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Role of chemokine ligand 2 in the protective response to early murine pulmonary tuberculosis

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SUMMARY

Chemokines play an important role in the development of immunity to tuberculosis. Chemokine ligand 2 (CCL2, JE, monocyte chemoattractant protein-1) is thought to be primarily responsible for recruiting monocytes, dendritic cells, natural killer cells and activated T cells, all of which play critical roles in the effective control of tuberculosis infection in mice. We show here that in mice in which the CCL2 gene was disrupted, low-dose aerosol infection with *Mycobacterium tuberculosis* resulted in fewer macrophages entering the lungs, but only a minor and transient increase in bacterial load in the lungs; these mice were still able to establish a state of chronic disease. Such animals showed similar numbers of activated T cells as wild-type mice, as determined by their expression of the $CD44^{hi}$ $CD62^{lo}$ phenotype, but a transient reduction in cells secreting interferon-γ. These data indicate that the primary deficiency in mice unable to produce CCL2 is a transient failure to focus antigen-specific T lymphocytes into the infected lung, whereas other elements of the acquired host response are compensated for by different ligands interacting with the chemokine receptor CCR2.

INTRODUCTION

Tuberculosis is the world's leading cause of death by a bacterial agent.[1] Although effective therapy is available, several factors have led to the resurgence of the disease as a worldwide problem, leading the World Health Organization (WHO) in 1993 to declare tuberculosis to be a global emergency. Among the reasons responsible for the resurgence are the increasing incidence of multidrug-resistance strains of *Mycobacterium tuberculosis*, human immunodeficiency virus (HIV) and the long-term treatment needed to effect cure.[2–4]

The nature of the host immune response to *M. tuberculosis* in the lungs is still poorly understood. Although the central T-cell populations have been identified,[5–7] and the key role of the cytokine interferon-γ (IFN-γ) has been established,[8,9] formation of the granulomatous response in the lungs and its control by host molecules has been analysed only minimally. For instance, although the pulmonary tuberculosis granuloma in the mouse has been described in detail,[10] it was surprising to observe that blocking the influx of macrophages from the bloodstream into the lungs did not affect the capacity of the animal to express protective immunity.[11] As a result of this, it was proposed[12] that this mechanism is cytokine driven, whereas the slower elements of the granulomatous response are predominantly controlled by chemokines.

A chemokine believed to be central to macrophage influx is chemokine ligand 2 (CCL2), which binds to the chemokine receptor 2 (CCR2) on macrophages, T cells and natural killer cells.[13–17] Expression of this chemokine is markedly increased, both in the mouse model[18,19] and in patients infected with tuberculosis.[20] However, it is unclear how important this chemokine is to the outcome of the acquired response, as mice lacking CCL2 were not found to be more susceptible to intravenous infection with *M. tuberculosis*.[21]

To address the issue of whether this also applied to direct infection of the lungs, we exposed CCL2 gene knockout (CCL2-KO) mice to an aerosol infection with *M. tuberculosis*. As shown below, while a considerable reduction in inflammation and macrophage influx into the lungs was observed, only a transient increase in the bacterial load was seen, which was concomitant with a reduction in antigen-specific IFN-γ-secreting cells entering this organ. These data suggest that recruitment of activated T cells into the lungs, rather than macrophage

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recruitment, is the key role of CCL2, at least in terms of the efficient expression of protective immunity.

MATERIALS AND METHODS

Mice

CCL2 gene-disrupted mice (kindly provided by Dr Barrett Rollins from the Dana Faber Cancer Institute) were generated by disruption of the *SCYA2* gene, as described previously.[21] All mice were maintained in specific pathogen-free conditions and used at 8–10 weeks of age. C57BL/6 mice of matching age and gender were used as the wild-type controls.

Bacterial strains

The *M. tuberculosis* strain, Erdman, originally obtained from the Trudeau Institute (Saranac Lake, NY), was grown in Proskauer-Beck liquid medium (containing 0·05% Tween-80) to mid-log phase and then frozen in aliquots at −70° until needed.

Aerosol infection

Mice were infected using a Glas-Col aerosol generator (Glas-Col, Terre Haute, IN), such that 100 bacteria were deposited in the lungs of each animal. The number of viable bacteria in target organs was determined at different time-points by plating serial dilutions of partial organ homogenates on nutrient Middlebrook 7H11 agar and counting colonies after 21 days of incubation at 37°.

