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Increased Levels of Soluble CD40L in African Tick Bite Fever: Possible Involvement of TLRs in the Pathogenic Interaction between *Rickettsia africae*, Endothelial Cells, and Platelets

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The pathophysiological hallmark of spotted fever group rickettsioses comprises infection of endothelial cells with subsequent infiltration of inflammatory cells. Based on its ability to promote inflammation and endothelial cell activation, we investigated the role of CD40L in African tick bite fever (ATBF), caused by *Rickettsia africae*, using different experimental approaches. Several significant findings were revealed. 1) Patients with ATBF (n = 15) had increased serum levels of soluble CD40 ligand (sCD40L), which decreased during follow-up. 2) These enhanced sCD40L levels seem to reflect both direct and indirect (through endothelial cell activation involving CX3CL1-related mechanisms) effects of *R. africae* on platelets. 3) In combination with sCD40L, *R. africae* promoted a procoagulant state in endothelial cells by up-regulating tissue factor and down-regulating thrombomodulin expression. 4) Although the *R. africae*-mediated activation of platelets involved TLR2, the combined procoagulant effects of *R. africae* and sCD40L on endothelial cells involved TLR4. 5) Doxycycline counteracted the combined procoagulant effects of *R. africae* and sCD40L on endothelial cells. Our findings suggest an inflammatory interaction between platelets and endothelial cells in ATBF, involving TLR-related mechanisms. This interaction, which includes additive effects between sCD40L and *R. africae*, may contribute to endothelial inflammation and hypercoagulation in this disorder. The Journal of Immunology, 2006, 177: 2699–2706.

African tick bite fever (ATBF) is a flulike illness frequently accompanied by multiple inoculation eschars, regional lymphadenitis, cutaneous rash, and aphthous stomatitis. ATBF is caused by *Rickettsia africae*, a newly identified spotted fever group (SFG) rickettsia, and is transmitted by ungulate ticks of the *Amblyomma* complex in large parts of rural sub-Saharan Africa (1). Paralleling the rapid expansion of international safari tourism, the incidence of travel-associated ATBF has recently increased significantly in Europe and elsewhere (2).

The pathophysiological hallmark of SFG rickettsioses comprises infection of endothelial cells and subsequent perivascular infiltration of T cells and macrophages, resulting in vasculitis (3). Indeed, systemic inflammation and T cell activation have been reported during severe forms of SFG rickettsioses (4–6), and we recently have shown raised plasma levels of von Willebrand factor (vWF) accompanied by increased levels of inflammatory cytokines such as IL-6 in patients with ATBF, suggesting endothelial activation and systemic inflammation also in this form of rickettsioses (7).

CD40L, a transmembrane protein structurally related to TNF-α, was originally identified on CD4+ T cells, but has also been found on mast cells, basophils, eosinophils, and platelets (8). Indeed, platelets release large amounts of soluble CD40 ligand (sCD40L) upon activation, and much attention has been focused on the role of platelet-derived CD40L in the inflammatory loop between platelets and other cells (9, 10). Thus, both membrane-bound and soluble forms of CD40L interact with CD40, which is constitutively expressed on various cells (e.g., B cells, monocytes/macrophages, and endothelial cells), resulting in several inflammatory responses. In fact, the CD40L–CD40 dyad has been suggested to play a pathogenic role in a wide range of inflammatory diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and atherosclerosis (10–12).

Based on its ability to promote inflammation and endothelial cell activation, sCD40L could potentially be an interesting mediator in ATBF. To study the possible role of sCD40L in ATBF, we have examined serum levels of sCD40L in patients with travel-associated ATBF and in healthy controls who had returned from the same areas without any illness. In a separate set of in vitro experiments, we also examined the capacity of heat-inactivated *R. africae* to promote release of sCD40L from platelets and PBMC as well as the ability of sCD40L to enhance *R. africae*-mediated responses in endothelial cells.

Materials and Methods

Patients and controls

Fifteen consecutively recruited patients, 3 women and 12 men, 20–57 (mean 39) years old, diagnosed with travel-associated ATBF at an outpatient clinic (Aker University Hospital, Oslo, Norway), were included in the study (7). All patients presented with flulike symptoms that manifested within the first 10 days after they had left rural areas in South Africa or Botswana; 11 patients had inoculation eschars, 12 had regional lymphadenitis, 3 had vesicular cutaneous rash, and 5 had aphthous stomatitis. Antirickettsial therapy with doxycycline (200 mg once daily for 7 days) was...
instituted in 12 case subjects. Fourteen sex- and age-matched healthy travelers who had returned to Norway from sub-Saharan Africa during the preceding 7 days served as control subjects. Informed consent for participation in the study was obtained from all individuals. All parts of the study were approved by the local ethical committee and conducted according to the ethical guidelines from the Helsinki declaration.

