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EDITORIAL

IMMUNOLOGY OF HUMAN RICKETTSIAL DISEASES

P. MANSUETO, G. VITALE, G. DI LORENZO, F. ARCOLEO[1], S. MANSUETO and E. CILLARI[1]

Dipartimento di Medicina Clinica e delle Patologie Emergenti, University of Palermo; [1]U.O. di Patologia Clinica, Azienda Ospedaliera "V. Cervello", Palermo, Italy

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Among human rickettsial diseases caused by micro-organisms of the genus Rickettsia (Order Rickettsiales; Family Rickettsiaceae), transmitted to human hosts through arthropod vectors, Mediterranean Spotted Fever, or Boutonneuse Fever, and Rocky Mountain Spotted Fever are considered to be important infectious diseases due to continued prevalence in the developed world, difficulties in early and accurate diagnosis, and potentially fatal outcome in severe cases. Proliferation of rickettsiae, at the site of the tick bite, results in focal epidermal and dermal necrosis (tache noire). Rickettsiae then spread via lymphatic vessels to the regional lymph nodes, and, via the bloodstream, to skin, brain, lungs, heart, liver, spleen and kidneys. The pathogen invades and proliferates in the endothelial cells of small vessels, target cells of rickettsial infection, together, destroying them, and spreading the infection to the endothelia of the vascular tree. The damage of the endothelium, and the subsequent endothelia dysfunction, is followed by the activation of acute phase responses, with alteration in the coagulation and in the cytokine network, together with a transient immune dysregulation, characterized by the reduction in peripheral CD4+ T lymphocytes.

The human rickettsial diseases are a variety of clinical entities caused by micro-organisms of the genus *Rickettsia* (Order *Rickettsiales*; Family *Rickettsiaceae*). The pathogen invades and proliferates in the endothelial cells (ECs) of small vessels, target cells of rickettsial infection, destroying them, and spreading the infection to the endothelia of the vascular tree. The damage of the endothelium, and the subsequent endothelia dysfunction, is followed by the activation of acute phase responses, with alteration in the coagulation and in the cytokine network, together with a transient immune dysregulation, characterized by the reduction in peripheral CD4+ T lymphocytes. The mechanisms of host defence are not yet completely understood, although cell-mediated immunity is believed to play a crucial role (1).

ENDOTHELIAL CELL INVASION AND INJURY

Vasculitis of small vessels of numerous organs (including central nervous system, lung, myocardium, liver, and kidney) is a typical event during Boutonneuse Fever (BF), as seen by histopathologic examination, and the complications are attributed to changes in vascular permeability and oedema due to endothelial injury (2).

Key words: rickettsial diseases, endothelial cells, T_H1 lymhocytes, T_H2 lymhocytes, cytokines, acute phase response

Mailing address: Dr Pasquale Mansueto, M.D.
Dipartimento di Medicina Clinica
e delle Patologie Emergenti,
Via del Vespro n° 141,
90127 Palermo, Italy
Tel: ++39 91 6552970 Fax: ++39 91 6555995
e-mail: pamansu@unipa.it

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EC invasion

Although some signalling events involved in rickettsial entry into the ECs have been documented (i.e. activation of Src-family tyrosine kinases, tyrosine phosphorylation of focal adhesion kinase, and β1-integrin activation), host proteins mediating entry are not known (2).

In some studies it has been reported that Ku70 subunit of DNA-dependent protein kinase is a receptor involved in *R. conorii* internalization (Fig. 1). Ku70 is recruited to *R. conorii* entry sites, and inhibition of Ku70 expression impairs *R. conorii* internalization. *R. conorii* infection also stimulates the ubiquitination of Ku70. In addition, an ubiquitin ligase, called c-Cbl, is recruited to *R. conorii* entry foci, and downregulation of c-Cbl blocks rickettsial invasion and Ku70 ubiquitination (3).