Media and reagents

Lung- and spleen-cell suspensions were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Cellgro, Herndon, VA) containing 10 mM *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES; Sigma, St. Louis, MO), 2 mM L-glutamine (Sigma), and MEM non-essential amino acids (Sigma) supplemented with 10% heat-inactivated, endotoxin-low fetal calf serum (FCS) (Gibco, Grand Island, NY).

Cell culture

Infected animals were killed by CO_2 asphyxiation, and lung, spleen and mediastinal lymph nodes were harvested from infected animals at the indicated time-points after infection. Lungs were perfused through the heart with cold saline containing 30 U/ml of heparin. After removal, the lungs were sectioned in ice-cold media using sterile razor blades. Dissected lung tissue was then incubated in DMEM containing collagenase IX (0·7 mg/ml; Sigma) and deoxyribonuclease (30 μg/ml; Sigma) at 37° for 30 min. Single-cell suspension from digested lung, spleen or lymph nodes was obtained by passing the organs gently through a 70-μm nylon cell strainer. Red blood cells were lysed using Gey's solution (0·15 M NH_4Cl, 10 mM $KHCO_3$). Cells were counted and either cultured with antigen (at 5×10^6 cells/ml) or analysed by flow cytometry.

Flow cytometry

A single-cell suspension of either spleen or lung was prepared, as described above, and resuspended in deficient RPMI (Irvine Scientific, Santa Ana, CA) containing 0·1% azide. Some cells were stimulated with anti-CD3 (clone 145-2C11), anti-CD28 (clone 37.51) and monensin for 4 hr prior to staining. Cells were incubated for at least 1 hr with dRPMI containing 0·1% azide and stained with specific antibody for 30 min at 4° in the dark. Cells were stained with antibodies recognizing CD4 [allophyocyanin- or phycoerythrin (PE)-labelled clone GK1.5], CD8 (allophyocyanin-labelled clone 53-6.7), CD44 [fluorescein isothiocyanate (FITC)-labelled clone IM7), and CD62 ligand (CD62L) (PE-labelled clone MEL-14) (all from BD Pharmingen, San Diego, CA). Control cells labelled with isotype antibodies were also prepared. After being stained on their surface, stimulated cells were permeabilized with Perm Fix/Perm Wash (BD Pharmingen) and the presence of intracellular IFN-γ was investigated using labelled antibody (FITC-labelled XMG1.2). Cells were analysed on a FACSCalibur flow cytometer (Becton-Dickinson, San Diego, CA). All antibodies were purchased from BD Pharmingen, including appropriate isotype-control antibodies that were used throughout the analysis. Cells were

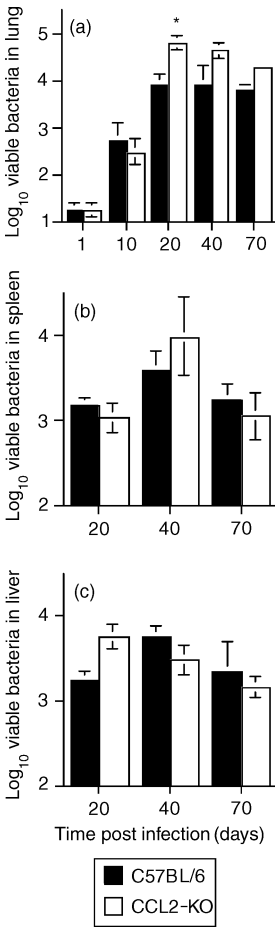

Figure 1. Mice lacking the gene for chemokine ligand 2 (CCL2) differ in their susceptibility to low-dose aerosol infection with *Mycobacterium tuberculosis*. Control (black bars) and CCL2 gene knockout (CCL2-KO) (white bars) mice were infected via the aerogenic route. The lungs (a), spleen (b) and liver (c) were evaluated for bacterial growth at different time points after infection. Data points represent the mean (± standard error of the mean) values from four mice. The value marked with an asterisk is significantly different from that of control mice at the same time point (*$P = 0{\cdot}019$, Students's *t*-test). Data are representative of three separate experiments.

collected in a FACSCalibur (Becton-Dickinson), dual-laser flow cytometer, with excitation at 488 nm and 633 nm, and analysed using CellQuest software (Becton-Dickinson). Lymphocytes were gated based on their forward- and side-scatter characteristics. Ten-thousand $CD4^+$ or $CD8^+$ lymphocytes were then analysed for their expression of the activation markers CD44 and CD62L.

