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Rickettsia conorii is a potent complement activator *in vivo* and combined inhibition of complement and CD14 is required for attenuation of the cytokine response *ex vivo*

Kari Otterdal[1], Aránzazu Portillo[2], Elisabeth Astrup[1,3], Judith K. Ludviksen[4], Camilla Schjalm[3,5], Didier Raoult[6], Juan P Olano[7], Bente Halvorsen[1,3,8], José A. Oteo[2], Pål Aukrust[1,3,8,9], Tom E. Mollnes[3,4,5,8,10,11], Per H. Nilsson[5,8*]

[1]Research Institute of Internal Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway

[2]Center of Rickettsioses and Arthropod-Borne Diseases, Department of Infectious Diseases, Hospital San Pedro-Center of Biomedical Research from La Rioja (CIBIR), Logroño, Spain

[3]Faculty of Medicine, University of Oslo, Oslo, Norway

[4]Research Laboratory, Nordland Hospital, Bodø, Norway

[5]Department of Immunology, Oslo University Hospital Rikshospitalet, Oslo, Norway

[6]Unité des Rickettsies, Université de la Mediterranée, Marseille, France

[7]Department of Pathology, University of Texas Medical Branch, Galveston, TX, USA

[8]K.G. Jebsen Inflammatory Research Center, University of Oslo, Oslo, Norway

[9]Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital Rikshospitalet, Oslo, Norway

[10]Faculty of Health Sciences, K.G Jebsen TREC, University of Tromsø, Tromsø, Norway

[11]Centre of Molecular Inflammation Research, Norwegian University of Science and Technology, Trondheim, Norway

* Corresponding author

E-mail: per.nilsson@rr-research.no

Address: Department of Immunology, Oslo University Hospital Rikshospitalet,

Songsvannsveien 20, 0372 Oslo, Norway

Telephone number: +47 23073066

Fax number: +47 23073510

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Abstract

Mediterranean spotted fever caused by ~~*R.*~~*Rickettsia conorii* is a potentially lethal disease characterized by vascular inflammation affecting multiple organs. Studies of *R. conorii* so far have focused on activation of inflammatory cells and their release of inflammatory cytokines, but complement activation has not been investigated in *R. conorii*-infected patients. Here, we did a comprehensive analysis of complement activation markers and the soluble cross-talking co-receptor CD14 (sCD14) in plasma from *R. conorii*-infected patients. The clinical data was ~~supplement~~supplemented with *ex vivo* experiments where the cytokine response was characterized in human whole blood stimulated with *R. conorii*. Complement activation markers at the level of C3 (C3bc, C3bBbP) and terminal pathway activation (sC5b-9), as well as sCD14, were markedly elevated ($p<0.01$ for all), and closely correlated ~~-~~($p<0.05$ for all), in patients at admission as compared to healthy matched controls. All tested markers were significantly reduced to baseline values at time of follow-up. *R. conorii* incubated in human whole blood was shown to trigger complement activation accompanied with release of the inflammatory cytokines IL-1β, IL-6, IL-8 and TNF. Whereas inhibition of either C3 or CD14 had only minor effect of released cytokines, combined inhibition of C3 and CD14 resulted in significant reduction, virtually to baseline levels, of the four cytokines ($p<0.05$ for all). Our data show that complement is markedly activated upon *R. conorii* infection and complement activation is together with CD14 responsible for a major part of the cytokine response induced by *R. conorii* in human whole blood.

Introduction

The clinical spectrum of spotted fever group (SFG) rickettsioses varies in severity from mild to potentially lethal disease with systemic multi-organ involvement such as in some cases of Mediterranean spotted fever (MSF) caused by *R. conorii.* The pathophysiological hallmark of SFG rickettsioses comprises infection of endothelial cells and subsequent perivascular infiltration of T cells and monocytes with vascular inflammation and increased microvascular permeability and in some cases, edema in vital organs (*e.g.* lung and brain) as consequences ~~(~~[1, 2~~)~~]. Although several inflammatory mediators have been suggested to play a role in the pathogenesis of SFG, the characterization of the different actors is still not fulfilled.