Experimental approaches have identified the rickettsial outer membrane protein B (OmpB) as a ligand for Ku70. Antibodies against OmpA and OmpB are able to protect against reinfection, but they do not play a key role in immunity against primary infection (4).

The rickettsiae induce internalization by phagocytosis, associated with phospholipase A_2 activity, apparently of rickettsial origin, and escaping from the phagosome into the cytosol by an unidentified mechanism, even if some experimental data suggest a role for the tlyC and pld genes, with potential membranolytic activities, in the process of phagosomal escape by *R. prowazekii* (5). In the cytosol, *R. conorii* stimulates the polymerization of host cell F-actin, controlled by the Arp2/3 complex, using the bacterial surface RickA protein, which propels the bacterium through the cytoplasm and across the cell membrane into the adjacent endothelial cell or extracellular space (Fig. 1) (6).

EC injury

The infection of cultured ECs causes the induction of oxidative stress mechanisms, as evidenced by accumulation of reactive oxygen species (ROS), induced by the combination of tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) production (7), altered levels of antioxidant enzymes, and depleted levels of intracellular reduced thiols, together with alterations in the levels of mRNA expression of selected antioxidant enzymes in *in vivo* experimental infection (8) (Fig. 2).

Production of cytokines and chemokines by ECs

Human umbilical vein endothelial cells (HUVECs), infected by *R. conorii*, actively secrete large amounts of IL-8 and IL-6, via an IL-1α dependent pathway (Fig. 3) (9).

IL-1α, produced by HUVECs, is mainly in a precursor form. Therefore, IL-1α precursor can be secreted and immediately bound to the type I IL-1 receptor (10). Alternatively, IL-1α can be active without being secreted and fixed on endothelial surface membranes in cell-cell membrane interaction. Thus, IL-1α can act as an autocrine factor on HUVECs. IL-1α activates ECs, and increases the expression of adhesion molecules on both ECs and leukocytes by inducing the production of other cytokines, such as IL-8 and IL-6 (10).

IL-8 and IL-6 might play a role in the development of vasculitis induced by rickettsial infection. IL-8 is a potent chemotactic agent for polymorphonuclear leukocytes, stimulates polymorphonuclear leukocyte transendothelial migration, and activates their functions, but it seems to have no effect on endothelial cells (11). IL-6, also secreted by *R. conorii*-infected HUVECs, may mediate the acute phase protein production associated with BF. IL-6 might also be implicated in the local differentiation and proliferation of T lymphocytes, through its stimulatory effects on IL-2 production and IL-2 receptor (IL-2R) expression in T cells, and in B lymphocyte stimulation (12).

In addition, the expression of the chemokines CXCL9 and CXCL10, known to target CD8+ and CD4+ T lymphocytes, is significantly high in ECs of C3H/HeN mice infected with *R. conorii*. The peak of expression of these chemokines occurred 4 days before CD8+ T cells infiltrated the infected tissues. Therefore, CXCL9 and CXCL10 may play a role early during the immune response against rickettsial infections (13). Similarly, the peak of expression of fractalkine (CX3CL1), another chemokine expressed mainly by endothelial cells, on day 3 of infection, coincided with the time of infiltration of macrophages into infected tissues and preceded the peak of rickettsial content in tissues (14).