Histology

The posterior lobe of the right lung was perfused with 10% paraformaldehyde in phosphate-buffered saline (PBS) and used for histological analysis. Haematoxylin and eosin staining was performed on sectioned tissues.

Statistical analysis

Four mice per group of three different experiments were used for all studies. The Student's *t*-test was used for comparisons between the groups of control and CCL2-KO mice.

RESULTS

Mice lacking CCL2 are more susceptible to pulmonary tuberculosis during the early stages of infection

To assess possible differences in susceptibility, control and CCL2-KO mice were exposed to approximately 100 bacteria by aerosol (Fig. 1). The uptake and initial bacterial load were similar, but by day 20 the CCL2-KO mice showed a 10-fold higher bacterial load compared with control animals. Despite this, the infection was then controlled, and at later time-points did not differ significantly between the two groups. Mice lacking CCL2 showed similar numbers of bacteria escaping to the spleen and liver, indicating that this event was not affected by the absence of this molecule.

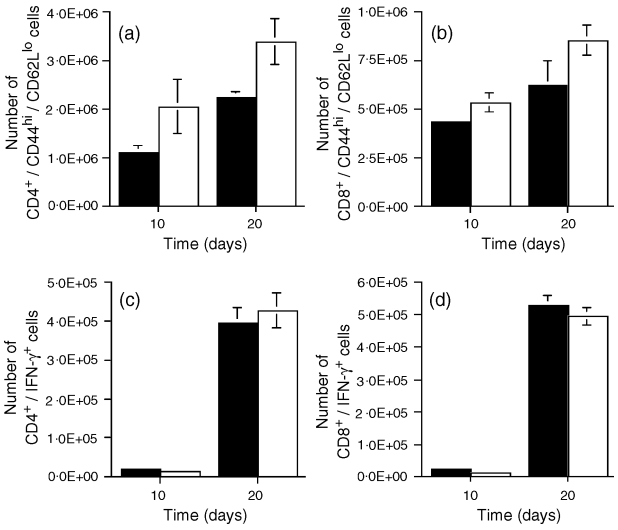

Figure 2. Mice lacking the gene for chemokine ligand 2 (CCL2) were able to activate T cells similar to control mice following aerogenic challenge with *Mycobacterium tuberculosis*. Control (black bars) or CCL2-knockout (white bars) mice were infected as described in the legend to Fig. 1, and splenocytes were analysed by flow cytometry for expression of $CD4^+$ $CD44^{hi}$ $CD62L^{lo}$ (a) and $CD8^+$ $CD44^{hi}$ $CD62L^{lo}$ (b). The number of cells expressing high levels of CD44 and low levels of CD62L per spleen is reported. Splenocytes were also stimulated with anti-CD3, anti-CD28 and monensin, and then analysed for intracellular interferon-γ (IFN-γ) production (c and d). The bars represent the mean values of four mice per group, and the graph shows one experiment representative of three.

Mice lacking CCL2 recruit $CD44^{hi}$ $CD62L^{lo}$ in comparable levels to control mice, but are less able to recruit IFN-γ-producing activated lymphocytes into the lungs

The activation of T cells was analysed by flow cytometry. As shown in Fig. 2, activated $CD4^+$ and $CD8^+$ T cells – defined by populations with high levels of expression of the CD44 molecule ($CD44^{hi}$) and low levels of the selectin, CD62L ($CD62^{lo}$) – increased as the infection progressed. This was associated with a parallel increase in the populations of $CD4^+$ and $CD8^+$ that expressed IFN-γ (Fig. 2c, 2d). CCL2-KO mice showed a tendency to have higher numbers of activated CD4 and CD8 T cells than wild-type (control) mice. The similar activation phenotype was further confirmed by stimulating spleen and mediastinal lymph node cells from infected mice with mycobacterial culture filtrate proteins (CFP) and measuring the production of IFN-γ. Cells from both strains produced equal amounts of IFN-γ (data not shown).

