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High serum CXCL10 in *Rickettsia conorii* infection is endothelial cell mediated subsequent to whole blood activation

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ABSTRACT

Background: The pathophysiological hallmark of *Rickettsia conorii* (*R. conorii*) infection comprises infection of endothelial cells with perivascular infiltration of T-cells and macrophages. Although interferon (IFN)-γ-induced protein 10 (IP-10)/CXCL10 is induced during vascular inflammation, data on CXCL10 in *R. conorii* infection is scarce.
Methods: Serum CXCL10 was analyzed in two cohorts of southern European patients with *R. conorii* infection using multiplex cytokine assays. The mechanism of *R. conorii*-induced CXCL10 release was examined *ex vivo* using human whole blood interacting with endothelial cells.
Results: (i) At admission, *R. conorii* infected patients had excessively increased CXCL10 levels, similar in the Italian (n = 32, ~56-fold increase vs controls) and the Spanish cohort (n = 38, ~68-fold increase vs controls), followed by a marked decrease after recovery. The massive CXCL10 increase was selective since it was not accompanied with similar changes in other cytokines. (ii) Heat-inactivated *R. conorii* induced a marked CXCL10 increase when whole blood and endothelial cells were co-cultured. Even plasma obtained from *R. conorii*-exposed whole blood induced a marked CXCL10 release from endothelial cells, comparable to the levels found in serum of *R. conorii*-infected patients. Bacteria alone did not induce CXCL10 production in endothelial cells, macrophages or smooth muscle cells.
Conclusions: We show a massive and selective serum CXCL10 response in *R. conorii*-infected patients, likely reflecting release from infected endothelial cells characterized by infiltrating T cells and monocytes. The CXCL10 response could contribute to T-cell infiltration within the infected organ, but the pathologic consequences of CXCL10 in clinical *R. conorii* infection remain to be defined.

1. Introduction

Rickettsia conorii (*R. conorii*) causes Mediterranean spotted fever (MSF); a severe form of spotted fever group (SFG) rickettsioses that

Abbreviations: HUVEC, human umbilical vein endothelial cells; IL-8, interleukin-8; IP-10, interferon (IFN)-γ-induced protein 10; MSF, Mediterranean spotted fever; [MCP]-1, monocyte chemoattractant peptide-1; *R. conorii*, *Rickettsia conorii*; RMSF, Rocky Mountain spotted fever; SFG, spotted fever group.

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in some cases cause substantial morbidity and mortality. Pathologically, the infection is characterized by endothelial cell activation with infiltration of T cells and macrophages and development of vasculitis [1,2]. While the immune response against *R. conorii* is important in order to eliminate the bacteria, it can also result in collateral damage and tissue destruction. This contributes to inappropriate inflammatory responses that can be harmful to the host [1,2]. To further elucidate these issues, studies on the cytokine profile during clinical infection may be useful, and in *R. conorii* infection, identification of the most important inflammatory mediators has not been accomplished.

Interferon (IFN)-γ-induced protein 10 (IP-10)/CXCL10 belongs to the CXC chemokine subfamily and its production is up regulated by IFN-γ during inflammation in various cells such as macrophages, endothelial cells and T cells. Through its interaction with CXCR3, CXCL10 recruits and activates the inflammatory Th1 cell subset with enhanced IFN-γ production as a consequence, thereby creating a self-amplifying inflammatory feedback loop [3]. Based on its postulated role in vascular inflammation [4,5], it is tempting to hypothesize that CXCL10 could play a central role in the pathogenesis of *R. conorii* infection. There is some evidence from experimental studies that CXCL10 could be involved in the early immune response against *R. conorii* [6], but data on CXCL10 levels in clinical *R. conorii* infection (*i.e.* MSF) are minimal to nil.

In the present study we aimed to investigate the role of CXCL10 in *R. conorii* infection by analysing the levels of CXCL10 in two cohorts of southern-European patients with MSF and to examine the mechanism of *R. conorii*-induced CXCL10 release in a novel *ex vivo* model of human whole blood interacting with endothelial cells. For comparison and to search for specificity in the CXCL10 response, a number of other cytokines, chemokines and growth factors were simultaneously investigated.

