Expression Analysis of the T-Cell-Targeting Chemokines CXCL9 and CXCL10 in Mice and Humans with Endothelial Infections Caused by Rickettsiae of the Spotted Fever Group

Gustavo Valbuena,* William Bradford,[†] and David H. Walker*

From the Department of Pathology,* University of Texas Medical Branch, Galveston, Texas; and the Department of Pathology,[†] Duke University Medical Center, Durham, North Carolina

Rocky Mountain spotted fever and other related diseases are systemic infections caused by rickettsiae. These obligatory intracellular bacteria target the endothelium, offering an appealing model to study the interactions between endothelial cells and T lymphocytes. We investigated the mRNA expression of chemokines known to target CD8⁺ T cells and CD4⁺ T-helper 1 cells in the lungs of C3H/HeN mice infected with *Rickettsia conorii* with the purpose of identifying evidence for a role of chemokines in the immune clearance of rickettsiae from the vasculature. The expression of the CXCR3 ligands CXCL9 and CXCL10 was significantly higher than the other chemokines investigated. We validated the relevance of these results in the animal model through the analysis of tissues from humans with Rocky Mountain spotted fever. We then characterized the kinetics and localization of expression of CXCL9 and CXCL10 in lungs, brain, and liver of mice infected with lethal or sublethal doses of *R. conorii* by a combination of quantitative real-time polymerase chain reaction and immunohistochemistry. Interestingly, the peak of expression of these chemokines occurred 4 days before CD8⁺ T cells infiltrated the infected tissues. Our results suggest that CXCL9 and CXCL10 may play a role early during the immune response against rickettsial infections. (Am J Pathol 2003; 163:1357–1369)

Spotted fever group rickettsiae are arthropod-borne (mainly by ticks), gram-negative, obligately intracellular bacteria, which primarily infect endothelial cells and spread from cell to cell because of their capacity for intracellular movement through stimulation of directional actin polymerization.^{1–4} They cause acute, potentially lethal diseases with systemic multiorgan involvement such as Rocky Mountain spotted fever (RMSF; caused by *Rickettsia rickettsii*), and boutonneuse fever (caused by

Rickettsia conorii).⁵ These diseases (particularly RMSF) can be fatal, even in young immunocompetent individuals, if not adequately treated with antibiotics early during the course of the illness. The incidence of these rickettsioses is expected to increase in parallel with a tendency for an active outdoor lifestyle and increased chances of contact with the vectors.

The clinical features of rickettsial diseases correspond to damage to the main target cells, endothelial cells, particularly importantly those in the lungs and brain. The initial symptoms are those of a flu-like syndrome; however, if untreated, severe manifestations can develop including noncardiogenic pulmonary edema/adult respiratory distress syndrome, interstitial pneumonia, acute renal failure, hemorrhagic phenomena, peripheral edema, and hypovolemic hypotension because of leakage of intravascular fluid into the extravascular space. Central nervous system manifestations include delirium, focal neurological deficits, seizures, and coma.^{6,7}

Studies in animal models have demonstrated that a successful anti-rickettsial immune response requires CD8⁺ T lymphocytes,⁸ and in terms of their effector functions both interferon (IFN)- γ production and cytolytic mechanisms are required.⁹ In humans, the presence of perivascular infiltrating CD8⁺ T cells has also been demonstrated in skin lesions of patients with spotted fever group rickettsioses.¹⁰ Also, endothelial cells become activated on rickettsial infection as evidenced by the production of cytokines and expression of adhesion molecules.^{11–14} The stable adhesion between cytotoxic lymphocytes and target cells through integrin activation is a requirement for the focalization of their effector mechanisms.¹⁵ In the majority of the studies addressing lymphocyte adhesion to the endothelium, this step is viewed as a requirement for the transmigration of leukocytes to the areas of inflammation beneath the endothelium. Hu-

Supported by a grant from the National Institutes of Health (grant RO1 AI21242 to D. H. W.), a College of American Pathologists Foundation Scholars Research Fellowship (to G. V.), and a James W. McLaughlin Predoctoral Fellowship (to G. V.).

Accepted for publication June 18, 2003.

Address reprint requests to David H. Walker, Professor and Chairman, Department of Pathology, University of Texas Medical Branch, 301 University Blvd., Galveston, Texas 77555-0609. E-mail: dwalker@utmb.edu.

man rickettsioses and the rickettsial mouse model offer the unique opportunity to study CD8+ T lymphocyte effector mechanisms in a relevant infectious disease in which endothelial cells themselves are presenting the antigen and are therefore the targets. The role of chemokines, particularly inflammatory chemokines that specifically target activated T cells through the CXCR3 receptor such as CXCL9 (Mig) and CXCL10 (IP-10), has not been explored in this regard; however, there is suggestive evidence from other systems including *in vitro* flow studies,¹⁶ transplantation studies, and other infectious diseases caused by intracellular organisms that do not target endothelial cells,^{17–22} that chemokines might play an important role in the anti-rickettsial immune response. We undertook the following studies to investigate the possible role of CXCL9 and CXCL10 in the immune response against spotted fever group rickettsiae.

Materials and Methods

Rickettsia

R. conorii (Malish 7 strain) is a human isolate from South Africa with an unknown number of passages in the yolk sacs of embryonated chicken eggs. For all of the experiments described in this study, a stock of *R. conorii* was produced by cultivation in specific pathogen-free embryonated chicken eggs. Yolk sacs from infected eggs with dead embryos were homogenized in a Waring blender, diluted to a 10% suspension in sucrose-phosphate-glutamate buffer (0.218 mol/L sucrose, 3.8 mmol/L KH₂PO₄, 7.2 mmol/L K₂HPO₄, 4.9 mmol/L monosodium L-glutamic acid, pH 7.0) and aliquoted for storage at –80°C after discarding the pellet produced by low-speed centrifugation (200 × *g*, 10 minutes). Rickettsial content of this stock was quantified by plaque assay,²³ and the LD₅₀ was determined experimentally in C3H/HeN mice.

Human Samples

Fourteen brainstem specimens from nine fatal pediatric cases of RMSF collected at Duke University between 1935 and 1978 were used for immunohistochemical analysis. Four brainstem samples from trauma cases were used as negative controls. No other tissues were analyzed because they were not available, and one case reported as negative for rickettsial antigen (see case 4 in Table 2) contained *R. rickettsii* in other tissues that were studied in the past.^{24,25} Two pathologists analyzed the samples and qualitatively graded each parameter according to the intensity of staining in a scale of 1+ to 4+.

Animal Model

The mouse model of endothelial-target spotted fever group rickettsioses consists of *R. conorii* infection of C3H/HeN mice and has been previously described in detail.^{26,27} All mice were housed in a biosafety level 3 facility and were infected intravenously (through the tail vein) with 0.25 LD₅₀ of *R. conorii* for the sublethal dose model

or 3 LD₅₀ of *R. conorii* for the lethal dose model (in a volume of 300 μ l in sucrose-phosphate-glutamate buffer). Animals infected with the higher dose usually died 6 to 7 days later. In three separate experiments, eight mice received a sublethal dose of *R. conorii*, and three mice received a lethal dose. One mouse from each group was euthanized every other day by cervical dislocation, and all organ tissues were harvested and divided for RNA preservation in RNAlater (Ambion, Austin, TX), and fixation in 10% buffered formalin or zinc fixative (BD Pharmingen, San Diego, CA). Tissues from mock-infected mice were similarly processed and evaluated as negative controls. Organs fixed in formalin were transferred to 70% ethanol after 24 hours to avoid overfixation.