The complement system consists of more than 50 membrane-bound and soluble proteins, comprising factors for activation, regulation and effector functions ~~(3).~~[3]. Activation can be initiated via three different pathways, typically triggered by antigen-antibody complexes by the classical pathway (CP), certain carbohydrate patterns by the lectin pathway (LP) and principally all surfaces lacking proper regulatory molecules by the alternative pathway (AP), thereby sensing a broad range of structures. An initiated response is in general multiplied via a potent amplification loop within the AP, responsible for a major part of the antimicrobial effects (*i.e.* generation of anaphylatoxins, opsonisation and complement-mediated lysis) ~~(4).~~[4]. A potent complement response contributes to elimination of invading microbes, but an excessive and inappropriate response can instead lead to complement driven tissue damage and organ dysfunction as sometimes seen in septicaemia ~~(~~[5, 6~~)~~].

CD14 is a pattern recognition receptor which acts as a co-receptor in conjunction with multiple of the Toll-like receptors (TLRs), *e.g.* the lipopolysaccharide (LPS)-sensing TLR4 ~~(~~[7, 8~~)~~]. CD14 is primarily expressed at the surface of monocytes and macrophages, whereas a soluble form (sCD14) is present in plasma and serum ~~(~~[9~~)~~]~~.~~. Both membrane bound and

sCD14 are functionally active [10]. Several inflammatory stimuli can induce elevation of sCD14 by promoting increased shedding of membrane CD14 and activate release of intracellularly stored CD14 from monocytes ~~(10),~~[11], and measuring sCD14 levels has a prognostic value in *e.g.* septicaemia ~~(11)~~[12].

Although complement activation and CD14 play an important role in the pathogenesis of ~~gram~~Gram negative bacterial infection such as *E. coli* sepsis ~~(12, [13),~~ 14], to the best of our knowledge, no studies have ever investigated these two mediator systems in the context of clinical *R. conorii* infection. While some experimental studies suggest that *R. conorii* is resistant to complement-mediated killing ~~(14, [15),~~ 16], there are no studies on the degree of complement activation in clinical *R. conorii.* Moreover, the literature is devoid of data on the regulation of CD14 in human *R. conorii* infection.

We hypothesized that complement and CD14 could play an important role in mediating the inflammatory response in human *R. conorii* infection. This hypothesis was explored by (*i*) measuring complement activation markers and sCD14 in patients with *R. conorii* infection and (*ii*), using an *ex vivo* human whole blood model to characterize the cytokine profile upon stimulation with *R. conorii*, with and without targeting complement C3 and CD14 individually or in combination.

Methods

Patients and controls

Blood samples from 36 patients (27 men and 9 women, 20-84 [mean 56.6] years of age) with confirmed MSF (all with characteristic signs of active MSF), admitted to the Infectious Diseases Department of the Hospital San Pedro, Logroño, Spain from 2004 to 2013 were prospectively collected for the study of pathogenic mechanisms in *R. conorii* infection. The duration of illness before diagnosis was less than one week. All patients were treated with oral doxycycline 100 mg/bid for 5-7 days. All patients had seroconversion with increases in the levels of anti-*R. conorii* antibodies as assessed by indirect immunofluorescence assay (Focus Diagnostics, Cypress, CA), six ~~patients~~of them also showed positive results for *Rickettsia* using PCR~~, and *R. conorii* was isolated in blood from two patients~~. Nine healthy subjects (5 women and 4 men, aged 35-58 years) recruited from the same area of Spain were included in the study as controls.

Patient blood sampling protocol

Blood was collected from all patients on admission (less than one week after the onset of the symptoms and before the specific treatment), and from four patients at follow-up (28-42 days). To avoid post-sampling complement activation, plasma samples were prepared according to the strict procedures for preparation of plasma for measurement of complement activation markers [17]. Peripheral venous blood was drawn into pyrogen-free vacuum blood collection tubes with EDTA and collection tubes without any additives (serum tubes). The EDTA vacutainer tubes were turned gently and immediately centrifuged at 4°C, 2000*g* for 20 minutes to obtain platelet-poor plasma. Blood in serum tube was allowed to clot for 60 minutes before centrifugation at 2000*g* for 10 minutes. EDTA-plasma and serum was stored at -80°C immediately after preparation and until analysis.