Subsequent analysis of T cells entering the lungs revealed a similar picture in terms of the numbers of cells with an activated

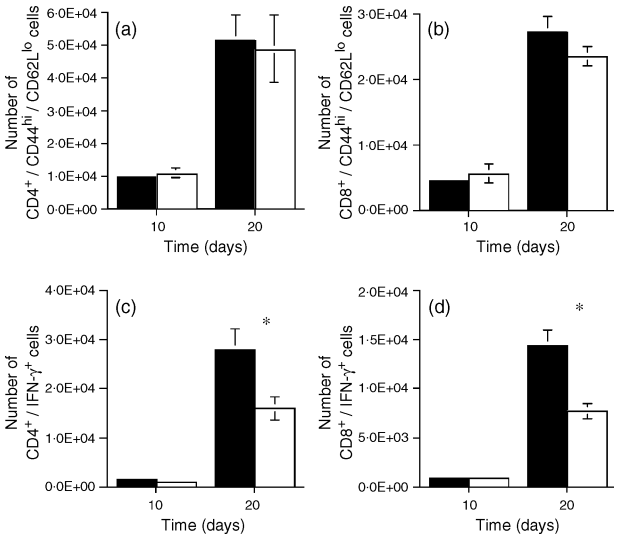

Figure 3. Mice lacking the gene for chemokine ligand 2 (CCL2-KO) fail to recruit as many interferon-γ (IFN-γ)-producing T lymphocytes to the lung as wild-type mice. Control (black bars) or CCL2-KO (white bars) mice were infected as described in the legend to Fig. 1, and lung cells were analysed by flow cytometry for expression of $CD4^+$ $CD44^{hi}$ $CD62L^{lo}$ (a) and $CD8^+$ $CD44^{hi}$ $CD62L^{lo}$ (b). The number of cells expressing high levels of CD44 and low levels of CD62L per lung is reported. Lung cells were also stimulated with anti-CD3, anti-CD28 and monensin, and then analysed for intracellular IFN-γ production (c and d). The bars represent the mean value of four mice per group, and the graph shows one experiment representative of three. The presence of an asterisk indicates statistical significance between control and CCL2-KO mice ($*P < 0{\cdot}05$, Student's *t*-test).

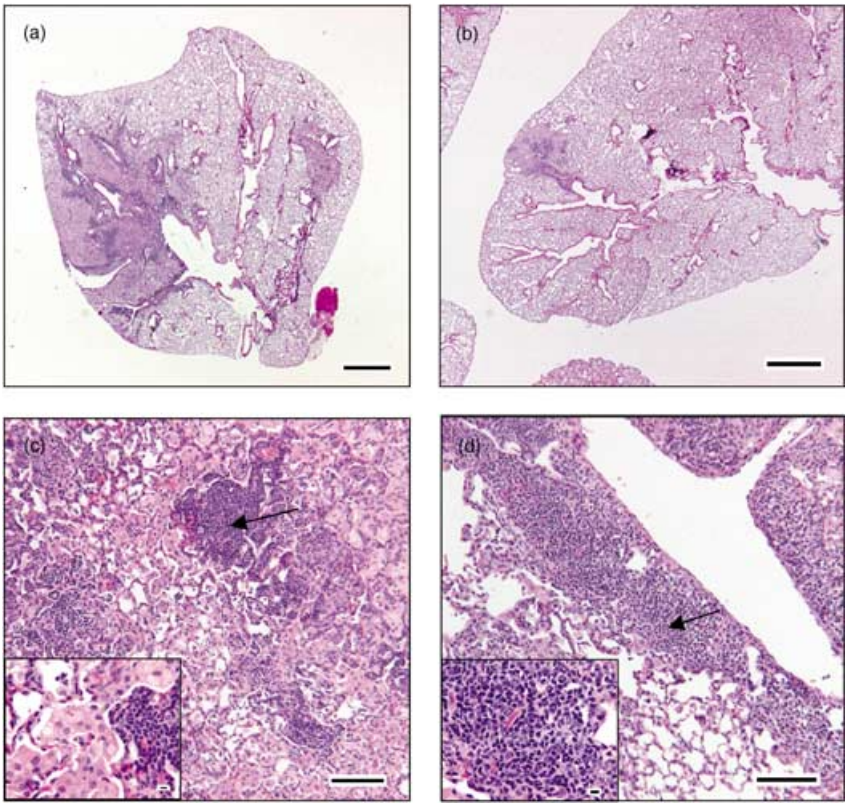

Figure 4. Representative micrograph showing lung lesions in C57BL/6 mice (a and c) and chemokine ligand 2 knockout (CCL2-KO) mice (b and d) 70 days after aerosol infection with *Mycobacterium tuberculosis* (haematoxylin and eosin staining). Size bars are 1-mm (a and b), 100-μm (c and d), and 10-μm insets. Note the macrophage-rich granulomatous response (asterisk and inset of c) in C57BL/6 mice, while in CCL2-deficient mice there is a predominant accumulation of lymphocytes (arrows and inset of d).

