate different cellular functions, such as leukocyte activation, chemoattraction and inflammation. The chemokine CXCL14 (BRAK) is a highly conserved gene among species and through evolution. It has been shown that CXCL14 is locally upregulated during viral infections, also, it has been found that this chemokine possesses direct antibacterial activities. Nonetheless, the exact role that CXCL14 plays during infection remains elusive.

Methodology: CXCL14 deficient mice were generated in a C57B6/129 background and followed by phenotypic characterization. Later, the effect of CXCL14 deficiency during influenza infection and *E. coli* challenge was assessed.

Results: Other than a slight weight reduction, CXCL14 deficient mice exhibited no phenotypic alterations. CXCL14 deficiency did not influence the outcome of influenza virus infection or challenge with *E. coli*, and no statistically significant differences in clinical signs, cellular responses and histopathological findings were observed.

Conclusions: CXCL14 does not seem to play a pivotal role during influenza and *E. coli* infections of the lung; these results are suggestive of functional overlap between CXCL14 and other chemokines that are present during lung infection.

Key words: CXCL14; BRAK; pneumonia; influenza.

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Introduction

Chemokines are a group of small proteins that are classified upon the position of four N-terminal cysteine residues and they interact with their cognate G-protein coupled receptors to activate specific cellular pathways. Their main role is to activate a number of leukocyte functions, mostly chemotaxis and leukocyte-dependent immune responses [1]. The CXCL14 receptor has not been characterized and only indirect information has been obtained. Given that the CXCL14 signaling is pertussis toxin-dependent, the receptor for this chemokine is probably a G protein-coupled receptor; moreover, due the lack of competition with the other chemokines, it has been ruled out that the CXCR1-4 molecules participate in the signaling of CXCL14 [2]. The characterization of this receptor is still an ongoing work that, when achieved, will allow researchers to delineate more precisely the cell subsets that are responsive to this chemokine and to identify the downstream signaling pathways that are involved.

The chemokine CXCL14 is highly conserved among different species [3] and it has been postulated to participate in a number of processes in which different leukocyte subsets play an essential role. CXCL14 has chemotactic properties for immature dendritic cells and it regulates their maturation; these properties, together with the expression of CXCL14 in a broad range of tissues, have been related with the immune surveillance network against tumors [2] and

pathogens. In a model of autoimmune arthritis, CXCL14 was found to be overexpressed in the inflamed joints, also, transgenic mice overexpressing CXCL14 exhibited exacerbated experimental arthritis with higher levels of autoantibodies and stronger Th1 responses [4]. Moreover, mice infected with neuro-invasive West-Nile virus (WNV) showed increased levels of CXCL14 in the brain and spleen 3 days post-infection [5], which suggests the involvement of this chemokine in the innate immune responses. Apart from leukocyte-specific functions, CXCL14 takes part in the developmental processes of the brain [6] and it also presents homeostatic functions in a hormone-like manner [7]. Interestingly, CXCL14 may enhance and suppress the growth of different types of tumors through a variety of mechanisms [2].

The purpose of this study was to better define the link between CXCL14 and the immune responses in the context of viral and bacterial infections. We generated CXCL14$^{-/-}$ genetically modified mice in a C57B6/129 background; the phenotypic characterization revealed that deficiency of murine CXCL14 causes weight reduction but it is not associated with any other recognizable phenotype or alterations in the markers of blood homeostasis. Later, these mice were used to study the effects of CXCL14 deficiency during infection with influenza virus and *E. coli* bacteria; no statistically significant alterations were found in terms of disease severity or inflammation profiles in the lung tissue. Therefore, CXCL14 in mice appears to be non-essential or redundant during the protective immune responses against influenza virus or bacterial infection.

Methodology

Mice

C57B6/129 mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). The animal use protocols were approved by the Animal Care Committee at the University Health Network.

Generation of CXCL14 knockout mice

A gene targeting strategy was designed to delete a fragment of the CXCL14 genomic DNA that includes the entire first and second exons (Figure 1A). The success of the deletion strategy was confirmed by Southern-blot (Figure 1B); also, the lack of CXCL14 expression was confirmed in splenocytes by RT-PCR (Figure 1D), as previously described [8].