Preparation of R. conorii

Preparation of heat-inactivated *R. conorii* is previously described ~~(16, 17)~~[18, 19]. Briefly, *R. conorii* (Malish strain) were grown in Vero cell monolayers. Heavily infected cells (5 days post-inoculation) were disrupted and harvested using sterile glass beads, thereafter pelleted by centrifugation at 10,000*g* for 15 minutes. Cell-free rickettsiae were collected on a sucrose gradient and resuspended in sterile water to a batch containing $1x10^8$ rickettsiae/mL as determined optically after Gimenez staining ~~(17).~~[19]. *R. conorii* was heat-inactivated at 60°C for 30 minutes.

Inhibitors and controls

For complement inhibition, compstatin (Cp40 analogue (*d*Tyr-Ile-[Cys-Val-Trp(Me)-Gln-Asp-Trp-Sar-His-Arg-Cys]-mIle-NH2) was used to specifically target C3 ~~(18). Linear compstatin was used as control for the cyclic Cp40. Cp40 and linear compstatin was a kind gift from John D. Lambris, University of Pennsylvania. CD14 was blocked using a mouse anti-human CD14 antibody (clone 18D11) and a mouse IgG1 isotype antibody (~~[20]. Linear compstatin was used as control for the cyclic Cp40. Cp40 and linear compstatin was a kind gift from John D. Lambris, University of Pennsylvania. Membrane bound and soluble CD14 were blocked using a mouse anti-human CD14 $F(ab')_2$ (clone 18D11) and an irrelevant mouse $F(ab')_2$ (from mouse IgG1, clone BH1) was used as control (both from Diatec AS, Oslo, Norway).

Ex vivo whole blood incubations

Whole blood incubations were performed using the lepirudin based human whole blood model described in detail previously ~~(19).~~[21]. Whole blood was obtained from nonimmune

healthy adult volunteers who had received no medication for at least 10 days. Blood was drawn from an 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. The inflammatory response in whole blood was studied by incubating *R. conorii* ($1x10^5$ bacteria/mL) in 1.8 mL round-bottom sterile polypropylene NUNC cryotubes (Nalgene NUNC) on rotation for two or four hours at 37°C. Following incubation, EDTA was added to a final concentration of 20 mM and the blood was centrifuged to platelet-poor plasma ~~(3000*g* for 20 minutes at 4°C) which~~at 3000*g* for 20 minutes at 4°C. The centrifugation protocol is slightly different from the patient plasma preparation (2000*g* vs. 3000*g*), however, both protocols are valid for plasma preparation and the *in vivo* and *ex vivo* data sets are independent from one another. Following centrifugation, plasma was immediately isolated and stored at -80°C until further analysis. In separate sets of experiments, whole blood with *R. conorii* was supplemented with Cp40 (20μM), anti-CD14 (20 μg/mL) or a combination thereof. The inhibitors were pre-incubated in whole blood for 5 minutes prior to the addition of bacteria.

Assays for complement activation markers and sCD14

The complement activation products ~~C1rs-C1INH, C4bc,~~ C3bc, C3bBbP~~ and~~, the soluble terminal complement complex (sC5b-9) and C1rs-C1INH were analysed in EDTA-plasma samples using enzyme-linked immunosorbent assays (ELISAs) as described in detail previously ~~(20).~~[22]. Briefly, the assays were either based on monoclonal antibodies detecting neo-epitopes exposed after activation (~~C4bc,~~ C3bc, sC5b-9) or complex formation (C1rs-C1INH) or pair of antibodies detecting complex formed between single components upon activation (C3bBbP), thus specifically measuring only components exposed or formed upon

activation. The amount of activation products present is related to an international standard defined to contain 1000 complement arbitrary units (CAU)/mL ~~(20).~~[22]. sCD14 was analysed in serum samples using the HK320 human sCD14 kit (Hycult, Uden, the Netherlands).