Microbiological diagnosis
As previously reported (7), all patients fulfilled the serological criteria for **R. africae** infection: IgG plus IgM (microimmunofluorescence assay) in which titers of Abs to **R. africae** were ≥2 dilutions higher than those to *Rickettsia conorii*; a Western blot profile that revealed only **R. africae**-specific Abs; or cross-absorption studies that demonstrated that the homologous Abs were directed against **R. africae**.

Blood sampling protocol
Blood was collected from patients with ATBF, both at first presentation (1–7 (mean 4) days after symptom onset) and at follow-up (11–21 (mean 17) days after symptom onset). Peripheral venous blood was drawn into pyrogen-free, vacuum blood collection tubes without any additives, immediately immersed in melting ice, and allowed to clot before centrifugation at 3000 × g for 10 min. Serum was stored at −80°C until analysis, and samples were thawed less than three times.

Bacteria
**R. africae** (strain ESF-5) were grown in Vero cell monolayers in 150-cm² tissue culture flasks and cultured in MEM (Invitrogen Life Technologies) supplemented with 4% FCS and 2 mM l-glutamine. Heavily infected cells (5 days postinoculation) were harvested with sterile glass beads and pelleted by centrifugation at 10,000 × g for 10 min. Serum was stored at −80°C until analysis, and samples were thawed less than three times.

**sCD40L in ATBF**

**FIGURE 1.** sCD40L in **R. africae** infection. A, Shows the serum levels of sCD40L in 15 patients diagnosed with travel-associated ATBF at first presentation (1–7 (mean 4) days after symptom onset) and at follow-up (11–21 (mean 17) days after symptom onset). Antirickettsial therapy with doxycycline (200 mg once daily for 7 days) was instituted in 12 of the patients. Fourteen sex- and age-matched healthy travelers who had returned to Norway from sub-Saharan Africa during the preceding 7 days served as control subjects. Data are mean ± SEM. *p < 0.05 vs healthy controls; #, p < 0.05 at follow-up vs baseline. B, Shows the effect of **R. africae** (10⁻³) on the release of sCD40L from platelets in unstimulated and SFLLRN-stimulated PRP. C, Shows the effects of neutralizing Abs against TLR2, TLR4, and TLR9 on the combined effects of **R. africae** and SFLLRN on the release of sCD40L in PRP. D, Shows the effect of synthetic TLR2 ligand PamCys (1 µg/ml) alone and with neutralizing Abs against TLR2 on the release of sCD40L from platelets in unstimulated and SFLLRN-stimulated PRP. In all platelet experiments (B–D), PRP was cultured for 120 min and the concentration of SFLLRN was 10 µM, representing a suboptimal dose. All neutralizing Abs were given at a concentration of 10 µg/ml, and no Ab (-ab) and Ab of the same isotype and concentration (IgG2a) were included as controls. E, Shows the effect of **R. africae** on the release of sCD40L in PBMC after culturing for 20 h. Data are mean ± SEM of six experiments. *p < 0.05 and **p < 0.01 vs unstimulated cells; #, p < 0.05 vs SFLLRN alone (B and D) or the combination of SFLLRN and **R. africae** (C).
neutralizing Abs against TLR2, TLR4, and TLR9 (D-18; Santa Cruz Biotechnology) or the isotype control Abs (all at a concentration of 10 μg/ml) were added to PRP 20 min before SFLLRN stimulation. In some experiments, PRP was also incubated with different concentrations of doxycycline (Sigma-Aldrich) and Pam3Cys (EMC; final concentration 1 μg/ml). The changes in sCD40L levels (ng/10³ platelets) were expressed as the concentration in platelet-free plasma (centrifugation of PRP for 10 min at 11,000 × g) at the end of the experiments minus the concentration at baseline. In the coculture experiments, HUVEC were stimulated with R. africae for 20 h and then washed twice with medium, before adding unstimulated and SFLLRN-stimulated PRP for 120 min. In a separate set of experiments, neutralizing Abs against CX3CL1 and CXCL12 or the isotype control Abs (all polyclonal goat IgG; R&D Systems) at a final concentration of 10 μg/ml were added to the HUVEC culture before coculturing with PRP.