Induction of cellular adhesion molecules

In vitro R. conorii infection of ECs, through the

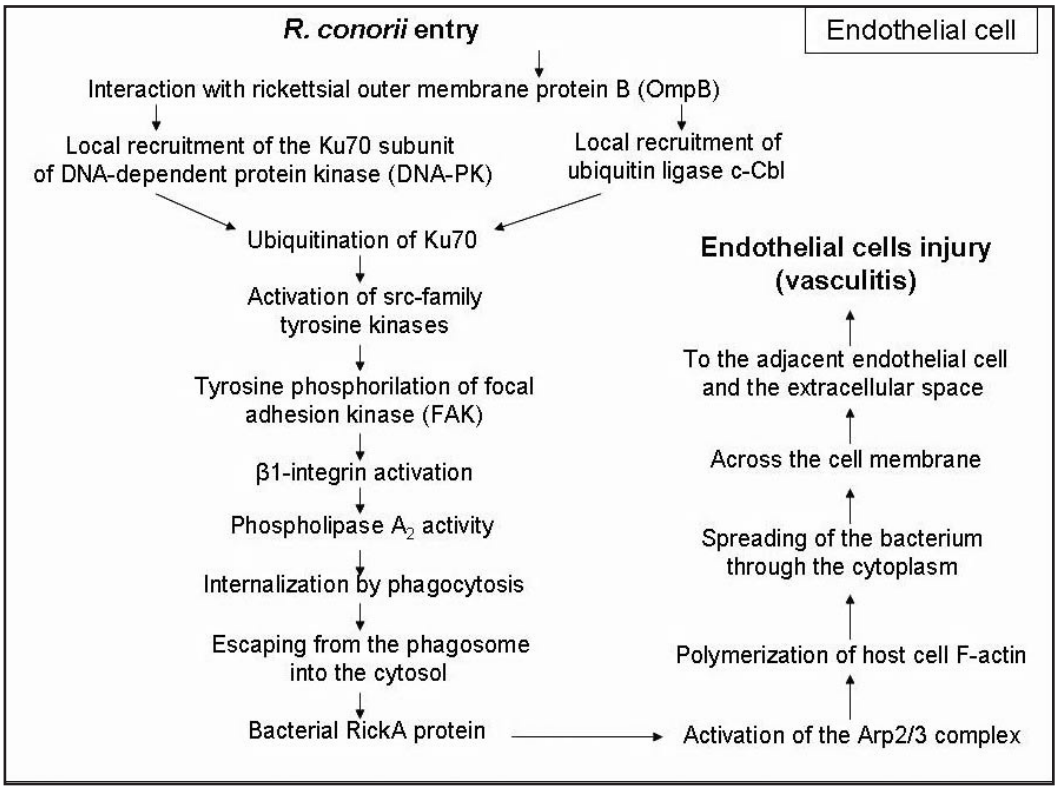

Fig. 1. *Endothelial cell invasion.*

release of the early response cytokines (IL-1α, IL-6, and TNF-α) and other components of the acute phase reaction, enhances the expression on the surface of ECs and polymorphonuclear leukocytes of cellular adhesion molecules (CAM), i.e. E-selectin, VCAM-1, and ICAM-1, which are responsible for enhanced rolling and the adherence of mononuclear cells to infected ECs (Fig. 3) (15).

In addition, during the acute phase of BF, soluble forms of these adhesion molecules (sL-selectin, sE-selectin, s-VCAM-1, and sICAM-1) are shed into the plasma by the proteolytic cleavage of their membrane-bound counterparts from activated leukocytes and ECs, and/or by the differential splicing of their mRNA (Fig. 3). The shed forms are functionally active and could be involved in the control of adhesive interactions between the cells. The persistence of high levels of this CAM has negative prognostic value, and could be useful to monitor disease evolution (15).

Platelet activation

In the acute phase of BF, through measurements of a major metabolite of thromboxane (TX) in the urine (11-dehydro-TXB_2), a marker of platelet activation, and of plasma prothrombin fragment 1+2, whose levels reflect activation of prothrombin to thrombin, biochemical evidence has been demonstrated for the occurrence of a TXA_2-dependent platelet activation and thrombin generation *in vivo*. These phenomena could, at least in part, account for clinical manifestations of BF, such as vasculitis and focal microthrombus formation (16).