Cell Line

The C3H/HeN mouse endothelial cell line SVEC4-10 was kindly provided by Dr. M. Edidin (Johns Hopkins University, Baltimore, MD).²⁸ This cell line was cultivated in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Carlsbad, CA) containing 2% fetal bovine serum (Hyclone Inc., Logan, UT). The cells were passaged twice weekly.

Chemokine Cloning

Standards for quantitative real-time polymerase chain reaction (PCR) were generated by cloning the chemokines and GAPDH cDNA into the vector pCR2 (Invitrogen, Carlsbad, CA) after PCR amplification using primers designed to encompass the entire reading frame. All of the inserts were confirmed by sequencing. The sequences of the primers are presented in Table 1. The relevant mRNA was obtained from splenic tissue of C3H/HeN mice infected for 5 days with 3 LD₅₀ of *R. conorii*. The mRNA was reverse-transcribed with Sensiscript (Qiagen, Valencia, CA) and oligo(dT) primers. cDNA for mouse CXCL9 and CXCL10 (cloned into pBluescript; Stratagene, La Jolla, CA) was a kind gift of P. Murphy (National Institutes of Health, Bethesda, MD).

RNA Methods

Optimal quality RNA was obtained by combining an organic extraction method (Totally RNA, Ambion) with the RNAqueous 4PCR kit from Ambion. Briefly, RNAlater-preserved samples (Ambion) were homogenized in denaturation solution using a mixer mill (Qiagen, Valencia, CA) and subsequently extracted with phenol/chloroform. The upper aqueous phase was mixed with an equal volume of 70% ethanol. This mixture was loaded into the filter cartridges of the RNAqueous kit and the protocol followed according to the manufacturer's instructions. The RNA concentration was adjusted to 0.2 mg/ml before DNase treatment (DNAfree, Ambion). In all cases, RNA quality was verified by gel electrophoresis before cDNA synthesis with Moloney murine leukemia virus reverse transcriptase (Superscript-II first strand synthesis system; Invitrogen, Life Technologies, Carlsbad, CA) and

Table 1. Primer and Probe Sequences

Sequences of primers for cloning	
CCL2 (MCP-1) forward	ATGCAGGTCCCTGTCATG
CCL2 reverse	GTTCACTGTCACACTGGTCA
CCL12 (MCP-5) forward	ATGAAGATTTCCACACTTCTATGC
CCL12 reverse	GCCTAGACATGAAGGTTCAAG
CCL3 (Mip-1 α) forward	ATGAAGGTCTCCACCACTG
CCL3 reverse	GGCATTTCAGTTCCAGGTC
CCL4 (Mip-1 β) forward	ACCATGAAGCTCTGCGTGT
CCL4 reverse	GTTCAACTCCAAGTCACTCAT
CX3CL1 (Fractalkine) forward	ATGGCTCCCTCGCCG
CX3CL1 reverse	TCACACTGGCACCAGGA
GAPDH forward	ATGGTGAAGGTCGGTGTGAA
GAPDH reverse	CTCCTTGGAGGCCATGTA
Sequences of primers for real-time PCR with SYBRgreen	
CCL2 (MCP-1) forward	CTTCTGGGCTGCTGTTC
CCL2 reverse	CCAGCCTACTCATTGGGATCA
CCL12 (MCP-5) forward	AGAATCACAAGCAGCCAGTGT
CCL12 reverse	ATCCAAGTGGTTTATGGAATCTTAAC
CCL3 (Mip-1 α) forward	GACTGCCTGCTGCTTCT
CCL3 reverse	GATCTGCCGTTTCTCTTAG
CCL4 (Mip-1 β) forward	CATGAAGCTCTGCGTGTCT
CCL4 reverse	CTGCCGGGAGGTGTAA
CX3CL1 (Fractalkine) forward	GCCGCGTTCTTCCATTG
CX3CL1 reverse	TGGGATTCTGTAGGTCATCTT
Sequences of primers and probes for real-time PCR with Taqman probes	
CXCL9 (Mig) forward	GAACCCTAGTGATAAGGAATGCA
CXCL9 reverse	CTGTTTGAGGTCTTTGAGGGATT
CXCL9 probe	CATCAGCACCAGCCGAGGCACG
CXCL10 (IP-10) forward	AGTGCTGCCGTCAATTTCTG
CXCL10 reverse	ATTCTCACTGGCCCGTCAT
CXCL10 probe	AGTCCCACTCAGACCAGCAGG
GAPDH forward	CAACTACATGGTCTACATGTTT
GAPDH reverse	CTCGCTCCTGGAAGATG
GAPDH probe	CGGCACAGTCAAGGCCGAGAATGGGAAGC
<i>rompB</i> forward	ACACATGCTGCCGAGTTACG
<i>rompB</i> reverse	AATTGTAGCACTACCGTCTAAGGT
<i>rompB</i> probe	CGGCTGCAAGAGCACCAGCCAACAA

oligo(dT) primers. In addition, mRNA was PCR-amplified directly (with no reverse transcriptase) to verify that there was minimal or no DNA contamination of the cDNA samples.

Quantitative real-time PCR was performed using the iCycler from Bio-Rad (Hercules, CA) and Stratagene's Brilliant quantitative PCR kit (La Jolla, CA). Two-step cycle parameters (95°C and 60°C) were used and the primers and probes used are presented in Table 1. Probes for CXCL9 and CXCL10 were labeled with FAM and Black Hole Quencher 1 (BHQ1), and the probe for GAPDH was labeled with TAMRA and Black Hole Quencher 2 (BHQ2) (Biosearch Technologies, Novato, CA). The primers used for real-time PCR with SYBRgreen (IQ SYBR green supermix; Bio-Rad, Hercules, CA) are presented in Table 1. Optimal magnesium concentrations obtaining efficiencies close to 100% were determined experimentally. In all cases 3 to 5 mmol/L Mg could be used. We used the delta delta method (comparative $C_T[\Delta C_T]$ method) for the quantitative analysis of gene expression.²⁹ The rickettsial load was determined by real-time PCR (with TaqMan probes) of the *Rickettsia*-specific gene *rompB*. For these experiments, the substrate of amplification was DNA-purified from RNAlater-preserved samples using the Dneasy tissue kit from Qiagen. The results were normalized to GAPDH in the same sample and expressed as copy number per 10^4 (for the lethal model) or 10^5 (for the

sublethal model) GAPDH copies (standard curves with more than 94% efficiency and linear amplification across 1 to 10^6 to 10^7 copies were used to obtain the copy number of the samples). The sequences of the primers and probes are presented in Table 1. The *rompB* probe was labeled with FAM and Black Hole Quencher 1 (BHQ1) (Biosearch Technologies).