Real-time quantitative RT-PCR

Total RNA was extracted from HUVEC using RNasea columns (Qiagen), subjected to DNase I treatment (RQI DNase; Promega), and stored in RNA storage solution (Ambion) at −80°C. Primers were designed using the Primer Express software, version 2.0 (Applied Biosystems), for tissue factor (TF); forward primer (FP), 5'-GGGCTTGCGACTACAAT-3' and reverse primer (RP), 5'-TTGCTTTTCAATCTCCTG-3'; plasminogen activator inhibitor type 1 (PAI-1); FP, 5'-AGGCTGACTTCACTGGCTGAT-3'; and thrombomodulin (TM); FP, 5'-CCCCACACCCAGCTAGCT-3' and RP, 5'-CGTCTAGTCCGCTGCAGT-3'. Quantification of mRNA was performed using the ABI Prism 7000 (Applied Biosystems) (15). Gene expression of the housekeeping gene β-actin (Applied Biosystems) was used for normalization.

Enzyme immunoassays (EIAs)

IL-6 and sCD40L were measured by EIAs obtained from R&D Systems and Bender MedSystems, respectively. TF, TM, and PAI-1 were determined by EIAs from American Diagnostica. Before analyzing cell-bound TF, the cells were completely lysed by 1% Triton X-100. F₁₋₂ were analyzed by EIA provided from Dade Behring. vWF was determined by EIA, as previously described (16). The intra- and interassay coefficients of variations were <10% for all EIAs.

Statistical analyses

Differences between groups were compared with the Mann-Whitney U rank sum test for unpaired data. Within group differences were analyzed with Wilcoxon rank sum test for paired data. The level of significance was set at p < 0.05.

Results

Serum levels of sCD40L in ATBF patients

As can be seen in Fig. 1A, the patient group had significantly raised levels of sCD40L compared with healthy controls when analyzing serum levels in the acute phase before starting doxycycline therapy. During follow-up, when the symptoms had resolved, we found a significant decrease in sCD40L levels (Fig. 1A). Although we have no data on R. africae antigenemia, these findings suggest an association between raised serum levels of sCD40L and R. africae-mediated disease.

Release of sCD40L from R. africae-stimulated platelets

Because platelets seem to be the major source of sCD40L in circulation (10, 17), we next examined the effect of R. africae on the release of sCD40L in SFLLRN (10 μM)-activated PRP after culturing for 120 min (Fig. 1B). Platelets have recently been reported to express TLR2, TLR4, and TLR9 (18–20), and notably, specific neutralizing Abs against TLR2 (21), but not specific neutralizing Abs against TLR4 (22), TLR9 (23), and isotype-matched control Abs, totally abolished the R. africae-mediated enhancement of sCD40L release in PRP (Fig. 1C). Moreover, the synthetic TLR2 ligand Pam3Cys also significantly enhanced the release of sCD40L in unstimulated and SFLLRN (10 μM)-activated PRP, with totally abolishing the effect of the neutralizing Abs against TLR2, further supporting that TLR2 activation can directly induce release of sCD40L from platelets (Fig. 1D).

Release of sCD40L from R. africae-stimulated PBMC

Another possible source for sCD40L in ATBF patients could be mononuclear blood cells (11, 12), and as seen in Fig. 1E, R. africae dose-dependently increased the release of sCD40L in PBMC supernatants. However, compared with the release from activated platelets, the concentrations of sCD40L in PBMC supernatants in general were rather low.

Modulatory role of sCD40L on R. africae-mediated inflammatory responses in endothelial cells

Endothelial cell activation is a hallmark of several SFG rickettsioses (7, 24–26). To elucidate potential pathogenic consequences of the raised sCD40L in AFTB, we examined the effect of R. africae, alone or in combination with rsCD40L, on the release of IL-6 and vWF from HUVEC, both mediators known to be elevated during AFTB (7). As shown in Fig. 2, heat-inactivated R. africae significantly increased the release of IL-6, but not of vWF, in HUVEC. Moreover, this stimulatory effect was markedly enhanced when rsCD40L was used as costimulus, showing additive effects not only on IL-6, but also on vWF (Fig. 2), suggesting that R. africae and sCD40L may act in concert in the inflammatory responses of ATBF in endothelial cells.