Routine genotyping of the mouse CXCL14 gene

DNA was extracted from mouse tail clips, as described elsewhere [9]. PCR was performed using Kapa HiFi HotStart DNA Polymerase (Kapa Biosystems, Wilmington, MA, USA), annealing temperature of 60°C and using the following pair of primers: CXCL14_Up5 5'-GGTGGCTTCGGACAGTA-3' and CXCL14_In2 5'-GTGGCATGGCACATTA-3'; these primers target sequences located in the 5' upstream and intron 2 regions, respectively, of the mouse CXCL14 gene. The wild type allele generates a 1,425bp amplicon and the fragment-deleted version of the gene produces a 699bp amplicon (Figure 1C).

Influenza infection

Influenza virus was expanded in embryonated chicken eggs. Turkey erythrocytes were used for hemagglutination and, for each strain, the 50% egg infectious dose (EID_{50}) was calculated. CXCL14$^{-/-}$ and wildtype C57B6/129 mice (WT) were infected with 10^4 EID_{50} of A/Mexico/4108/2009 and 10^3 EID_{50} of A/Puerto Rico/8/1934 (ATCC #VR-95) (PR8), as previously described [10].

E. coli pneumonia model

Experimentally induced mouse pneumonia was carried out as previously described [11]. Briefly, live *E. coli* (strain DH5α at 1×10^9 CFU in 50 µL of PBS) was administered intratracheally to CXCL14$^{-/-}$ and C57B6/129 WT mice. After 18 hours, mice were euthanized and the bronchoalveolar lavage (BAL) and lung tissues were collected.

Blood and BAL analysis

As part of the phenotypic characterization of the knockout mice (KO) mice, hematological and biochemical analysis of blood samples were performed by using an automated cell counter Cell-Dyn 3500 (Abbott Laboratories, Abbott Park, IL, USA) and an automated chemistry analyzer Hitachi 911 (Roche Diagnostics, Mississauga, ON, Canada), respectively. Peripheral blood mononuclear cells (PBMCs) were incubated with antibodies anti-CD115-PE, CD11c-FITC, F4/80-PerCP, anti-CD11c-PE and isotype controls (BD Biosciences, Mississauga, ON, Canada), and analyzed using a FACSCalibur flow cytometer (BD Biosciences) and the Flowjo software (Tree Star, Ashland, OR, USA).

BALs were collected 18 hours after *E. coli* inoculation and processed to obtain single-cell suspensions. Cells were labeled with anti-CD3-FITC,

CD4-PerCP, CD8-PerCP and CD14-PE antibodies (BD Biosciences) and analyzed by flow cytometry.

Histopathology

Lung pathology was evaluated in formalin-fixed, paraffin-embedded tissue slides stained with hematoxylin and eosin.