Mechanism of intracellular killing of R. conorii

Perivascular infiltrations of CD4+ and CD8 T lymphocytes, macrophages, natural killer (NK) cells, infected ECs themselves, and marginated elements of blood secrete cytokines and chemokines that activate infected ECs, by paracrine and autocrine stimulation, to kill intracellular rickettsiae. Human cells might be capable of controlling rickettsial infections intracellularly, the most relevant location in these infections, by a cytokine- and chemokine-dependent, nitric oxide-dependent mechanism, and, in particular, by one or a combination of these three

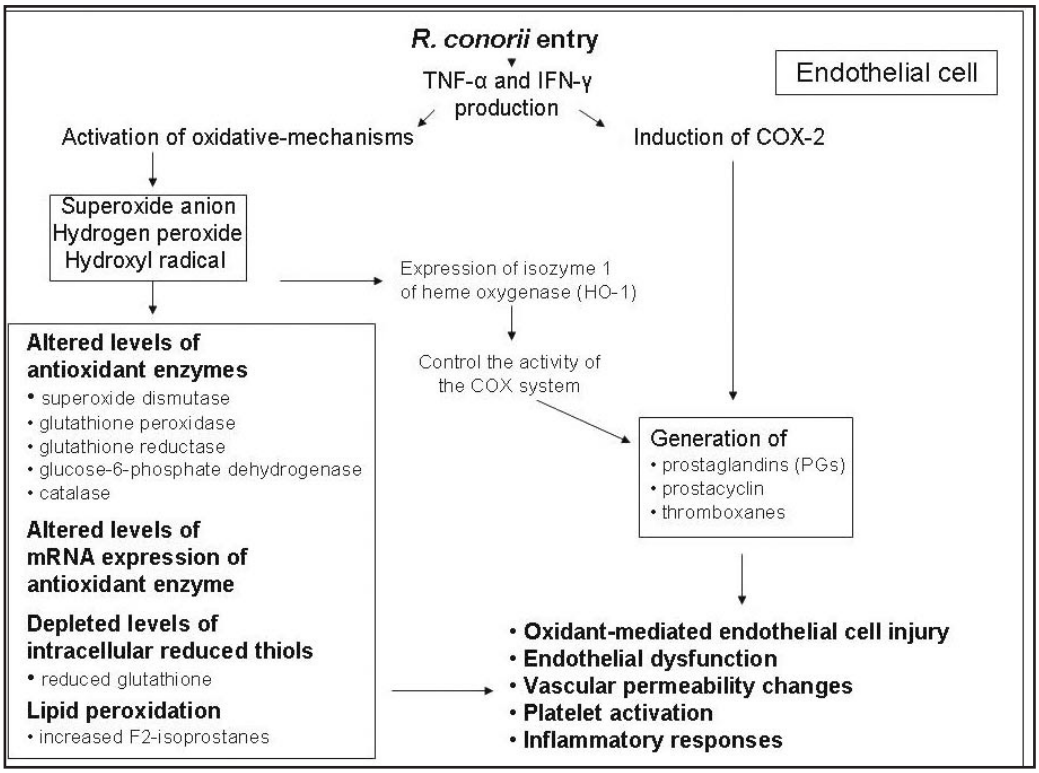

Fig. 2. *Endothelial cell injury.*

mechanisms involving: 1) nitric oxide synthesis; 2) hydrogen peroxide production; 3) tryptophan degradation, resulting in its intracellular depletion and starvation for the parasite (17).

ACUTE PHASE RESPONSE

In BF, the invasion and proliferation of rickettsiae in the ECs is the early event responsible for acute phase response activation. Some acute phase responses appear to be involved in the promotion of inflammatory events (IL-1α, IL-8, IFN-γ, complement proteins, and fibrinogen), while others appear to moderate them (ceruloplasmin, and α_1-antitrypsin). After the resolution of the infection, all mediators reach the normal range, acute phase response subsides, and the homeostatic balance is restored (Fig. 4) (18).

The analysis of cytokine serum levels indicates that IL-1α is not detectable in the blood of acute BF patients, which probably depends on the fact that stimulation in ECs causes the production of IL-1α predominantly in cell-associated form, thus contributing to the localized procoagulant and inflammatory responses which occur in the course of rickettsial disease. The undetectable serum levels of IL-8 may be due to its prevalent concentrations on endothelial protrusions, either in vesicles or on the plasma membrane (18).