Modulatory role of sCD40L on R. africae-mediated effects on pro- and anticoagulant proteins in endothelial cells

Activation of the coagulation cascade has been recognized as an important pathogenic feature in several infectious diseases, including SFG rickettsioses (24–28). To further examine any possible
pathogenic role of sCD40L in ATBF, we examined the ability of rsCD40L to modulate procoagulant (i.e., TF and PAI-1) and anticoagulant (i.e., TM) responses of *R. africae* in HUVEC. As shown in Fig. 3, A–D, *R. africae* markedly enhanced the gene and protein expression of TF and PAI-1 in HUVEC. In contrast, rsCD40L had no effect on these procoagulant proteins either alone or in combination with *R. africae* (Fig. 3, A–D). However, while neither *R. africae* nor rsCD40L had any effect on TM expression when given alone, the combination of these stimuli markedly suppressed the expression of this anticoagulant mediator as shown both at the gene and protein level (Fig. 3, E and F). These findings, showing a synergic down-modulatory effect of *R. africae* and rsCD40L on TM expression, suggest an enhanced prothrombotic potential in endothelial cells when these stimuli are acting together, as during ATBF. Indeed, when heparinized human plasma (29) was added to the HUVEC cultures, *R. africae* alone, and particularly in combination
with rsCD40L, significantly enhances thrombin formation, as assessed by increased levels of the prothrombin fragments $F_1 + 2$ in plasma (Fig. 3G). This finding underscores the pathogenic significance of the *R. africae*-induced modulation of TF, PAI-1, and TM expression in endothelial cells.

*R. africae* promotes an endothelial cell-mediated increase in the release of sCD40L from platelets

Platelets may interact with endothelial cells, resulting in an inflammatory loop (30), and we next examined whether *R. africae* could modulate this interaction. When coincubating PRP for 120 min with HUVEC that had been preactivated by *R. africae* for 20 h, we found significantly increased release of sCD40L as compared with coincubating with unstimulated endothelial cells (Fig. 4A). Particularly enhancing effects of *R. africae*-stimulated HUVEC on sCD40L release were seen when HUVEC were coincubated with SFLLRN-activated PRP, using a suboptimal dosage of the PAR-1 agonist (i.e., 10 mM) (Fig. 4B). *R. africae*-stimulated HUVEC could theoretically directly contribute to the increased release of sCD40L in the coculture experiment, but when HUVEC were exposed to *R. africae*, we found no detectable protein levels of sCD40L after culturing for 6 and 24 h, as assessed by EIA (data not shown). These findings suggest that *R. africae* could promote increased release of sCD40L from platelets, not only directly (Fig. 1B), but also indirectly through its ability to promote endothelial cell-mediated platelet activation.

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** *R. africae* promotes endothelial cell-mediated activation of platelets. The release of sCD40L in unstimulated (A) and SFLLRN-stimulated (10 mM, suboptimal dose) (B) PRP, which was coincubated for 120 min with washed HUVEC that had been cultured for 20 h with and without *R. africae* (10^−1) before coculturing with PRP. In some experiments, neutralizing Abs against CX3CL1 and CXCL12 were added to *R. africae*-activated HUVEC before coculturing with PRP. No Ab (-ab) and Ab of the same type and concentration (goat IgG) were included as controls. sCD40L levels were measured with ELISAs in cell and platelet-free supernatants. Data are mean ± SEM of six experiments. *, $p < 0.05$ and **, $p < 0.01$ vs unstimulated HUVEC. #, $p < 0.05$ vs no Ab.

R. *africae*-activated endothelial cells enhance platelet activation through CX3CL1-related mechanisms

We next examined the possible nature of the signals, provided by *R. africae*-pretreated HUVEC, that could subsequently induce enhanced release of sCD40L from PRP. Based on the followings, we hypothesized that the chemokines CX3CL1 and CXCL12 could be involved in this process: 1) Both chemokines are released in large amount from activated endothelial cells (31). 2) The receptors for both these chemokines are expressed on the platelet surface (i.e., CX3CR1 and CXCR4, respectively) (32, 33). 3) As for CX3CL1, there are some reports on the activation of this chemokine in endothelial cells during rickettsia infection (34). As shown in Fig. 4, when Abs against CX3CL1, but not against CXCL12, were added to the *R. africae*-activated HUVEC culture before coculturing with PRP, there was a marked decrease in the release of sCD40L in the coculture experiment in both unstimulated and SFLLRN-stimulated PRP. Thus, although several endothelial-derived mediators may be involved, these findings suggest that HUVEC-derived CX3CL1 is involved in the endothelial-mediated activation of PRP when HUVEC is pretreated with *R. africae*.