Immunohistochemistry

Sections (4 to 6 μ m) from paraffin-embedded tissues fixed in 10% formalin in phosphate-buffered saline (PBS) were antigen-retrieved in citrate pH 6.0, stained with relevant primary antibodies, and detected with appropriate biotinylated secondary antibodies, ABC-peroxidase and diaminobenzidine as a substrate (Vector Laboratories, Burlingame, CA). After digestion with Protease I (Ventana Medical Systems, Tucson, AZ) for 10 minutes at room temperature, rabbit anti-spotted fever group rickettsia antibody was detected with anti-rabbit alkaline phosphatase antibody (Jackson Immunoresearch Laboratories, West Grove, PA) and Vector Red substrate. The strong red fluorescence produced by this substrate localized the rickettsiae using a fluorescence microscope equipped with a wide band-pass fluorescein-isothiocyanate filter (Olympus Optical, Melville, NY).

In the mouse model, CXCL9 and CXCL10 were detected with affinity-purified goat polyclonal antibodies (R&D Systems, Minneapolis, MN). In human cases, CXCL9 and CXCL10 were detected with rat monoclonal antibodies (clones 6/D4/D6/G2 and B8-11, respectively; BD Pharmingen). The anti-human CXCR3 antibody was also obtained from BD Pharmingen (clone 1C6/CXCR3) and the anti-human CD8 mAb (clone 4B11) from Novocastra Laboratories (Burlingame, CA). Mouse zinc-fixed tissues were used for the detection of CD8+ T cells with a rat anti-mouse CD8 monoclonal antibody (clone 53-6.7, BD Pharmingen) and biotinylated F(ab')₂ fragments of mouse-absorbed mouse anti-rat IgG (Jackson ImmunoResearch) as a secondary reagent. The anti-human CXCR3 antibody served as isotype-negative control for anti-human CXCL9 (they are both IgG1), and the anti-human CD8 antibody served as a negative control for anti-human CXCL10 (they are both IgG2) because they produced a clearly distinguishable pattern of staining. In addition, in the absence of primary antibody, no signal was detected. For mouse samples, noninfected mice that do not express the antigen (rickettsia and chemokines) served as negative controls.

Intracellular Cytokine Staining

Confluent monolayers of the mouse endothelial cell line SVEC4-10 were infected with 4×10^3 pfu/cm² of *R. conorii*, mock-infected as a negative control, or stimulated with 5000 U/ml human tumor necrosis factor (TNF)- α (known to stimulate mouse TNF- α receptors) and 500 U/ml mouse IFN- γ (Roche Applied Science, Indianapolis, IN) for 12, 24, or 48 hours. Four hours before fixation with 4% paraformaldehyde in PBS for 20 minutes, the cells were treated with Brefeldin A (Golgi Stop, BD Pharmingen) to paralyze the cellular secretory mechanisms and in this way permit detection of CXCL9 and CXCL10 inside the cells. Goat polyclonal antibodies against mouse CXCL9 and CXCL10 were used (R&D Systems) for detection.

Western Blot

To confirm the identity of the proteins detected with the goat polyclonal antibodies against CXCL9 and CXCL10, confluent monolayers of SVEC4-10 cells treated with TNF- α and IFN- γ for 24 hours were lysed with a buffer consisting of 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate in PBS supplemented with protease inhibitor cocktail (Roche Applied Science). Total protein was quantified with the bicinchononic acid (BCA) reagent from Pierce (Bradford, IL). Thirty μ g of protein per sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes. Chemokines were detected with the same antibodies used for immunohistochemistry and intracellular cytokine staining and were detected with an alkaline phosphatase system (Kierkegaard & Perry Laboratories, Gaithersburg, MD).

Results

Screening for T-Cell-Targeting Chemokines in an Animal Model of Spotted Fever Group Rickettsioses

We initially screened for ligands of chemokine receptors present in effector and memory CD8+ T lymphocytes because these cells are essential in the immune clearance of rickettsiae from the infected vasculature of the host.^{8,9} The chemokines investigated were CCL2 (MCP-1), CCL12 (MCP-5), CCL3 (Mip-1 α), CCL4 (Mip-1 β), CXCL9 (Mig), CXCL10 (IP-10), and CX3CL1 (Fractalkine). The entire open reading frame of each one of these chemokines was PCR-amplified and cloned into the TOPO TA vector pCR2. The identity of the cloned products was verified by sequencing. These plasmid DNA samples were used to optimize primers for SYBRgreen real-time PCR, and in all cases the efficiency of the reaction was more than 95% with linear amplification across 6 log₁₀ of target concentration (data not shown). We subsequently analyzed the expression of all these chemokines by real-time PCR of cDNA from the lungs of C3H/HeN mice infected with lethal (3 LD₅₀) and sublethal (0.25 LD₅₀) inocula of *R. conorii* (Figures 1 and 3; CX3CL1 data not shown). Our study focused on CXCL9 and CXCL10 because they had the highest level of up-regulation after rickettsial infection among all chemokines analyzed and because they are known to target activated T cells.³⁰

Expression of CXCR3 Ligands in Humans with RMSF

Before further characterizing the expression of these chemokines in the animal model of spotted fever group rickettsioses, we wanted to determine the relevance of these findings to human disease by characterizing the expression of CXCL9 and CXCL10 in nine fatal cases of RMSF. The expression of these chemokines, their receptor (CXCR3), CD8 antigen, and rickettsial antigen was analyzed by immunohistochemistry of samples taken from the brainstem (Figure 2). They are all from pediatric autopsies collected between 1935 and 1978. The results are summarized in Table 2. CXCL9 and CXCL10 were detected in all cases, and their levels of expression almost paralleled each other. More importantly, the majority of the signal was found in endothelial cells (Figure 2; a, b, and d), although occasional astrocytes and infiltrating leukocytes did express CXCL9 and CXCL10 (Figure 2c, and data not shown). Despite the fact that many examples of expression of these chemokines in rickettsia-infected endothelial cells were found (Figure 2d), many vascular beds expressing CXCL9 and CXCL10 had no detectable rickettsial antigen (Figure 2, a and b). This may be explained by immune- and/or antibiotic-mediated clearance of the rickettsiae. Infiltrating CD8+ and CXCR3+ cells around vessels with no detectable rickettsiae might also reflect this effect, and the converse situation, rickettsia-infected endothelium with perivascular

Figure 1. Quantitative real-time PCR with SYBRgreen of cDNA from lungs of mice intravenously infected with either sublethal (S1 to 21) or lethal doses (L1 to L5) of *R. conorii*, and sacrificed every other day. The levels of chemokine expression were normalized to the levels of GAPDH expression, and the results were expressed as fold increase over control (C), which has a value of 1. All data points correspond to the means of three simultaneous amplifications with negligible standard deviations.

CD8+ and CXCR3+ cells, was frequently found (Figure 2, e and f). In all cases many microvessels that did not express CXCL9 or CXCL10 served as an internal negative control. In addition, brainstem samples from non-infectious disease cases were negative throughout. It is also worth pointing out that the use of a fluorescent substrate for the detection of rickettsial antigen enhanced our ability to identify areas with very few rickettsiae (Figure 2, g and h).