As for as IFN-γ, its increase has been detected in the firsts week, followed by its normalization. Furthermore, IFN-γ appears to be associated with complement protein modifications. In fact, IFN-γ is the main inducer of C4 gene expression (18-19). Complement could prove useful for the elimination of infected, damaged or compromised cells, exerting anti-inflammatory activities (18).

The detection of high levels of serum fibrinogen, in the first week of BF infection, constitutes the primary event inducing a local activation of haemostasis, through platelet activation, and it is a cursory indicator of inflammation in most human infections (18).

Also anti-inflammatory mediators (i.e. ceruloplasmin and α_1-antitrypsin) are detected in patients affected by BF. The strong release

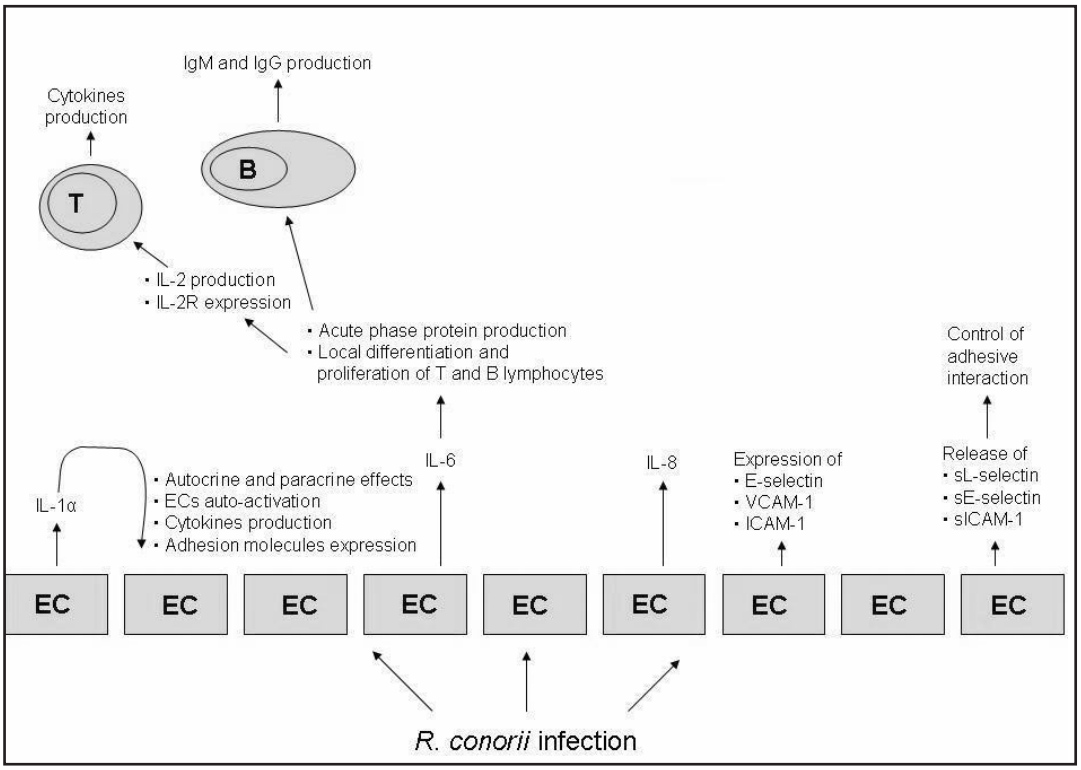

Fig. 3. *Production of cytokines by ECs and induction of cellular adhesion molecules.*

of ceruloplasmin might minimize the level of inflammatory response, acting as scavenger of oxygen free radicals, or ROS, produced by leukocytes (18). α_1-antitrypsin persists at high levels in BF patients until the third week and could indicate that this proteinase inhibitor is required for a long period, both to neutralize the lysosomal hydrolases released following the infiltration of macrophages and, more importantly, to induce the release of anti-inflammatory cytokines (18).