Cytokine assays

Plasma samples from whole blood *ex vivo* incubated with *R. conorii* were analysed using a 27-Plex Panel multiplex cytokine assay comprising interleukin (IL)-1β, IL-1 receptor antagonist (IL1-ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, interferon (IFN)-γ and tumor necrosis factor (TNF) as well as the chemokines IL-8/CXCL8, eotaxin1/CCL11, IFN-γ inducing protein 10 (IP-10/CXCL10), monocyte chemotactic protein-1 (MCP-1/CCL2), and macrophage inflammatory proteins 1α (MIP-1α/CCL3) and MIP-1β/CCL4 by a multiplex cytokine assay (Bio-Plex Human Cytokine 27-Plex Panel; Bio-Rad Laboratories Inc., Hercules, CA, USA). After the initial screening using the 27-plex panel, a 4-plex subpanel containing IL-1β, IL-6, IL-8 and TNF (Bio-Rad Laboratories) was employed to analyse plasma samples from whole blood *ex vivo* incubations with *R. conorii* supplemented with inhibitors.

Ethics statement

The study was designed and performed according to the ethical guidelines from the declaration of Helsinki. Informed written consent for participation in the study was obtained from all individuals. The study was approved by the regional ethical committee of South-Eastern Norway Regional Health Authority.

Statistical analyses

All data was compiled using Prism version 6.0 (GraphPad Software, La Jolla CA). For the patient data, differences between patients at admission were compared against controls and patients at follow-up with the Kruskal-Wallis test ~~(C3bc, C3bBbP, sC5b-9, sCD14) or between admission and follow-up using the Mann-Whitney test (C1rs-C1INH, C4bc) when lacking reliable control data.~~. Correlations between activation parameters were analysed using Spearman's rank order correlation. Data from the *ex vivo* experiments were initially tested for statistical difference between whole blood with *R. conorii* and the buffer controls using paired *t*-test (consistent differences: sC5b-9, IL-8; consistent ratios: IL-1β, IL-6, TNF). If significantly separated ($p<0.05$~~). The~~), the *R. conorii*-samples were further compared to *R. conorii*-whole blood supplemented with inhibitors using repeated measures one-way ANOVA followed by Dunnett´s multiple comparisons test.

Results

In vivo complement activation during R. conorii infection

Plasma samples from 36 patients with confirmed MSF were analysed for ~~five~~four different complement activation markers reflecting activation at different stages of the complement system. On admission, three of these markers were significantly increased as compared to the matched healthy controls (n=9) (Fig. 1). Thus, C3bc ($p<0.001$), a marker of C3-activation, and the alternative pathway C3-convertase (C3bBbP) ($p<0.001$), were both significantly increased on admission, and dropped to normal levels at follow-up. Importantly, the terminal complement complex (sC5b-9) was also significantly increased at admission ($p<0.0001$) and significantly lowered at follow-up indicating that the complement cascade was activated to the very end, including release of C5a, in patients with MSF. All three markers showed strong internal correlation, C3bc correlated to C3bBbP ($r=0.84$, $p<0.0001$) and to sC5b-9 ($r=0.67$, $p<0.0001$) and C3bBbP correlated to sC5b-9 ($r=0.71$, $p<0.0001$). ~~We have established reference values for these assays (20),~~We have established reference values for these assays [22], and all the healthy controls and patients at follow-up presented levels within the range of the reference values for respective assays.

~~Activation markers C1rs-C1INH and C4bc, representing activation of the classical pathways and C4bc in addition reflecting activation of the lectin pathway, were both markedly elevated on admission as compared to reference values for each assay, and were significantly lowered at follow-up (C1rs-C1INH: $p<0.01$, C4bc: $p<0.05$) (Fig. 2). For these two assays, the samples from the healthy control population were above the stated reference values, and therefore not included in the analyses. Positive significant correlation was found between C1rs-C1INH and C4bc ($r=0.54$, $p<0.01$) and for C4bc against C3bc ($r=0.42$, $p<0.05$) but not against other complement activation markers.~~

We then examined whether the classical pathway could be the initial trigger of the complement activation observed. For this purpose we use the C1rs-C1INH assay. Patients at admission had markedly elevated C1rs-C1INH complexes than at follow-up ($p<0.05$) (Fig. 1D). The control population also had higher levels of C1rs-C1INH complexes than the reference range, and these did not differ from the values seen in patients at admittance.