Expression of CXCR3 Ligands in C3H/HeN Mice Infected with *R. conorii*

The findings in human cases of RMSF prompted us to characterize the animal model of spotted fever group rickettsioses with respect to the expression of CXCL9 and CXCL10. Both the mRNA and protein expression of these chemokines were analyzed in the most important target organs, namely brain and lungs, as well as liver, by real-time PCR with TaqMan probes (to ensure specificity) and by immunohistochemistry. For real-time PCR, the levels of expression of CXCL9 and CXCL10 were normalized against the levels of expression of GAPDH in each organ, and the samples were compared by the delta delta method assigning a value of 1 to the control (Figure 3). In all cases the immunohistochemical results closely paralleled the real-time PCR results.

In the lungs and liver of animals infected with a sublethal dose of *R. conorii*, the mRNA expression of both CXCL9 and CXCL10 peaked on day 3 with levels that were dramatically higher than the control (Figure 3). At

the protein level, both CXCL9 and CXCL10 were detected in multiple foci in the sinusoids of the liver 24 hours after the infection (Figure 4a), and subsequently, on day 3, the signals for both chemokines were found globally throughout the sinusoids of the liver (Figure 4b). Between days 5 and 7, the expression of CXCL9 and CXCL10 became focal again (Figure 4c) and eventually became undetectable between days 13 and 21. In the lungs, CXCL9 and CXCL10 proteins were also detectable in multiple foci between days 1 and 3 (Figure 4d); however, the peak of protein expression was not globally distributed as in the liver although it clearly involved the vasculature as well as infiltrating leukocytes (Figure 4, e and f).

C3H/HeN mice infected with 3 LD₅₀ of *R. conorii* usually died at approximately 6 days after infection. The pattern of expression of CXCL9 and CXCL10 protein in the lung and liver of mice infected with a lethal inoculum was not very different from what was observed in the sublethal infection model; however, at the mRNA level, the expression of CXCL9 was not as high as in the lungs of the sublethal model, and the peak of expression of CXCL10 in the liver of the lethally infected animals was on day 1 (Figure 3).

CXCL10 is found in the brain, both at the protein and mRNA levels, but only beginning on day 3 after a sublethal rickettsial challenge, which is the peak of expression (Figure 3). In the case of CXCL9, the peak of expression is on day 7. Both chemokines are subsequently down-regulated in a similar manner as in the lungs and liver (Figure 3). In the lethal model of spotted fever group rickettsiosis, both CXCL9 and CXCL10 were detected 24

Table 2. Semiquantitative Observations of *R. rickettsii* Infiltrating Cell Types, and Endothelial Chemokines in the Brain in Human Rocky Mountain Spotted Fever

Patient	Age/sex	Days of illness	Rickettsial antigen	CD8+ cells	CXCR3+ cells	CXCL9 (Mig)	CXCL10 (IP-10)
1	6/M	13	+	+++	—	+	+
2	7/M	12	+	++++	+++	++	+++
3	7/M	9	+	++	+	++++	++++
4	9/F	8	—	—	—	+++	+++
5	13/M	7	+	—	—	+++	++++
6	3/F	9	+++	+	+	++	++
7	7/M	9	+++	++	+	++	+++
8	7/F	15	++	+++	+++	+	++
9	7/M	18	+	+	—	+	+

hours after the infection, and the levels had increased on day 3. At the protein level, CXCL9 and CXCL10 were detected in multiple foci of the vasculature, but the protein expression was not generalized at any point as it was in the liver sinusoids (data not shown).

Interestingly CXCL9 and CXCL10 were detected on the luminal side of kidney tubular epithelial cells at very early time points after rickettsial infection (Figure 4g). This distribution might reflect tubular reabsorption of these small proteins that are likely filtered through the glomerulus. Therefore, the analysis of the kidney for small cytokines and chemokines such as CXCL9 and CXCL10 might be useful as a reflection of systemic production.

Kinetics of CD8+ T-Cell Infiltration in C3H/HeN Mice Infected with *R. conorii*

The peak of expression of CXCL9 and CXCL10 on day 3 was surprising because these chemokines have been shown to preferentially target mature effector and memory T cells and these cells usually infiltrate infected tissues at later times. For this reason we decided to investigate the kinetics of T-cell infiltration into rickettsia-infected tissues by immunohistochemistry. CD8+ T lymphocytes are first observed in the parenchyma of all organs analyzed on day 6, and they are not found in infected tissues at earlier time points (Figure 4, h and i).

Kinetics of Rickettsial Growth in C3H/HeN Mice Infected with *R. conorii*

We investigated the rickettsial load in lungs, liver and brain of the same mice used for the chemokine expression studies by both real-time PCR of the rickettsial gene *rompB* (using TaqMan probes and immunohistochemistry). The PCR in these experiments was performed using DNA as a target, and the data were normalized against the eukaryotic gene encoding GAPDH. For these experiments, the results were expressed as number of *rompB*

copies per 10^4 (lethal model) or 10^5 (sublethal model) GAPDH copies. Mice receiving either a lethal or a sublethal dose of *R. conorii* had a progressive increase in rickettsial load peaking on day 5 (Figure 5; lung and brain results not shown). The animals receiving a lethal inoculum had ~10-fold higher rickettsial loads than mice receiving sublethal inocula, which is consistent with the 10-fold difference in the size of the inocula. In the sublethal model, the rickettsial load progressively decreased after day 5 with a dramatic decline between days 5 and 7. This is consistent with the time when infiltrating CD8+ T cells are first detected in infected tissues (day 6). The immunohistochemical results validated the real-time PCR results (data not shown); however, we noticed a difference in our capacity to detect rickettsial antigen in paraffin-embedded tissue sections depending on the type of fixative used. Rickettsial antigen can be detected at lower concentrations (2 to 3 days earlier) in tissues fixed with a zinc-based preparation as compared to tissues fixed in 10% formalin despite the fact that in both cases the antigen was retrieved through an enzymatic treatment.

Expression of CXCR3 Ligands in a Mouse Endothelial Cell Line

We also investigated whether rickettsial infection of the endothelium can directly trigger the expression of CXCL9 and CXCL10. The mouse endothelial cell line SVEC4-10 was infected with 4×10^4 PFU/cm² of *R. conorii*, and the expression of these chemokines was analyzed at the protein level by intracellular cytokine staining at various time points between 8 and 72 hours after infection. CXCL9 and CXCL10 were not detectable at any point; however, SVEC4-10 cells stimulated with IFN- γ or a combination of TNF- α and IFN- γ expressed both chemokines (data not shown). The identity of the proteins detected with the antibodies used was confirmed by Western blot of lysates from SVEC4-10 cells stimulated with TNF- α and IFN- γ for 24 hours (data not shown).

Figure 2. Immunohistochemical analysis of CXCL9, CXCL10, CD8, CXCR3, and spotted fever group rickettsial antigen in human brains from fatal cases of RMSF. Rickettsial antigen was detected with alkaline phosphatase and a red substrate. All of the other markers were detected with an HRP system and diaminobenzidine as substrate yielding a brown product. CXCL10 (a) and CXCL9 (b) localized to the surface and perinuclear area of endothelial cells. Occasional astrocytes stained for CXCL10 (c), and by double immunohistochemistry rickettsia-infected endothelial cells were found to express CXCL10 (d). CD8+ cells (e) and CXCR3+ cells (f) infiltrated the perivascular space around rickettsia-infected microvessels. The bright red fluorescence of the Vector Red substrate significantly enhanced our ability to localize the rickettsial antigen (g and h demonstrate the same field under transmitted light and fluorescent microscopy, respectively; rickettsiae are shown with arrows). Original magnifications, $\times 400$.