TOLL-LIKE RECEPTORS AND THE INNATE IMMUNE SYSTEM

One of the mechanisms by which the innate immune system senses the invasion of pathogenic microrganisms is through the pattern recognition receptors (PRRs), which are germ line-encoded receptors, including transmembrane Toll-like receptors (TLRs), that can sense pathogen-associated molecular patterns, and initiate signalling cascades that lead to an innate immune response. The Toll-like receptor 4 (TLR4) plays an important role in inflammation and immunity. It has been demonstrated that the +869G single-nucleotide polymorphism of the TLR4 gene, known to attenuate receptor signalling, decrease endotoxin responsiveness and determine poor outcome from sepsis, is overexpressed in BF patients, with significant differences, in the frequency of TLR genotypes and alleles, compared with age-matched controls. These data might be interpreted as one of the hypothetical bases for a genetic predisposition to BF (20).

IMMUNOLOGIC ALTERATIONS

Rickettsial-dendritic cell interaction

The role played by dendritic cells (DCs), known as specialized antigen presenting cells, initiators and orchestrators of the immune response, remains unclear in rickettsial infections. It seems that a vigorous proinflammatory response of DCs, associated with up-regulation of CD40, CD80, CD86, and major histocompatibility complex class II, production of IL-2, IL-12, and IL-23, and

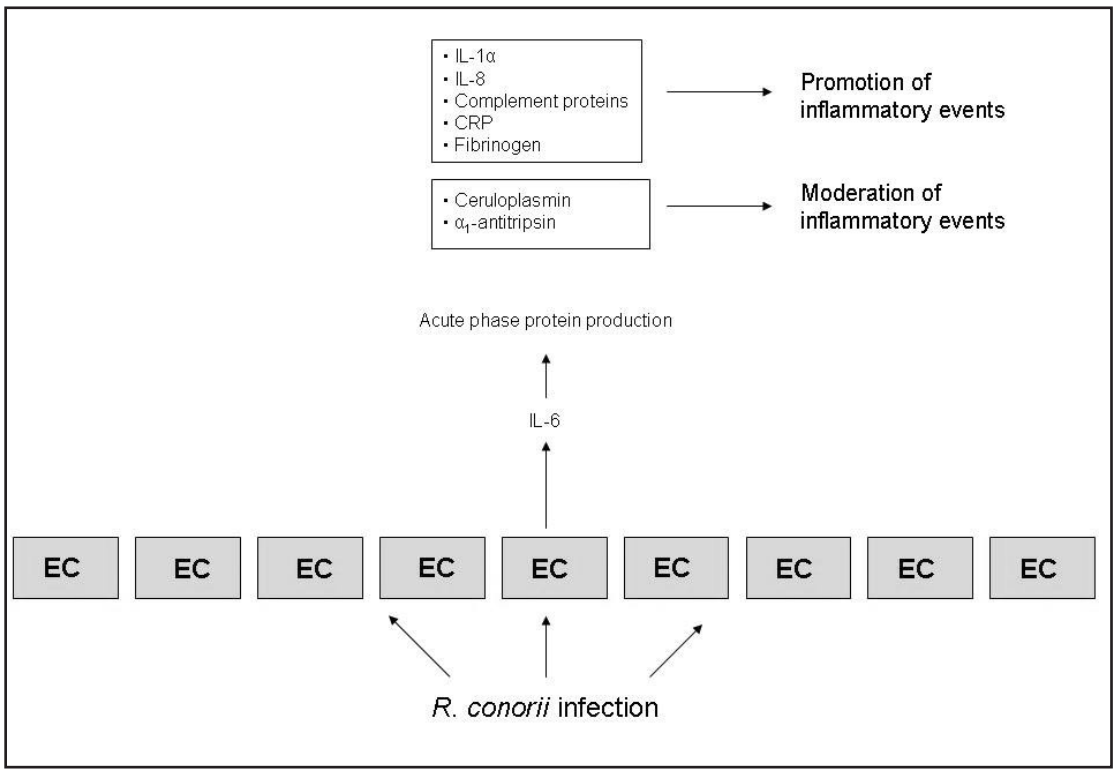

Fig. 4. *Acute phase response.*

production of antigen-specific IFN-γ in CD4+ T cells, is involved in the appearance of a protective immunity to rickettsiae (21).