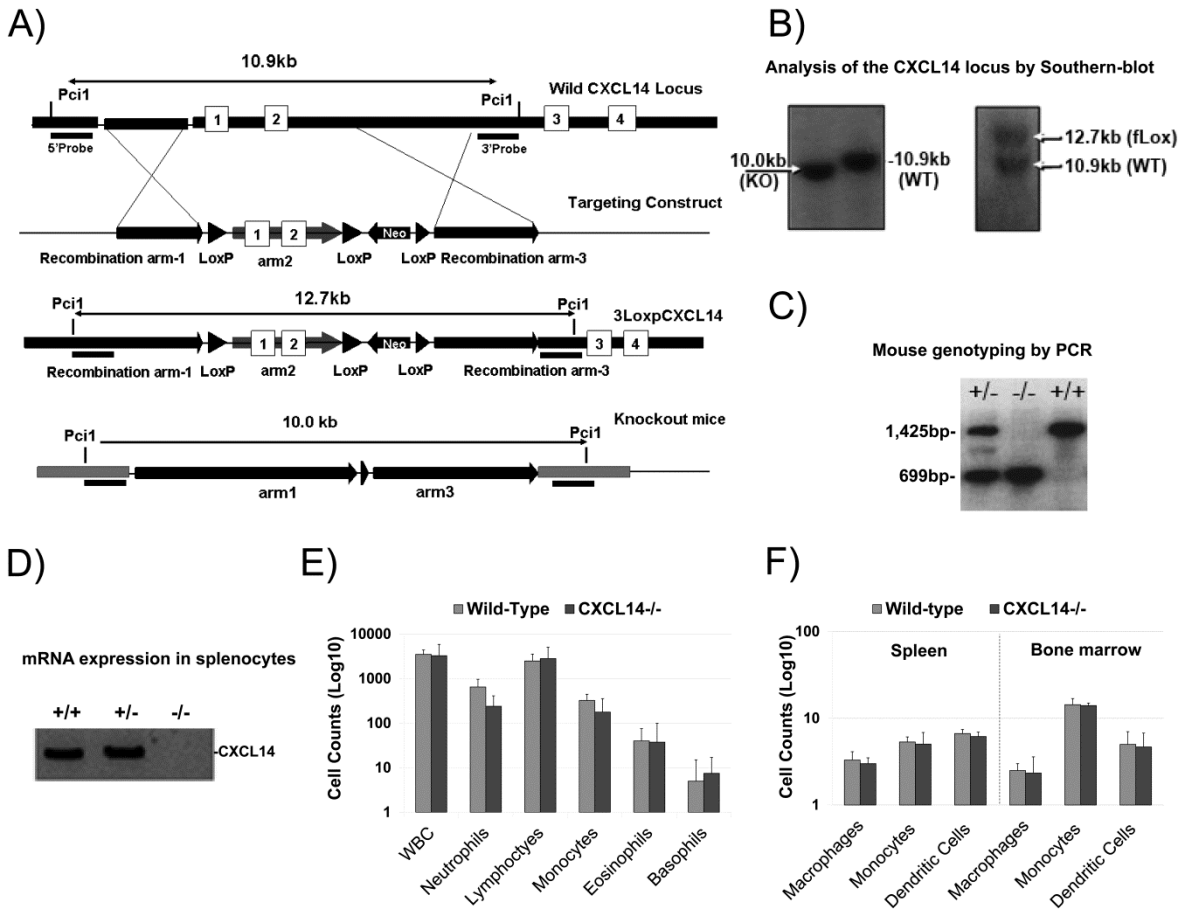
Results and discussion

Generation and phenotypic characterization of CXCL14$^{-/-}$ mice

To dissect the role of CXCL14 as part of the immune responses against infections, we generated CXCL14 knockout mice. CXCL14 expression was suppressed completely by a gene-targeted construct, which was designed to delete exons 1 and 2 of the CXCL14 gene that encode 55 amino acids including signal peptides, initiation codon and conserved cysteine residues (Figure 1A). Deletion of the targeted areas was confirmed by Southern blot (Figure 1B), and lack of CXCL14 expression in homozygous mice was assessed at the mRNA level (Figure 1D).

CXCL14-deficient mice, either heterozygous or homozygous mice, breed and grow normally without presenting any gross abnormalities. Nevertheless, CXCL14$^{-/-}$ mice were healthy but smaller than age-

Figure 1. Generation of CXCL14 deficient mice in a C57B6/129 background and phenotypic characterization. A) The gene targeting construct was engineered in a triple LoxP pKO backbone vector. Exons 1–2, which encode 55 amino acids of CXCL14 that includes signal peptides, initiation codon and conserved cysteines (open boxes in arm2), were flanked by two of three loxP sites (black triangles). The neomycin resistance cassette (Neo) introduced in the intron 2 of CXCL14 sequence upstream of the loxP site prior exon 3. The third loxP site was positioned upstream of the Neo cassette for future removal of this cassette. Black rectangles indicated the three homologous arms in our targeting construct. As shown in the figure, arm1 includes part of genomic DNA upper to exon 1, arm 2 contains exon 1–2 and arm3 comprises the intron upstream to exons 3-4 of CXCL14. Crossed lines indicated double homologous recombination incident that happens among arms 1 and 3 of the gene targeting construct and the identical sequence of the wild type genome. **B)** Detection approach for the CXCL14 genomic locus. Digestion of genomic DNA with Pci1 and analyzed by Southern hybridization blot using a probes that were generated at 5' and 3' of arm1 and arm 3, respectively, differentiates the wild type C57B6/129 (10.9kb), the CXCL14 knockout (10.0kb), and the floxed genotype (12.7kb). **C)** Genotyping of the CXCL14 locus in mice by PCR. **D)** The expression of CXCL14 was analyzed in the RNA extracted from splenocytes in homozygous, heterozygous and wild type mice. **E)** The patterns of leukocyte subsets in the blood were analyzed using an automated cell counter. **F)** Distribution of macrophages, monocytes and dendritic cells in the spleen and bone marrow was analyzed by FACS. Mean ratios of total normal cells data were showed as ± SEM from 5 to 8 mice in three distinct experiments.