In vivo sCD14 during R. conorii infection

At admission serum levels of sCD14 in patients with confirmed *R. conorii* infection were significantly raised as compared to the levels in matched healthy controls ($p<0.01$), and at time of follow-up, sCD14 had returned to normal (Fig. ~~3~~2). Levels of sCD14 showed significant positive correlation to ~~four~~all of the complement activation markers: C1rs-C1INH ($r=0.59$, $p<0.01$), C3bc ($r=0.42$, $p<0.05$), C3bBbP ($r=0.39$, $p<0.05$) and sC5b-9 ($r=0.61$, $p<0.001$~~), but not against C4bc ($r=0.24$, $p=0.21$).~~).

Ex vivo whole blood complement activation and cytokine release in response to R. conorii

R. conorii was incubated in human whole blood *ex vivo*, after which complement activation was measured as generation of sC5b-9 after two hours, and the levels of 27 different cytokines were analysed after 4 hours by multiplex technology. *R. conorii* induced significant complement activation ($p<0.01$) as compared to the buffer control (Fig ~~4A~~3A). When analysing a broad array of cytokines, nine out of 27 were found increased 2-fold or more in *R. conorii* as compared to the buffer control (Fig. ~~4B~~3B). The overall cytokine profile was strongly balanced towards an inflammatory state, exhibiting ~~TNF~~IL-1β, IL-6~~ and,~~ IL-8 and ~~IL-1β as~~TNF among the ~~top four~~markedly induced analytes~~ measured as highest concentrations~~ upon stimulation with *R. conorii*.

Modulation of R. conorii-induced inflammation ex vivo by targeting C3 and CD14

R. conorii-induced inflammation *ex vivo*, evaluated as release of ~~the~~ four of the most potent cytokine responders, *i.e.*, IL-1β, IL-6, IL-8 and TNF (cfr. Fig ~~4B~~3B), was then targeted by applying inhibitors directed against the central complement component C3, and the TLR-co-receptor CD14. As in the first experiment, *R. conorii* induced significant increase in IL-1β, IL-6, IL-8 and TNF (all $p<0.001$) compared to the buffer control (Fig. ~~5~~4). Targeting of C3 alone significantly lowered the level of IL-8 only ($p<0.05$), whereas CD14 inhibition alone did not inhibit any of the four cytokines ~~(.Fig. 5).~~ In contrast, targeting of C3 and CD14 in combination resulted in significant reduction of all the four analytes with a mean attenuation by 75% for IL-1β ($p<0.05$), by 84% for IL-6 ($p<0.0001$), by 83% for IL-8 ($p<0.001$) and by 70% for TNF ($p<0.01$) (Fig ~~5~~4).

Discussion

In the present study, for the first time, we demonstrate that patients with confirmed *R. conorii* infection have enhanced complement activation *in vivo* as assessed by increased plasma levels of several complement activation markers, including sC5b-9 showing that the complement cascade was activated to the very end. Complement activation in patients was accompanied and correlated with increased serum levels of sCD14 as a marker of monocyte activation. Our *ex vivo* experiments suggest that *R. conorii* directly activate the complement system and induce release of several inflammatory cytokines and chemokines. Combined inhibition of CD14 and complement activation at the level of C3, but not inhibition of CD14 or C3 alone, markedly attenuated the *R. conorii*-induced inflammatory response *ex vivo*. Our findings suggest that activation of complement and CD14-related pathways are involved in the generation of inflammatory responses during *R. conorii* infection, and that targeting these pathways could be beneficial to dampen overwhelming and harmful inflammatory responses in MSF patients.