Figure 3. Quantitative real-time PCR with TaqMan probes of cDNA from lungs, livers, and brains of mice intravenously infected with either sublethal (S1 to S21) or lethal doses (L1 to L5) of *R. conorii*, and sacrificed every other day. The levels of chemokine expression were normalized to the levels of GAPDH expression, and the results were expressed as fold increase over control (C), which has a value of 1 (brain CXCL10 results were compared against S17, the lowest level found). All data points correspond to the means of three simultaneous amplifications with negligible standard deviations. The data presented are representative examples of two separate experiments for cDNA analysis (three separate experiments were analyzed by immunohistochemistry).

Discussion

Human rickettsioses are underdiagnosed and underreported bacterial diseases that have recently started to generate more attention because two of them, *R. rickettsii* and *R. prowazekii*, are select agents that can potentially be used as bioweapons. Rickettsiae are divided in two groups: spotted fever group rickettsiae include the agents of RMSF (*R. rickettsii*) and Mediterranean spotted fever (*R. conorii*) among others. Typhus group includes the agents of louse-borne epidemic typhus (*R. prowazekii*) and murine typhus (*R. typhi*). The spotted fever group rickettsiae differ from the typhus group rickettsiae in their capacity to stimulate host cell actin polymerization and the presence of the rickettsial outer membrane protein OmpA.¹⁻³ These bacteria are strict intracellular parasites that principally infect endothelial cells in the vasculature. Because of their cytoplasmic

location, the cellular immune response plays a preeminent role in the clearance of the infection. Indeed, we have previously shown that CD8⁺ T cells, IFN- γ , and cytolytic mechanisms are all important in the effective anti-rickettsial immune response.^{8,9,27,31}

The main target cells, endothelial cells, also play an important role in immunity. Experiments *in vitro* with cultured endothelial cells (mainly human umbilical vein endothelial cells) show that they become activated after rickettsial infection with concomitant production of cytokines such as interleukin (IL)-1 α , IL-6, and IL-8^{11,12} and expression of adhesion molecules such as ICAM-1 and VCAM-1.¹³ Rickettsiae directly induce the activation of transcription factors of the nuclear factor- κ B family, which regulate the expression of cytokines, chemokines, adhesion molecules, and other early response genes,^{32,33} and may initiate these responses during rick-

ectisial infection. Activation of endothelial cells in rickettsial infection is reflected not only by the expression of markers such as the ones just discussed but more importantly by the capacity for intracellular killing of rickettsiae through nitric oxide produced by inducible nitric oxide synthase (iNOS or NOS2) after IFN- γ and TNF- α stimulation.³¹

The experimental evidence indicates that a fine equilibrium between the activation of rickettsia-infected endothelial cells and the effector mechanisms of rickettsia-specific CD8+ T cells is necessary to achieve clearance of the infection. Because chemokines are known to play an important role in inducing a stop signal in lymphocytes rolling on the surface of endothelial cells through increased integrin affinity,^{34,35} we hypothesized that chemokines that target T cells might be important in the localization and focalization of anti-rickettsial CD8 T-cell effector functions.

As a first step we evaluated the expression of CCR2, CCR5, CX3CR1, and CXCR3 ligands in lungs of C3H/HeN mice infected with both lethal and sublethal doses of *R. conorii* (a well-established model of human spotted fever group rickettsioses).^{8,9,26,27} We studied the chemokines binding to these receptors because they are expressed mainly on mature Th1 and CD8+ T lymphocytes,^{36–40} which are critical in the clearance of rickettsiae from the infected vasculature. The ligands for CCR2 are CCL2 (MCP-1) and CCL12 (MCP-5); CCL3 (MIP-1 α) and CCL4 (MIP-1 β) are the ligands for the chemokine receptor CCR5; CX3CL1 (Fractalkine) is the ligand for CX3CR1; and CXCL9 (Mig) and CXCL10 (IP-10) are ligands for CXCR3. Real-time PCR analysis with SYBRgreen revealed that most of these chemokines had a peak of expression on day 3 after either a lethal or sublethal challenge with *R. conorii* (Figure 1); however, the CXCR3 ligands, CXCL9 and CXCL10, had much higher levels of mRNA induction with respect to control than all of the other chemokines (data for SYBRgreen-PCR not shown; Figure 3 shows results with TaqMan probes). These CXCR3 ligands have been implicated in CD8+ T-cell-mediated responses not only in infectious diseases, but also in models of allograft rejection and autoimmunity. For instance, in experimental cardiac allograft rejection, blocking of CXCL9,¹⁷ CXCL10¹⁸ or its receptor CXCR3¹⁹ with neutralizing antibodies prolongs allograft survival. Furthermore, CXCR3 gene knockout mice are resistant to development of acute allograft rejection. Also, knockout of the CXCR3 gene in the RIP-GP background delays the development of insulinitis and diabetes.⁴¹ More importantly, the role of these chemokines has been demonstrated in different infectious disease models. Antibody neutralization of CXCL10 in a mouse model of *Toxoplasma gondii* results in increased parasite burden and mortality.²⁰ In fact, anti-CXCL10 treatment reduces the number of antigen-specific splenic CTLs. Also, CXCL9 and CXCL10 neutralization drastically reduces the number of protective infiltrating CD4+ and CD8+ T cells into the CNS of mice with acute encephalomyelitis caused by mouse hepatitis virus.^{21,22}

Our mRNA data, together with the findings from other models in which CD8+ T cells are essential, prompted us

to focus our analysis on the chemokines CXCL9 and CXCL10; however, before proceeding any further with our animal model, we wanted to validate the relevance of the mouse mRNA expression data by investigating the expression of these chemokines in human cases of RMSF. A collection of brainstem samples (where rickettsial lesions are particularly numerous)⁴² from nine pediatric cases of fatal RMSF was analyzed by immunohistochemistry using specific monoclonal antibodies against CXCL9, CXCL10, CD8, and CXCR3 (Table 2). Unfortunately no other tissues were available for analysis; however, we believe that despite the heterogeneous nature of the endothelium of different organs the endothelial chemokine response may be similar in different vascular beds as reflected by the immunohistochemical detection of these chemokines in different organs of rickettsia-infected mice (Figure 4). Although it is not possible to make firm conclusions based on the retrospective analysis of relatively few human samples that were subjectively analyzed by two pathologists, we observed that 1) the amount of rickettsial antigen detected in human cases was variable (presumably owing to anti-microbial treatment and/or the host immune response) and did not correlate with the expression of any of the other markers studied; 2) CD8+ and CXCR3+ cells were few or not detectable in cases with a short duration of illness (< 9 days) and were more abundant at later times (> 12 days). This observation is consistent with the time required for maturation of the adaptive immune response; and 3) fewer CXCR3+ cells were detected than CD8+ cells. Down-regulation of CXCR3 after CXCL9/10/11 binding to this receptor could explain this finding.⁴³ Perhaps more importantly, the data obtained from human samples validated the usefulness of our mouse model of spotted fever group rickettsioses for pathophysiological and immunological studies that may be relevant to the human spotted fever group rickettsioses.