Analysis of lymphocyte subpopulations

In acute BF patients there is a reduction of circulating T cells and, in particular, of CD4+ (helper/inducer T cells), CD4+/CD45RO+ (memory T cells), and CD4+/CD45+ (naive T cells) subsets. These modifications may be due to the cell adhesion to vascular endothelium, followed by their entrance into the sites of inflammation. Also apoptosis, spontaneous and activation-induced, could have a role in this decrease. The other lymphocytic subsets (CD8+ [suppressor/cytotoxic T cells], CD16+ [NK cells], and CD20+ [B cells]) tend to decrease in a non-significant way, whereas monocytes/macrophages (CD14+/HLA-DR+) are significantly increased. All cell subsets return to the normal levels after successful treatment, except for monocytes, that are persistently high after recovery (22).

The inflammatory and immunologic responses, mediated by the increase in T_H1-type (TNF-α and IFN-γ), and in T_H2-type cytokines (IL-10, and IL-6), appear to be important for the healing process (Fig. 5). In detail, TNF-α, IFN-γ, IL-10, and IL-6 are significantly increased in the blood of patients with acute BF compared with those detected from control healthy subjects (22).

In addition to the above, also in mild/moderate Mountain Spotted Fever (MSF) a strong and balanced intralesional proinflammatory and anti-inflammatory response is demonstrable, with a dominant type 1 immunity, whereas severe MSF is associated with increased expression of chemokine, i.e. RANTES, mRNA (23).

The dramatic increase in TNF-α, detected in the first week after the onset of symptoms, together with IFN-γ, is one of the early events observed in acute BF patients, followed by the increase of IL-10 and IL-6 (Fig. 5) (22). Activated antigen presenting cells (i.e. activated macrophages) produce high levels of TNF-α. TNF-α induces IFN-γ production from activated CD4+ T cells and NK cells. One important