2. Materials and methods

2.1. Patients and controls

2.1.1. The Italian cohort

The Italian MSF cohort consisted of 32 patients (17 women and 15 men, 19–90 [mean 61.5] years of age) admitted to Termini Imerese Hospital Palermo, Palermo, Italy during the summer (June to September) of 2005 [7]. Patients were included if they had two or three of the typical symptoms, including fever, eschar, or rash, *and* seroconversion in IgG to Rickettsia conori by indirect immunofluorescence (IFA) and enzyme-linked immunosorbent assay (ELISA) [7]. All patients had signs and symptoms of active MSF with disease duration <2 weeks before diagnosis [8]. The control group consisted of 24 healthy individuals (9 women and 15 men, 21–67 years of age) recruited from the same part of Italy. Serum samples from the controls were stored at −80 °C approximately one year before the analysis.

2.1.2. The Spanish cohort

Thirty-eight patients (28 men and 10 women, 20–84 [mean 56.6] years of age) with confirmed MSF, admitted to the Infectious Diseases Department of the Hospital San Pedro, Logroño, Spain from June 2004 to July 2011, were retrospectively included in the study. They all had characteristic signs of active MSF. The duration of illness before diagnosis was less than 1 week. For all patients the first serum sample was obtained either before, or with no more than one dose before administration of any active drug against *Rickettsia* spp. All patients with MSF had seroconversion with increased levels of anti-*R. conorii* antibodies as assessed by indirect IFA (Focus Diagnostics, Cypress, CA) and six of them also showed positive results for *Rickettsia* using molecular biology tools (PCR and sequencing). *R. conorii* was isolated from blood in two cases. All patients recovered although two of them had severe manifestations of the disease. Nine healthy subjects (5 women and 4 men, 35–58 years of age) recruited from the same area of Spain were included in the study as controls. Blood samples from the control group were obtained during October 2012, and their sera were stored at −80 °C.

2.2. Patient blood sampling

Blood was collected both at the initial visit (less than two weeks after the onset of the symptoms and before the specific treatment), and one time during follow-up (7–21 days [Italian cohort] and 28–42 days [Spanish cohort]). Two specimens for each patient were available. Peripheral venous blood was drawn into pyrogen-free, vacuum blood collection tubes without any additives and allowed to clot before centrifugation at 2000*g* for 10 min. Serum was stored at −80 °C until analysis.

2.3. Whole blood sampling for ex vivo experiments

Whole blood was obtained from healthy adult volunteers who had received no medication for at least 10 days. Blood was drawn from the antecubital vein into 4.5 ml sterile polypropylene cryotubes (Nalgene NUNC, Roskilde, Denmark) containing the thrombin specific inhibitor lepirudin (Refludan; Pharmion ApS, Copenhagen, Denmark) at a final concentration of 50 μg/ml. Lepirudin was used as anticoagulant in all *ex vivo* experiments [9].

2.4. Cell cultures

The method for endothelial cell culture has previously been described [10] using human umbilical vein endothelial cells (HUVECs) from umbilical cord veins [10]. HUVECs were grown to confluence for 3–5 days. The HUVECs were utilized at passage levels 4–9 (passage induced by treatment with 0.05% trypsin-EDTA (Gibco, Grand Island, NY).

The human monocytic cell line THP-1 cells were kept in RPMI-1640 (American Type Culture Collection, Rockville, MD) with 10% fetal calf serum (FCS; Sigma, St. Louis, MO, penicillin-streptomycin (50 U/ml–0.05 mg/ml, Sigma), 2 mmol/l L-glutamine (Sigma). Prior to experimental start, the cells were seed in 12-wells plates (1×10^6 per ml, Costar, Cambridge, MA) and differentiated into macrophages by a 24-h incubation with phorbol myristate acetate (PMA, 100 nmol; Sigma). Thereafter, the macrophages were treated with recombinant tumor necrosis factor (TNF; 10 ng/ml, R&D Systems, Minneapolis, MN) in growth medium containing 2.5% FCS for 48 h before the pre-treated cells were stimulated with or without heat-inactivated *R. conorii* (1×10^5 bacteria/ml) for 20 h.

Human aortic smooth muscle cells (SMC) were obtained from PromoCell GmbH (Heidelberg, Germany) and grown in SMC Growth Medium 2 with complete supplement mix (PromoCell). At 90% confluence, the culture was trypsinized and replated. The day prior to experiments, cells were seeded in 24-well plates (1.5×10^5 cells/ml; Costar) and grown in the same medium. At experimental start, the cells were cultured in OptiMem with Glutamix (Invitrogen, Carlsbad, CA) alone or with heat-inactivated *R. conorii* (1×10^5 bacteria/ml) for 20 h.

2.5. Preparation of R. conorii

Preparation of *R. conorii* was performed as previously described [11] using Malish strain grown in monolayers of Vero cells. Five days post-inoculation heavily infected cells were harvested and pelleted (centrifugation at 10,000*g* in 15 min) before being