The expression of CXCL9 and CXCL10 in C3H/HeN mice infected with *R. conorii* was analyzed at both the mRNA and protein levels. We used real-time PCR with TaqMan probes to analyze cDNA for CXCL9 and CXCL10 in lungs, liver, and brain throughout the course of the disease in mice undergoing both lethal and sublethal infections with *R. conorii*. The same tissues were also analyzed by immunohistochemistry with the objectives of validating the mRNA data and localizing the protein in the tissues analyzed. Indeed the protein data mirrored the mRNA results. We felt that the immunohistochemical approach was necessary (in contrast with other methods such as enzyme-linked immunosorbent assay for serum samples) because many chemokines, including CXCL9 and CXCL10, bind to glycosaminoglycans on the surface of endothelial cells,⁴⁴ and this is the fraction that is most likely to be physiologically relevant.

In general, the peak of expression of both CXCL9 and CXCL10 occurred 3 days after intravenous injection of either lethal or sublethal inocula of *R. conorii* (Figure 3). The major exception was the expression of CXCL9 in the brain peaking at day 7 of infection in the sublethal model. On day 3 of rickettsial infection the protein signal was multifocal and extensive in both lungs and brain and

Figure 5. Quantitative real-time PCR with TaqMan probes of DNA from livers of mice intravenously infected with either sublethal (S1 to S21) or lethal doses (L1 to L5) of *R. conorii* or uninfected control mice (C), and sacrificed every other day. The number of copies of the rickettsial *rompB* gene was normalized by expressing this value relative to 10⁴ or 10⁵ copies of the eukaryotic gene GAPDH. All data points correspond to the means of three simultaneous amplifications with negligible standard deviations. The data presented are representative examples of two separate experiments.

essentially generalized in the liver at the level of the sinusoids (Figure 4). In all cases the signal localized to endothelial cells, although it was also found in infiltrating leukocytes. At earlier and later times, the signal was more focal. Unfortunately CXCL9 and CXCL10 can only be detected in formalin-fixed tissues and not in tissues fixed with precipitant fixatives such as zinc-based preparations, which allow the more sensitive detection of rickettsial antigen. Our method for detection of rickettsial antigen in formalin-fixed tissues involves enzymatic digestion of the tissue and does not identify the signal before day 4 in the sublethal rickettsial infection model (whereas zinc fixation allows the detection of rickettsial antigen on day 1). In fact, the peak of the rickettsial load in lungs and liver occurs at approximately day 5 as assessed by real-time PCR for the rickettsial gene *rompB* (Figure 5). We hypothesize that the focal expression of CXCL9 and CXCL10 on day 3 in the lungs and brain and day 1 in the liver corresponds to vascular beds infected with rickettsiae.

This is, to the best of our knowledge, the first study addressing the kinetics and localization of expression of T-cell-targeting chemokines throughout the natural history of an infectious disease. One of the most interesting observations was that the peak of expression of these chemokines, which have been regarded as specific for memory/effector Th1 and CD8 T cells, occurred at a time when no infiltrating CD8+ T cells were found in the tissues of rickettsia-infected mice (Figure 3 and Figure 4, h and i). Because CXCR3, the receptor for CXCL9 and CXCL10, is also expressed by natural killer (NK) cells,⁴⁵ it is possible that this early peak of chemokine expression might be important for the effector functions of the innate immune system. On the other hand, our data from a mouse endothelial cell line suggest that rickettsial infection does not directly trigger the expression of CXCL9 and CXCL10 and that IFN- γ or a combination of IFN- γ and TNF- α are necessary to elicit their expression. We

have previously found that both cytokines are detectable in the sera of rickettsia-infected mice on the second day of infection.⁴⁶ A potential early source of these chemokines might be NK cells, and, if this is the case, they could be part of an early positive feedback loop. Unfortunately it was not possible to evaluate the presence of NK cells in our animal model because NK cells of C3H/HeN mice do not have exclusive markers that could allow for their identification in tissues; however, we have shown in two other models of rickettsioses that NK cells become activated.⁴⁶ Another yet more intriguing possibility is that rickettsia-infected endothelial cells directly prime antigen-specific CD8+ T cells very early after infection, which can produce an early rise in IFN- γ before they proliferate and become detectable as infiltrating cells in infected tissues. This hypothesis is plausible because there is evidence that endothelial cells can prime T-cell responses.^{47–52} This is, therefore, a subject that should be addressed in the rickettsial model because this system offers the unique opportunity to study lymphocyte-endothelial interactions in the context of an intracellular bacterial infection that targets these cells specifically.

It is also possible that the early rise in the expression of CXCL9 and CXCL10 may participate in the process of Th1 polarization and effector cell generation that are so important in the clearance of rickettsiae from infected hosts. There is evidence that these chemokines, particularly CXCL10, play that role in models of arthritis,⁵³ *T. gondii* infection,²⁰ and in CXCL10 gene knockout mice.⁵⁴ These knockout mice exhibit decreased Th1 responses including decreased contact hypersensitivity and all-or-none responses, defective clearance of a virus (mouse hepatitis virus), and decreased numbers of effector CD8+ T lymphocytes.

Although we do not know the importance of endothelial proliferation and angiogenesis in the repair process during a rickettsial infection, given the fact that CXCR3 ligands are angiostatic chemokines,^{55,56} it is possible that

Figure 4. Immunohistochemical analysis of the expression of CXCL9 or CXCL10 (brown) in liver (a–c), lungs (d–f), and kidney (g) of C3H/HeN mice intravenously infected with a sublethal dose of *R. conorii*, and immunohistochemical detection of infiltrating CD8+ T cells (brown) in the lungs (h) and testis (i) of *R. conorii* (red)-infected mice. CXCL9 in the liver on days 1 (a), 3 (b), and 6 (c) after a sublethal rickettsial infection. The liver from mock-infected mice did not have any visible signal (not shown). d and e: CXCL9 in the lungs on days 3 (d), and 6 (e) after a sublethal rickettsial infection. The CXCL9 signal localizes to endothelial cells (f, arrows). The CXCL10 results were analogous to the CXCL9 results (not shown). g: CXCL10 on the apical border of kidney tubular epithelial cells early during the course of a rickettsial infection. Original magnifications: $\times 100$ (a, e); $\times 400$ (b–d, f–i).

high levels of CXCL9 and CXCL10, such as the ones found in our study, might have a detrimental effect in the repair process of the vasculature. This is also an issue that merits consideration for future studies.