**Involvement of TLRs and NF-κB in *R. africae*-mediated effects in endothelial cells**

We next examined the involvement of TLR2 and TLR4 in the *R. africae*-mediated effects on endothelial cells. In contrast to the effects on platelets, blocking Abs against TLR4, but not against TLR2, nearly abolished the enhancing effect of *R. africae* on TF expression as well as the suppressive effect of *R. africae* on TM expression when costimulated with rsCD40L (Fig. 5, A and B). In parallel experiments, we found that LPS from *E. coli*, but not the TLR2 ligand Pam3Cys, markedly enhance TF expression and suppress TM expression in HUVEC, and this effect was totally abolished by the specific Abs against TLR4 (data not shown). These findings further suggest that in contrast to the *R. africae*-mediated effect on sCD40L release from platelets, the *R. africae*-mediated effects on TF and TM expression in HUVEC involve TLR4, but not TLR2. Moreover, pyrroloidine dithiocarbamate (75 μM), an antioxidant inhibitor of NF-κB, also markedly attenuated the effects of *R. africae* in combination with rsCD40L on TF and TM expression (Fig. 5, C and D).

**The effect of doxycycline on *R. africae*-mediated effects in endothelial cells**

Doxycycline is regarded as the primary choice in the treatment of *R. africae* and other SFG rickettsioses (1). In addition to its antibacterial activity, this drug has also been shown to directly modulate endothelial cell function (35). We therefore examined the ability of doxycycline to modulate the prothrombotic effects of *R. africae* in endothelial cells. Although this medication (50 μM) had no effects on cell-bound TF or TM, it nearly abolished the effects of *R. africae* in combination with rsCD40L on the release of TF and TM into HUVEC supernatants (Fig. 6, A and B).

**The effects of doxycycline on sCD40L release**

The ability of doxycycline to modulate the inflammatory response to *R. africae* could suggest that the decrease in serum levels of sCD40L in patients with ATBF during follow-up is a direct effect of doxycycline therapy (Fig. 1A). However, such a decrease was also seen in those three patients who did not receive this medication (data not shown). Moreover, although we found that doxycycline could modulate the effect of *R. africae* on the expression of TM and TF in HUVEC, this medication (5–500 μM) had no effect on the release of sCD40L in unstimulated or SFLLRN-activated (10 μM) platelets in healthy controls ($n = 5$) (Fig. 6C), suggesting...
that the decline in serum levels of sCD40L during follow-up is not merely an effect of this medication.

**Discussion**

Platelet activation is often reported during acute and chronic infections (36), and there are also a few reports of enhanced platelet activation during SFG rickettsioses (28, 37). In the present study, we showed raised serum levels of sCD40L during ATBF, with a subsequent decrease during follow-up, suggesting an association between raised sCD40L levels and R. africae-mediated disease. Our in vitro experiments suggest that this finding could reflect both direct effects of R. africae on platelets as well as indirect effects through its ability to promote an inflammatory interaction between endothelial cells and platelets, involving CX3CL1-related mechanisms. This inflammatory loop could contribute to vascular inflammation, acting as a vicious cycle in various forms of SFG rickettsioses (24–26, 28).

Recently, much attention has been focused on the role of platelet-induced CD40L in the inflammatory interaction between platelets and other cells (9, 10). In the present study, we found raised serum levels of sCD40L in patients with ATBF. Even more importantly, we show that R. africae and sCD40L may act in concert in the inflammatory responses of ATBF in endothelial cells, resulting in enhanced release of IL-6 and vWF, mediators that we previously have reported to be increased in this group of patients (7). One might argue that the concentration of sCD40L used in the in vitro experiments was higher than those that were found in serum during ATBF. However, within the endothelial microenvironment, involving interactions between endothelial cells, activated platelets, and leukocytes, the sCD40L levels may be much higher, potentially resulting in relevant inflammatory responses. Moreover, while the biological effects of sCD40L as compared with the membrane-bound form of this ligand have been debated (10, 17), our findings nevertheless illustrate the inflammatory potential of CD40 engagement during ATBF.