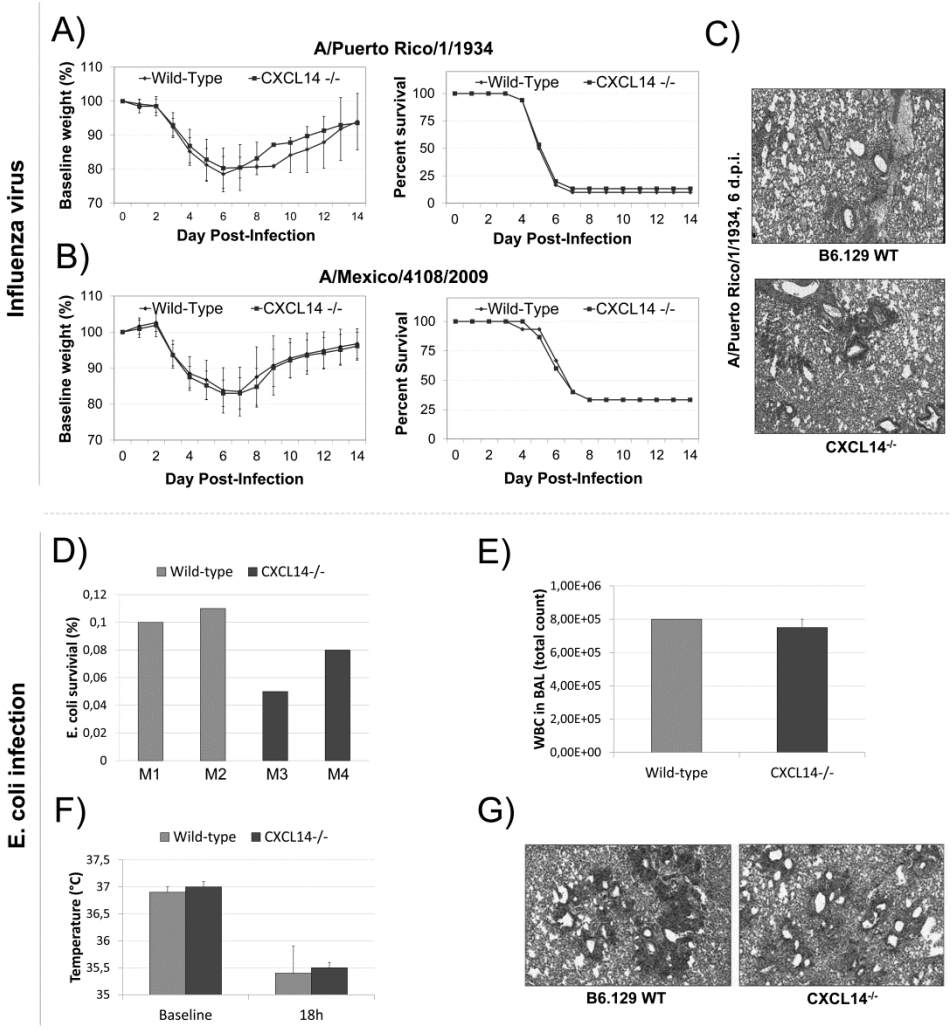
matched WT mice (weight reduction of 10-20%, approximately); these results are in accordance with a previous study in which a different CXCL14$^{-/-}$ mouse strain was independently developed [8]. This reduction in the body weight may support the previously postulated hormone-like roles of CXCL14 [7].

In addition to chemotaxis, chemokines also participate in homeostatic and hematopoietic processes. Constitutive expression in a variety of tissues and high conservation of CXCL14 through evolution supports a homeostatic role of this cytokine. Analysis of the blood, bone marrow and spleen revealed no differences in any leukocyte subsets as compared to WT controls (Figure 1E-F). Moreover, blood biochemistry revealed no abnormalities in CXCL14$^{-/-}$ mice when compared to WT mice (data not shown). Therefore, these results indicate that CXCL14 deficiency does not affect the hematopoietic processes.