The generalized expression of CXCL9 and CXCL10 in the sinusoids of the liver and the almost generalized expression of these chemokines in the lungs 3 days after rickettsial infection were surprising findings as well because this pattern of expression does not help explain the precise targeting and localization of T cells around infected endothelial cells. It is possible, however, that most of these chemokines on the surface of endothelial cells are partially or totally inactivated by enzymatic modification. Recent evidence indicates that this is a plausible explanation because dipeptidyl peptidase IV (CD26) modifies CXCR3 ligands to become antagonists or low-potency agonists.⁵⁷

In conclusion, we have shown that among several different T-cell-targeting chemokines the CXCR3 ligands CXCL9 and CXCL10 have the highest levels of expression during endothelial-target infections caused by spotted fever group rickettsiae. Our findings in a mouse model were validated in fatal human cases of RMSF. Also, the morphological evidence showed that endothelial cells are an important source of these chemokines. The fact that the timing and distribution of expression of CXCL9 and CXCL10 do not seem to agree with their expected role as chemoattractants for effector T cells in this rickettsial infection model opens interesting new questions in regard to the function of the early rise and wide distribution of these chemokines. It is likely that they also play a role during the induction phase of the adaptive immune response and/or in guiding effector cells of the innate immune system including macrophages and NK cells. In the future it will be important to determine whether these chemokines are intact or modified by CD26 or other peptidases and whether functional blocking with specific antibodies can affect the outcome of the infection. It will also be important to identify the source of early IFN- γ production during an experimental rickettsial infection because this is the most likely stimulus that triggers the production of CXCR3 ligands and other chemokines.

References

- Heinzen RA, Hayes SF, Peacock MG, Hackstadt T: Directional actin polymerization associated with spotted fever group rickettsia infection of Vero cells. *Infect Immun* 1993, 61:1926–1935
- Heinzen RA, Grieshaber SS, Van Kirk LS, Devin CJ: Dynamics of actin-based movement by Rickettsia rickettsii in Vero cells. *Infect Immun* 1999, 67:4201–4207
- Van Kirk LS, Hayes SF, Heinzen RA: Ultrastructure of Rickettsia rickettsii actin tails and localization of cytoskeletal proteins. *Infect Immun* 2000, 68:4706–4713
- Teyssie N, Chiche-Portiche C, Raoult D: Intracellular movements of Rickettsia conorii and R. typhi based on actin polymerization. *Res Microbiol* 1992, 143:821–829
- Walker DH: Rocky Mountain spotted fever: a seasonal alert. *Clin Infect Dis* 1995, 20:1111–1117
- Walker DH, Lane TW: Rocky Mountain spotted fever: Clinical signs, symptoms, and pathophysiology. *Biology of Rickettsial Diseases*. Edited by DH Walker. Boca Raton, CRC Press, 1988, pp 63–78
- Kaplowitz LG, Fischer JJ, Sparling PF: Rocky Mountain spotted fever: a clinical dilemma. *Current Clinical Topics in Infectious Diseases*. Edited by JS Remington, MN Swartz. New York, McGraw-Hill Book Co., 1981, pp 89–108
- Feng H, Popov VL, Yuoh G, Walker DH: Role of T lymphocyte subsets in immunity to spotted fever group rickettsiae. *J Immunol* 1997, 158:5314–5320
- Walker DH, Olano JP, Feng HM: Critical role of cytotoxic T lymphocytes in immune clearance of rickettsial infection. *Infect Immun* 2001, 69:1841–1846
- Herrero-Herrero JI, Walker DH, Ruiz-Beltran R: Immunohistochemical evaluation of the cellular immune response to Rickettsia conorii in taches noires. *J Infect Dis* 1987, 155:802–805
- Kaplanski G, Teyssie N, Farnarier C, Kaplanski S, Lissitzky JC, Durand JM, Soubeyrand J, Dinarello CA, Bongrand P: IL-6 and IL-8 production from cultured human endothelial cells stimulated by infection with Rickettsia conorii via a cell-associated IL-1 α -dependent pathway. *J Clin Invest* 1995, 96:2839–2844
- Sporn LA, Marder VJ: Interleukin-1 α production during Rickettsia rickettsii infection of cultured endothelial cells: potential role in autocrine cell stimulation. *Infect Immun* 1996, 64:1609–1613
- Dignat-George F, Teyssie N, Mutin M, Bardin N, Lesaulle G, Raoult D, Sampol J: Rickettsia conorii infection enhances vascular cell adhesion molecule-1- and intercellular adhesion molecule-1-dependent mononuclear cell adherence to endothelial cells. *J Infect Dis* 1997, 175:1142–1152
- Sporn LA, Lawrence SO, Silverman DJ, Marder VJ: E-selectin-dependent neutrophil adhesion to Rickettsia rickettsii-infected endothelial cells. *Blood* 1993, 81:2406–2412
- Henkart PA: Cytotoxic T lymphocytes. *Fundamental Immunology*. Edited by WE Paul. Philadelphia, Lippincott-Raven, 1999, pp 1021–1049
- Piali L, Weber C, LaRosa G, Mackay CR, Springer TA, Clark-Lewis I, Moser B: The chemokine receptor CXCR3 mediates rapid and shear-resistant adhesion-induction of effector T lymphocytes by the chemokines IP10 and Mig. *Eur J Immunol* 1998, 28:961–972
- Miura M, Morita K, Kobayashi H, Hamilton TA, Burdick MD, Strieter RM, Fairchild RL: Monokine induced by IFN- γ is a dominant factor directing T cells into murine cardiac allografts during acute rejection. *J Immunol* 2001, 167:3494–3504
- Hancock WW, Gao W, Csizmadia V, Faia KL, Shemmeri N, Luster AD: Donor-derived IP-10 initiates development of acute allograft rejection. *J Exp Med* 2001, 193:975–980
- Hancock WW, Lu B, Gao W, Csizmadia V, Faia K, King JA, Smiley ST, Ling M, Gerard NP, Gerard C: Requirement of the chemokine receptor CXCR3 for acute allograft rejection. *J Exp Med* 2000, 192:1515–1520
- Khan IA, Maclean JA, Lee FS, Casciotti L, DeHaan E, Schwartzman JD, Luster AD: IP-10 is critical for effector T cell trafficking and host survival in Toxoplasma gondii infection. *Immunity* 2000, 12:483–494
- Liu MT, Chen BP, Oertel P, Buchmeier MJ, Armstrong D, Hamilton TA, Lane TE: The T cell chemoattractant IFN-inducible protein 10 is essential in host defense against viral-induced neurologic disease. *J Immunol* 2000, 165:2327–2330
- Liu MT, Armstrong D, Hamilton TA, Lane TE: Expression of Mig (monokine induced by interferon- γ) is important in T lymphocyte recruitment and host defense following viral infection of the central nervous system. *J Immunol* 2001, 166:1790–1795
- Walker DH, Cain BG: The rickettsial plaque. Evidence for direct cytopathic effect of Rickettsia rickettsii. *Lab Invest* 1980, 43:388–396
- Bradford WD, Croker BP, Tisher CC: Kidney lesions in Rocky Mountain spotted fever: a light-, immunofluorescence-, and electron-microscopic study. *Am J Pathol* 1979, 97:381–392
- Roggli VL, Keener S, Bradford WD, Pratt PC, Walker DH: Pulmonary pathology of Rocky Mountain spotted fever (RMSF) in children. *Pediatr Pathol* 1985, 4:47–57
- Walker DH, Popov VL, Feng HM: Establishment of a novel endothelial target mouse model of a typhus group rickettsiosis: evidence for critical roles for gamma interferon and CD8 T lymphocytes. *Lab Invest* 2000, 80:1361–1372
- Feng HM, Popov VL, Walker DH: Depletion of gamma interferon and tumor necrosis factor α in mice with Rickettsia conorii-infected endothelium: impairment of rickettsicidal nitric oxide production re-