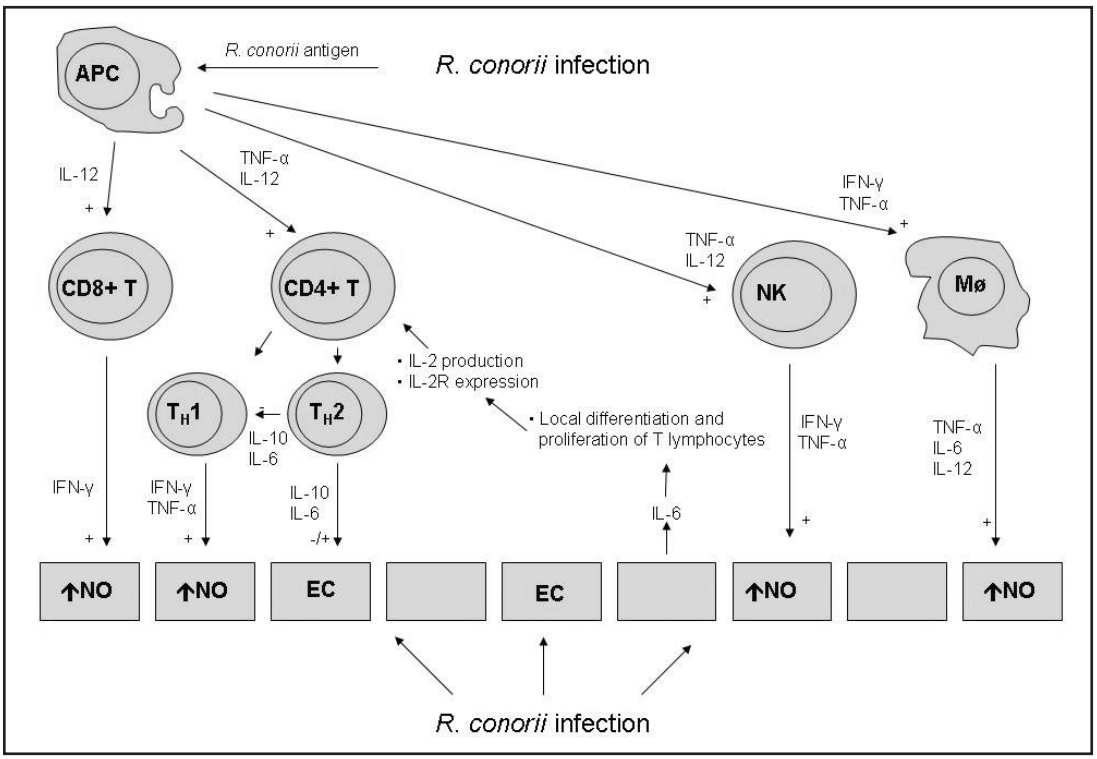

Fig. 5. *Immunologic alterations.*

effect of IFN-γ on macrophages is the induction of receptors for TNF-α, so that the binding of TNF-α on macrophages might further activate these cells by an autocrine TNF-α loop, thus increasing their anti-rickettsial activity, via the induction of NO synthesis and incretion (24). Moreover, the persistence of high serum levels of TNF-α, in recovered patients, could be a sign of residual local lesions. A relationship has also been observed between serum TNF-α and a high production of one the two soluble TNF receptors, sTNF-RI; persisting high values of sTNF-RI, in BF, could be indicator of disease activity (25).

Because IL-10 (a T_H2-type cytokine) suppresses the ability of IFN-γ-activated macrophages to produce inflammatory mediators, the persistence of high levels of IL-10 could be due to its ability to down-regulate the potential tissue-damaging effects, mediated by the increase in T_H1-type cytokines (26).

The profile of serum IL-6 (another T_H2-type cytokine) production, in patients with acute BF and after recovery, is similar to that of IL-10. In acute BF, IL-6 could acts as an anti-inflammatory cytokine, rather than as a growth and differentiation factor for B cell and immunoglobulin production (27).

Therefore, in BF patients, T_H1- and T_H2-type responses are not characteristically polarised, as both activating (i.e. TNF-α, IFN-γ) and suppressive (i.e. IL-10 and IL-6) cytokines are increased. In BF, as in any other inflammatory environment, counterbalancing mechanisms are normally produced to curtail the process, thus IL-10 and IL-6 could be capable of derailing T_H1-type responses and deactivating macrophages, thereby, moderating tissue injury (28).

Finally, in BF patients, IL-12, predominantly produced by monocytes, appears to act very early, favouring T_H1 differentiation and T_H1-type cytokine production, primarily of IFN-γ from T cells and NK, acting as a growth factor for activated T cells and NK, enhancing cytotoxic activity of NK, and contributing to the phagocyte cell activation and the innate resistance to intracellular pathogens (29).

ROLE OF CD8+ T CELLS

CD8+ T lymphocytes contribute to

protective immunity to rickettsiae by both Major Histocompatibility Complex (MHC)-I-restricted Cytotoxic T-lymphocyte (CTL) activity and production of IFN-γ. However, CTL activity may be more critical for the recovery from rickettsial infection than the effects of IFN-γ production (Fig. 5) (30).

Infected cells are eliminated by apoptosis associated with CTL activity. Although apoptotic loss of endothelium could be considered a pathologic event, it favours the host, more than the survival or the necrosis of an infected cell. Survival of infected ECs would permit further growth of the rickettsiae, while necrosis would release rickettsiae, allowing their spread to establish foci of infection in other cells. In contrast, rickettsiae-containing apoptotic bodies would be phagocytised rapidly and digested intracellularly (30).

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