phenotype (Fig. 3a, 3b). However, in contrast to the spleen-cell data, significantly fewer cells present in the lungs at day 20 in CCL2-KO mice stained positive for IFN-γ (Fig. 3c, 3d; *P*-value $< 0{\cdot}05$).

Histological analysis of CCL2-KO mice

The morphology of the infected lungs was followed over the first 70 days in order to allow the granulomatous response to fully develop. As shown in Fig. 4, by day 70 postinfection the granulomatous inflammatory response in the control mice was substantially more florid than that seen in the CCL2-KO mice. Moreover, the distribution of mononuclear cells was clearly different, with many fewer macrophages seen in the CCL2-KO mice. There were no significant differences in the histopathology observed in the lungs prior to day 70.

DISCUSSION

The results of this study show that mice unable to express the CCL2 chemokine exhibited a transient increase in bacterial load in the lungs during the first month of infection with *M. tuberculosis*, but were still able to establish a state of chronic disease. It was notable that this event occurred despite a visually evident reduction in the number of macrophages arriving in the lung tissues during this early time period of infection. Examination of the T-cell response showed that the animal was fully capable of generating activated CD4 and CD8 T cells in the draining lymph nodes and spleen, but there was a transient reduction, observed on day 20, in the numbers of cells accumulating in the lungs that were capable of secreting IFN-γ.

It is reasonable to hypothesize therefore that the transient increase in bacterial load in the lungs was caused by the accumulation of fewer IFN-γ^+ cells. The data also confirms our previous observations,[11] that mice lacking the capacity to efficiently recruit monocytes from the blood into areas of infection does not, in fact, directly prevent the animal from expressing protective immunity. In this regard, we have used such information to propose[12] that protective immunity is a mechanism separate from the influx of monocytes and development of the granulomatous response.

In fact, while CCL2 is regarded as a major chemokine for macrophages and monocytes, the data obtained here seem to indicate that in the context of pulmonary tuberculosis a more important role for this molecule is in the efficient recruitment of antigen-specific IFN-γ-secreting T cells into the lungs. The mice used here retain fully functional CCR2 receptors and therefore are responsive to other members of the monocyte chemoattractant protein (MCP) family, such as CCL7, CCL8 and CCL12,[22] suggesting that CCL2 may play a selective role in recruiting T cells into the lungs. This may be organ-specific, as loss of the ability to produce CCL2 has been shown[21] not to

affect the course of tuberculosis in mice given a high-dose intravenous infection.

In contrast to this latter study, a defect or transient delay in priming T cells to secrete IFN-γ and to focus in the lungs was observed in another high-dose intravenous-infection model in which mice lacking the CCR2 receptor were used.[18] Because of the high dose, these mice were rapidly killed, but defects in both macrophage influx and the capacity of T cells to secrete IFN-γ were observed. In a more realistic model, using low-dose aerosol infection, CCR2-deficient mice were capable of controlling the infection despite a reduced influx of macrophages, and again defects in T-cell priming and influx were observed.[23] These latter observations are therefore highly consistent with those of the present study.

One minor difference between CCR2-KO mice and CCL2-KO mice was the observation, in the CCR2-deficient mice, of reduced T-cell priming and activation, whereas in the present study this parameter, measured in terms of the arrival of $CD44^{hi}$ $CD62^{lo}$ phenotype cells, showed no differences. A simple explanation for this is that ligands for the CCR2, other than CCL2, play a role in this event.

The controlled influx of immune cells into sites of bacterial implantation in the lungs, where immunity must be expressed, is clearly both a complex and sustained process, and in terms of chemoattractant ligands and their corresponding receptors there are considerable levels of redundancy that makes analysis difficult. Better understanding of such mechanisms is obviously needed, however, as control of the granulomatous process by chemokines is a central event in tuberculosis, and therapeutic modulation of this process may help to limit the eventual lung damage caused by these structures as well as potentially modify breakdown and the onset of reactivation disease.

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