A few experimental *in vitro* studies have suggested that *R. conorii* can evade complement mediated clearance via recruitment of the complement regulator factor H to the bacterial surface and by evading C5b-9 mediated killing through interacting with the terminal complement complex inhibitor vitronectin ~~(15, 21)~~[16, 23]. In the present *in vivo* study we thoroughly investigated complement activation by measuring activation markers at different levels of the system and we could, for the first time, show enhanced complement activation in MSF patients with increased levels of the terminal complement complex, sC5b-9, ~~as the major finding. Markers~~and markers reflecting ~~C3 (~~alternative pathway activation, *i.e.* C3bc and C3bBbP~~) and C4 activation (C4bc) were also markedly elevated in patients in addition~~. The alternative pathway can be activated directly by a foreign surface or be amplified irrespective of which initial pathway that is triggered. We measured the C1rs-C1INH

complexes to investigate whether the classical pathway ~~specific C1rs-C1INH complex which was~~ could be activated. There was indeed a markedly and significantly ~~elevated in patients on admission as compared to~~ higher level of C1rs-C1INH at admittance than at follow-up ~~which indicate that complement activation, at least in some patients, was triggered by the~~ , suggesting classical pathway~~.~~ activation, but these data should be interpreted with caution due to a higher level in the control population than expected. Thus, ~~although~~though *in vitro* studies suggest that *R. conorii* could bind factor H and thereby avoid complement-mediated clearance this mechanism seems not to be ~~operating~~fully applicable *in vivo* in MSF patients. Our *ex vivo* studies suggest that *R. conorii* itself can activate the complement system to the very end (*i.e.* generation of C5b-9). If *R. conorii* binds vitronectin from host plasma and thereby counteract C5b-9 mediated killing of the bacteria ~~could~~, the enhanced complement activation in MSF patients could have harmful effect on the host through induction of bystander tissue damage and inflammation. ~~-~~Still, with the plethora of different strategies that microbes have developed to evade complement recognition and clearance, one can speculate that rickettsial hijacking of complement regulators could prolong bacterial lifetime in blood long enough to enable intracellular escape. Nevertheless, the highly significant sC5b-9 and C3-activation found in patients rule out that *R. conorii* infection occur without complement activation.

Increased serum level of sCD14 is primarily thought to reflect enhanced activation of monocytes but sCD14 can also ~~could~~ be derived from neutrophils ~~(10).~~[11]. Monocyte activation has been reported during *R. conorii* infection, contributing to the adherence of monocytes to the activated endothelium in *R. conorii*-infected endothelial cells ~~(22)~~[24]. Our finding in the present study with increased serum levels of sCD14 in MSF patients on admission to the hospital, further support that monocyte activation is a characteristic of these

patients. sCD14 was significantly correlated with sC5b-9 levels, suggesting concurrent activation of both complement and monocytes upon infection.

The pathophysiological significance of enhanced complement activation in MSF patients is at present not clear. However, our *ex vivo* findings suggest that activation of the complement cascade could contribute to *R. conorii*-mediated inflammation. Exposure of human whole blood to *R. conorii* induced elevation of several inflammatory cytokines, of which IL-1β, IL-6, IL-8 and TNF were among the most abundant. Inhibition of C3 attenuated *R. conorii*-mediated IL-8 release, potentially attributed to C5 dependent mechanisms (*i.e.* C5a and C5b-9). ~~Moreover, when combined with CD14 inhibition, C3 inhibition markedly attenuated the response of all tested inflammatory cytokines (*i.e.*, IL-1β, IL-6 and TNF in addition to more profound inhibition of IL-8).~~Although blockade of CD14 alone did not lower any of the four cytokines, anti-CD14 enhanced the inhibitory effects seen when C3 was blocked. In fact, the combined C3 and CD14 inhibition was the only regimen that significantly reduced rickettsia-induced release of all four cytokines. IL-1β was statistically increased by anti-CD14 alone. The reason for this is uncertain, but most likely due to methodological type I error, since it was not seen for any of the other cytokines, and in particular since anti-CD14 potentiated the inhibitory effect of C3 to significant level, close to the background, as seen for the other cytokines as well. CD14 is a promiscuous protein primarily known as an accessory molecule facilitating LPS transfer from LPS-binding protein to TLR4-MD2 complexes. Recent studies indicate considerable cross-talk between complement and TLR4 activation, and we have shown reduced inflammation upon combined targeting of CD14 and complement in various experimental models, even when the inflammation is predominantly LPS-induced and therefore CD14 dependent ~~(12, 23)~~[13, 25]. Our findings in the present study suggest that similar mechanisms could be operating in *R. conorii* infection.