In addition to vasculitis, thrombus formation related to endothelial cell activation is an important clinical manifestation of SFG rickettsioses (3, 24). Although the mechanisms resulting in this elevated coagulability have not been fully clarified, there are several reports, including both in vivo (e.g., plasma from patient groups) and in vitro studies (e.g., HUVEC exposed to R. conorii and R. rickettsii), that show enhanced expression of PAI-1 and TF in SFG rickettsioses (24, 38–40). Such a procoagulant potential was also shown in the present study analyzing the effect of R. africae on HUVEC. In contrast to the reports on PAI-1 and TF, the reports of TM expression during rickettsioses are somewhat conflicting, showing both enhanced and decreased levels (24, 25). In this study, we found no effect of either R. africae or sCD40L on TM expression in HUVEC. However, when these stimuli were combined, as may reflect the in vivo situation in patients with ATBF, there was a significant down-regulation of this endothelial anticoagulant cofactor. The prothrombotic effect of R. africae-stimulated endothelial cells was further supported by their ability to enhance thrombin formation in plasma, as assessed by increased levels of the prothrombin fragments F1 + 2, particularly when combined with rsCD40L. If such mechanisms also are operating within the infected endothelium in vivo, this interaction could further contribute to a procoagulant state in patients suffering from ATBF and other rickettsioses. Moreover, this decrease in TM levels could not only promote thrombus formation. In fact, recent studies suggest that TM may exert potent anti-inflammatory properties involving protein C-dependent and independent mechanisms (41). This synergistic down-regulation of TM during exposure to R. africae and sCD40L could therefore also contribute to endothelial inflammation, resulting in further enhancement of platelet activation and release of sCD40L, representing a vicious circle in the pathogenesis of ATBF and possibly also other rickettsioses.

TLRs are probably the most important class of pattern-recognition receptors. Recognition of pathogen-associated molecular patterns by TLRs, either alone or in heterodimerization with other TLR or non-TLR receptors, induces the production of signals that are responsible for the activation of genes important for an effective host defense, especially those of inflammatory cytokines (42). In this study, we show that the R. africae-mediated induction of a procoagulant state in endothelial cells, showing up-regulation of TF and down-regulation of TM, involves TLR4, but not TLR2 activation. TLRs have also been detected recently on platelets (18–20), and somewhat surprisingly, an opposite pattern for TLR neutralization was found for the R. africae-mediated effects of platelet activation. Thus, while TLR4 previously has been suggested to
play a role in modulation of LPS-induced thrombocytopenia (19, 20), we found that anti-TLR2, but not anti-TLR4, totally abolished the enhancing effects of \textit{R. africae} on the release of sCD40L in SFLRN-activated platelets. Although it is unknown whether TLRs on platelets are derived from intracellular stores or whether they arise from adsorption of free TLRs in plasma or carryover expression from megacytocytes (18, 43), our findings clearly suggest that TLR2 is biologically active in SFLRN-activated platelets. We have at present no conclusive data on what \textit{R. africae}-related ligand(s) is interacting with TLR2 on platelets, but we have recently completed the genome sequence of \textit{R. africae} (D. Raoult, unpublished observation), and interestingly, it includes genes for peptidoglycans, previously described as potent TLR2 ligands (44). Moreover, although LPS has been described as a classic TLR4 ligand, there is some evidence suggesting that LPS from selected Gram-negative bacteria also could represent TLR2 ligands (45). Nevertheless, our findings indicate a link between TLR activation and thrombus formation that includes both platelet activation and the promotion of a procoagulant state in endothelial cells, involving CD40L-related mechanisms.

Doxycycline is regarded as the first choice of therapy in the management of SFG rickettsioses, and in addition to its antirickettsial properties, this drug has been shown to possess anti-inflammatory and endothelial cell-modulating properties (35, 46). Although doxycycline had no effect on the release of sCD40L from activated platelets, we found that this medication significantly counteracted the combined procoagulant effects of \textit{R. africae} and rsCD40L on TF and TM in HUVEC. Previously, doxycycline has been shown to inhibit matrix metalloproteinase activity and to decrease NO production, possibly involving MAPK-related pathways (35, 46). In the present study, we found that doxycycline modulated the release of TF and TM, but not the cell-bound concentration of these mediators, suggesting effects on release mechanisms. Although the exact mechanisms remained to be defined, these findings suggest that this medication also possesses anticoagulant properties. However, whether such effects also are operating in vivo, under doxycycline concentrations that are obtained in patients treated for SFG rickettsioses, is far from clear.

In the present study, we show raised serum levels of sCD40L in patients with ATBF, most probably reflecting an enhanced inflammatory interaction between platelets and endothelial cells, involving TLR-related mechanisms. This interaction, which includes additive or even synergistic effects between sCD40L and \textit{R. africae}, may contribute to endothelial cell inflammation and hypercoagulation in this disorder.

Disclosures
The authors have no financial conflict of interest.

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