- sulting in fatal, overwhelming rickettsial disease. *Infect Immun* 1994, 62:1952–1960
28. O'Connell KA, Edidin M: A mouse lymphoid endothelial cell line immortalized by simian virus 40 binds lymphocytes and retains functional characteristics of normal endothelial cells. *J Immunol* 1990, 144:521–525
29. Belperio JA, Keane MP, Burdick MD, Lynch III JP, Xue YY, Li K, Ross DJ, Strieter RM: Critical role for CXCR3 chemokine biology in the pathogenesis of bronchiolitis obliterans syndrome. *J Immunol* 2002, 169:1037–1049
30. Farber JM: Mig and IP-10: CXC chemokines that target lymphocytes. *J Leukoc Biol* 1997, 61:246–257
31. Walker DH, Popov VL, Crocquet-Valdes PA, Welsh CJ, Feng HM: Cytokine-induced, nitric oxide-dependent, intracellular antirickettsial activity of mouse endothelial cells. *Lab Invest* 1997, 76:129–138
32. Sahni SK, Van Antwerp DJ, Ereemeeva ME, Silverman DJ, Marder VJ, Sporn LA: Proteasome-independent activation of nuclear factor kappaB in cytoplasmic extracts from human endothelial cells by Rickettsia rickettsii. *Infect Immun* 1998, 66:1827–1833
33. Sporn LA, Sahni SK, Lerner NB, Marder VJ, Silverman DJ, Turpin LC, Schwab AL: Rickettsia rickettsii infection of cultured human endothelial cells induces NF-kappaB activation. *Infect Immun* 1997, 65:2786–2791
34. Moser B, Loetscher M, Piali L, Loetscher P: Lymphocyte responses to chemokines. *Int Rev Immunol* 1998, 16:323–344
35. Kim CH, Broxmeyer HE: Chemokines: signal lamps for trafficking of T and B cells for development and effector function. *J Leukoc Biol* 1999, 65:6–15
36. Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B, Mackay CR: The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J Clin Invest* 1998, 101:746–754
37. Kim CH, Rott L, Kunkel EJ, Genovese MC, Andrew DP, Wu L, Butcher EC: Rules of chemokine receptor association with T cell polarization in vivo. *J Clin Invest* 2001, 108:1331–1339
38. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A: Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med* 1998, 187:875–883
39. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borzatti A, Sozzani S, Allavena P, Gray PA, Mantovani A, Sinigaglia F: Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 1998, 187:129–134
40. Nishimura M, Umehara H, Nakayama T, Yoneda O, Hieshima K, Kakizaki M, Dohmae N, Yoshie O, Imai T: Dual functions of fractalkine/CX3C ligand 1 in trafficking of perforin+/granzyme B+ cytotoxic effector lymphocytes that are defined by CX3CR1 expression. *J Immunol* 2002, 168:6173–6180
41. Frigerio S, Junt T, Lu B, Gerard C, Zumsteg U, Hollander GA, Piali L: Beta cells are responsible for CXCR3-mediated T-cell infiltration in insulinitis. *Nat Med* 2002, 8:1414–1420
42. Lillie RD: The Pathology of Rocky Mountain Spotted Fever. National Institute of Health Bulletin No. 177. Washington, D.C., United States Government Printing Office, 1941, pp 1–46
43. Sauty A, Colvin RA, Wagner L, Rochat S, Spertini F, Luster AD: CXCR3 internalization following T cell-endothelial cell contact: preferential role of IFN-inducible T cell alpha chemoattractant (CXCL11). *J Immunol* 2001, 167:7084–7093
44. Nelson PJ, Krensky AM: Chemokines, chemokine receptors, and allograft rejection. *Immunity* 2001, 14:377–386
45. Taub DD, Sayers TJ, Carter CR, Ortaldo JR: Alpha and beta chemokines induce NK cell migration and enhance NK-mediated cytotoxicity. *J Immunol* 1995, 155:3877–3888
46. Billings AN, Feng HM, Olano JP, Walker DH: Rickettsial infection in murine models activates an early anti-rickettsial effect mediated by NK cells and associated with production of gamma interferon. *Am J Trop Med Hyg* 2001, 65:52–56
47. Savinov AY, Wong FS, Stonebraker AC, Chervonsky AV: Presentation of antigen by endothelial cells and chemoattraction are required for homing of insulin-specific CD8+ T cells. *J Exp Med* 2003, 197:643–656
48. Kreisel D, Krupnick AS, Gelman AE, Engels FH, Popma SH, Krasinski AM, Balsara KR, Szeto WY, Turka LA, Rosengard BR: Non-hematopoietic allograft cells directly activate CD8+ T cells and trigger acute rejection: an alternative mechanism of allorecognition. *Nat Med* 2002, 8:233–239
49. Kreisel D, Krupnick AS, Balsara KR, Riha M, Gelman AE, Popma SH, Szeto WY, Turka LA, Rosengard BR: Mouse vascular endothelium activates CD8+ T lymphocytes in a B7-dependent fashion. *J Immunol* 2002, 169:6154–6161
50. Limmer A, Ohl J, Kurts C, Ljunggren HG, Reiss Y, Groettrup M, Momburg F, Arnold B, Knolle PA: Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. *Nat Med* 2000, 6:1348–1354
51. Marelli-Berg FM, Scott D, Bartok I, Peek E, Dyson J, Lechler RI: Antigen presentation by murine endothelial cells. *Transplant Proc* 2001, 33:315–316
52. Marelli-Berg FM, Scott D, Bartok I, Peek E, Dyson J, Lechler RI: Activated murine endothelial cells have reduced immunogenicity for CD8+ T cells: a mechanism of immunoregulation? *J Immunol* 2000, 165:4182–4189
53. Salomon I, Netzer N, Wildbaum G, Schiff-Zuck S, Maor G, Karin N: Targeting the function of IFN-gamma-inducible protein 10 suppresses ongoing adjuvant arthritis. *J Immunol* 2002, 169:2685–2693
54. Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD: IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *J Immunol* 2002, 168:3195–3204
55. Luster AD, Greenberg SM, Leder P: The IP-10 chemokine binds to a specific cell surface heparan sulfate site shared with platelet factor 4 and inhibits endothelial cell proliferation. *J Exp Med* 1995, 182:219–231
56. Romagnani P, Annunziato F, Lasagni L, Lazzeri E, Beltrame C, Francalanci M, Uguccioni M, Galli G, Cosmi L, Maurenzi L, Baggiolini M, Maggi E, Romagnani S, Sero M: Cell cycle-dependent expression of CXC chemokine receptor 3 by endothelial cells mediates angiostatic activity. *J Clin Invest* 2001, 107:53–63
57. Proost P, Schutyser E, Menten P, Struyf S, Wuyts A, Opdenakker G, Dethoux M, Parmentier M, Durinx C, Lambier AM, Neyts J, Liekens S, Billiau A, Van Damme J: Amino-terminal truncation of CXCR3 agonists impairs receptor signaling and lymphocyte chemotaxis, while preserving antiangiogenic properties. *Blood* 2001, 98:3554–3561