The present study has some limitations such as a low number of patients during follow-up, and although we in our lab have established reference values for the actual complement parameters, the numbers of healthy controls were rather low. On the other side, a broad spectrum of complement activation markers were analysed all showing the same pattern.

In conclusion, we demonstrate enhanced activation of the complement cascade and increased sCD14 in MSF patients, and our *ex vivo* findings suggest that targeted therapy against complement activation and CD14 could be of interest in severe *R. conorii* infection.

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Transparency declaration

The authors declare no conflicts of interest

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Figure legends

Fig. 1. ~~Complement~~Levels of complement activation markers ~~reflecting C3, alternative and terminal pathways~~in MSF patients. Plasma levels for **A**. C3-activation (C3bc), **B**. alternative pathway C3-convertase formation (C3bBbP~~) and~~), **C**. terminal pathway activation (sC5b-9) ~~is~~and **D**. C1rs-C1INH are shown for 36 patients (33 for C1rs-C1INH) at admission, 4 patients at follow-up (28-42 days after symptom onset) and 9 healthy controls. Normal population reference values for each marker are shown in grey area between dotted lines. Data are given as means and 95% confidence intervals. Statistical difference between patients at admission were compared against controls and patients at follow-up with the Kruskal-Wallis test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001~~.~~, ns=not significant.

Fig.

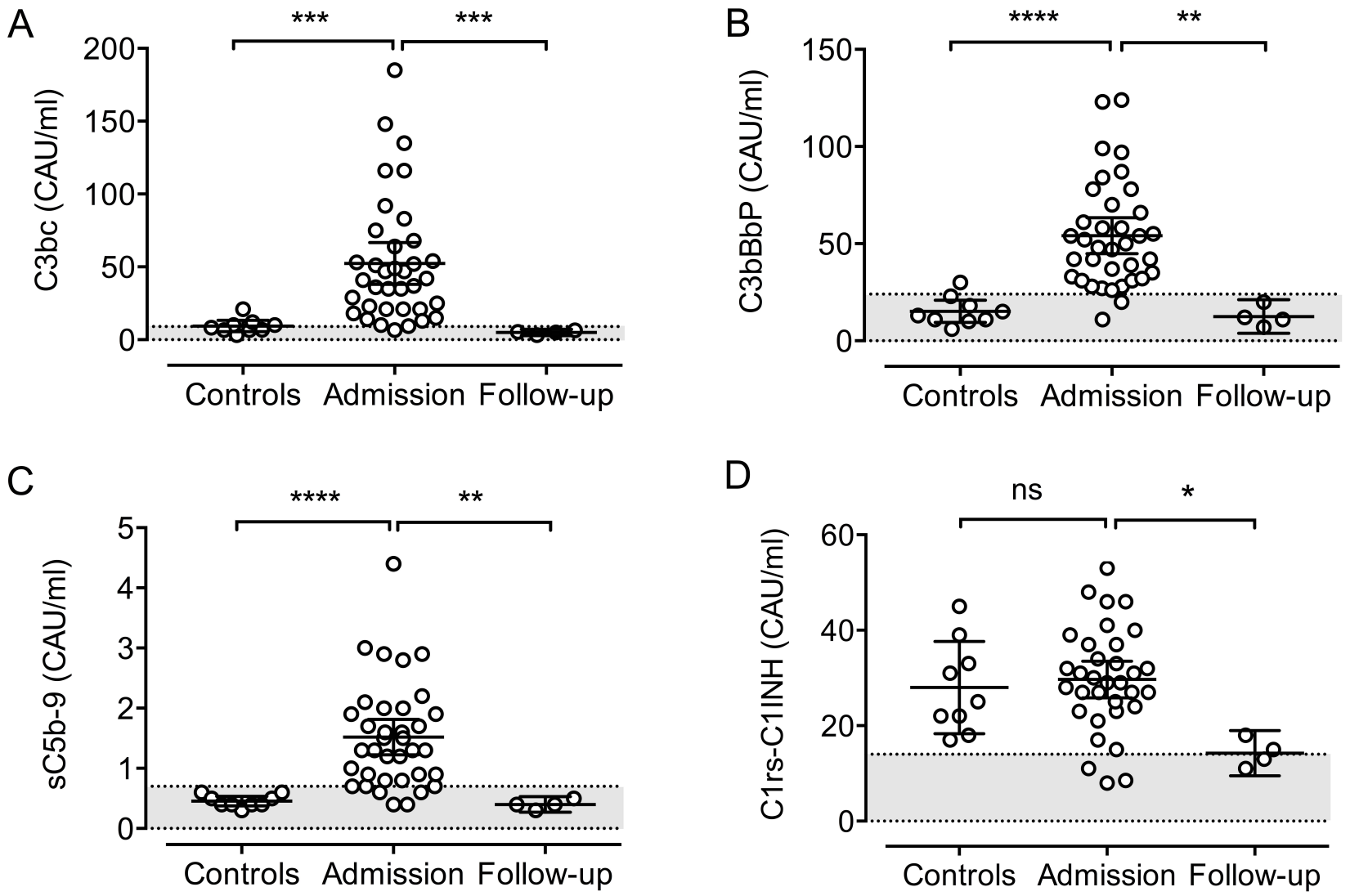
~~**Fig. 2. Complement activation markers reflecting classical pathway (C1rs-C1INH) and classical- and lectin pathway (C4bc).** Plasma levels of **A**. classical pathway activation (C1rs-C1INH) and **B.** C4-activation (C4bc) are shown for 33 and 36 patients respectively at admission, and for 4 patients at follow-up (28-42 days after symptom onset). Normal population reference values for each marker are represented in grey area between stippled lines. Data are given as means and 95% confidence intervals. Statistical difference between admission and follow-up was tested with the Mann-Whitney test, *p<0.05, **p<0.01.~~