CXCL14 deficiency does not impact the outcome of influenza infection

Influenza A virus causes a great burden of disease in the human populations. During the course of the infection, cytokines and chemokines exert a precise control of the activation of the immune responses; while insufficient immune activation leads to failure to resolve the process and excessive activation or dysregulation may lead to lung injury. As part of

Figure 2. Deficiency of CXCL14 does not affect the severity of the infection with either influenza virus or E. coli. CXCL14-deficient and wild type control mice were intranasally infected with **A)** 10^3 EID_{50} of influenza PR8 (n=12 per group), and **B)** 10^4 EID_{50} of influenza A/Mexico/4108/09 (n=12 per group); the weight loss and the survival rate were monitored over the first 14 days post infection. **C)** Histopathological evaluation of the lung tissue from mice infected with PR8, 6 days post-infection. Using an experimental model of mouse pneumonia, mice were intratracheally infected with live E. coli (strain DH5α at 1×10^9 CFU in 50 µL of PBS). **D)** E. coli was able to colonize the lungs of both CXCL14$^{-/-}$ and wild type mice, however, no significant differences were found between CXCL14$^{-/-}$ and the wild type mice in terms of: **E)** counts of total white blood cells in the brochoalveolar lavage (BAL) 18 hours post-infection, **F)** body core temperature and **G)** histopathology of the lung tissue.



another study published by our group, microarray analysis revealed the presence of different chemokines in the lungs of mice infected with influenza, including CXCL9, CXCL10 and CXCL14 (Gene Expression Omnibus accession GSE31022) [12]. While abundant biological information is available for CXCL9 and CXCL10 [13], only limited information is available about the role of CXCL14 in the context of infectious diseases. Hence, we decided to explore the effect of CXCL14 deficiency in a model of influenza infection in mice. No statistically significant differences were found in the weight loss and mortality profiles between CXCL14$^{-/-}$ and WT mice after infection with two different influenza strains: A/Mexico/4108/2009 and A/Puerto Rico/8/1934 (Fig. 2A-B). Effector cells such as macrophages, neutrophils and NK cells are recruited into the lungs as part of the innate immune responses [14]; here, histopathological examination showed that the deficit of CXCL14 did not alter the localization of the inflammatory infiltrate and its extent was neither increased or diminished (Figure 2C). Despite the local upregulation of CXCL14 during viral infection [12], CXCL14 does not seem to have a pivotal role in regulating the inflammation in the lung, a process that is governed by a different subset of cytokines and chemokines.

CXCL14$^{-/-}$ mice show identical susceptibility and histopathological status after infection with E. coli

It has been reported that CXCL14 exerts direct antimicrobial activity against *E. coli* and other bacteria [15]. Also, CXCL14 is constitutively expressed in a variety of tissues, such as lung, liver, spleen, blood, skin and heart (data not shown). To test whether the presence of CXCL14 in the lung participates in the host responses against bacterial pneumonitis, CXCL14$^{-/-}$ and WT mice were inoculated intratracheally with live *E. coli*. We found that *E. coli* was able to colonize the lung tissue of both experimental groups (Figure 2D), however, further investigation is required to determine whether the difference in the levels of bacteria in the lungs is statistically significant and biologically relevant. CXCL14$^{-/-}$ and WT mice were monitored for changes in core body temperature (Figure 2F), total white blood cell counts in BAL collected 18 hours after infection (Figure 2E), distribution of CD4, CD8 and CD14 cells in BAL (data not shown) and histological changes in the lung tissue (Figure 2G). None of those parameters revealed differences between CXCL14$^{-/-}$ and WT during E. coli-induced pneumonia. Experimental evidence from different studies suggest that constitutively-expressed CXCL14 may be part of the first barrier against opportunistic infections in different tissues by means of its direct bacterial activity; however, once the bacterial infection has been established, CXCL14 does not seem to play a pivotal role in regulating acute inflammation of the lung, and its presence in the WT mice may not accelerate the bacterial clearance either.

Conclusions

Previous studies showed that CXCL14 exerts chemoattractanct properties over different cell types *in vitro*, however, functional redundancy with other chemokines makes it difficult to establish the real contribution of CXCL14 during an acute inflammatory process *in vivo*. Despite the negative results obtained when trying to link CXCL14 with the host responses during viral and bacterial infection in mice, we hope that the CXCL14$^{-/-}$ mice generated as part of this study will play a decisive role to dissect the biological functions of CXCL14 in future studies. The fact that the receptor molecule of CXCL14 remains unidentified constitutes the main barrier for the study of the functional roles of this chemokine. The receptor would be the key to identify the target cells and to determine which signaling pathways are involved; this would allow researchers to better elucidate the roles of CXCL14 as part of the immune responses, organogensis and other biological processes.

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