~~**Fig. 3. Levels of sCD14 in the patients.**~~**2. Levels of sCD14 in MSF patients.** Serum levels of sCD14 are shown for 36 patients at admission, 15 patients at follow-up (28-42 days after symptom onset) and 9 healthy controls. Data are presented on a logarithmic scale. Statistical

difference between patients at admission were compared against controls and patients at follow-up with the Kruskal-Wallis test; **p<0.01, ****p<0.0001.

Fig. ~~4~~3. Complement activation and cytokine response in human whole blood after incubation with *R. conorii.* A. *R. conorii* was incubated for two hours in whole blood and complement activation was measured as levels of sC5b-9. Data are presented as means and 95% confidence intervals (n=6). The effect of *R. conorii* was statistically tested against the buffer control using paired t-test, **p<0.01. **B.** *R. conorii* was incubated in whole blood for 4 hours, levels of 27 cytokines, chemokines and growth factors were measured in plasma after incubation. Cytokines that showed a ~~two-~~mean fold ~~or higher~~ increase of two or more from three individual experiments in the presence of *R. conorii*~~,~~ (dark grey), compared to whole blood incubated without bacteria~~,~~ (light grey), are in the figure depicted with mean levels +/- standard deviation (n=3).

Fig. ~~5~~4. Effect of C3- and CD14-inhibition on pro-inflammatory cytokines in response to *R. conorii* incubated in human whole blood *ex vivo*. Incubation of *R. conorii* in the presence or absence of a complement C3 inhibitor (compstatin Cp40~~)),~~), an ~~antibody~~F(ab´)$_2$-fragment blocking CD14, or a combination thereof. The inflammatory response was evaluated a subset of pro-inflammatory cytokines represented by **A**. IL-1β, **B**. IL-6, **C**. IL-8 and **D**. TNF. Data are presented as means and 95 % confidence intervals (n=6). Effect of *R. conorii* was statistically tested against the basal control using student's t-test ($^{###}$p<0.001). Effect of respective inhibition was statistically tested against *R. conorii* using repeated measures one-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ns=not significant.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***
***
C3bc (CAU/ml)
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150
100
50
0
Controls
Admission
Follow-up
B
****
**
C3bBbP (CAU/ml)
150
100
50
0
Controls
Admission
Follow-up
C
****
**
sC5b-9 (CAU/ml)
5
4
3
2
1
0
Controls
Admission
Follow-up
D
ns
*
C1rs-C1INH (CAU/ml)
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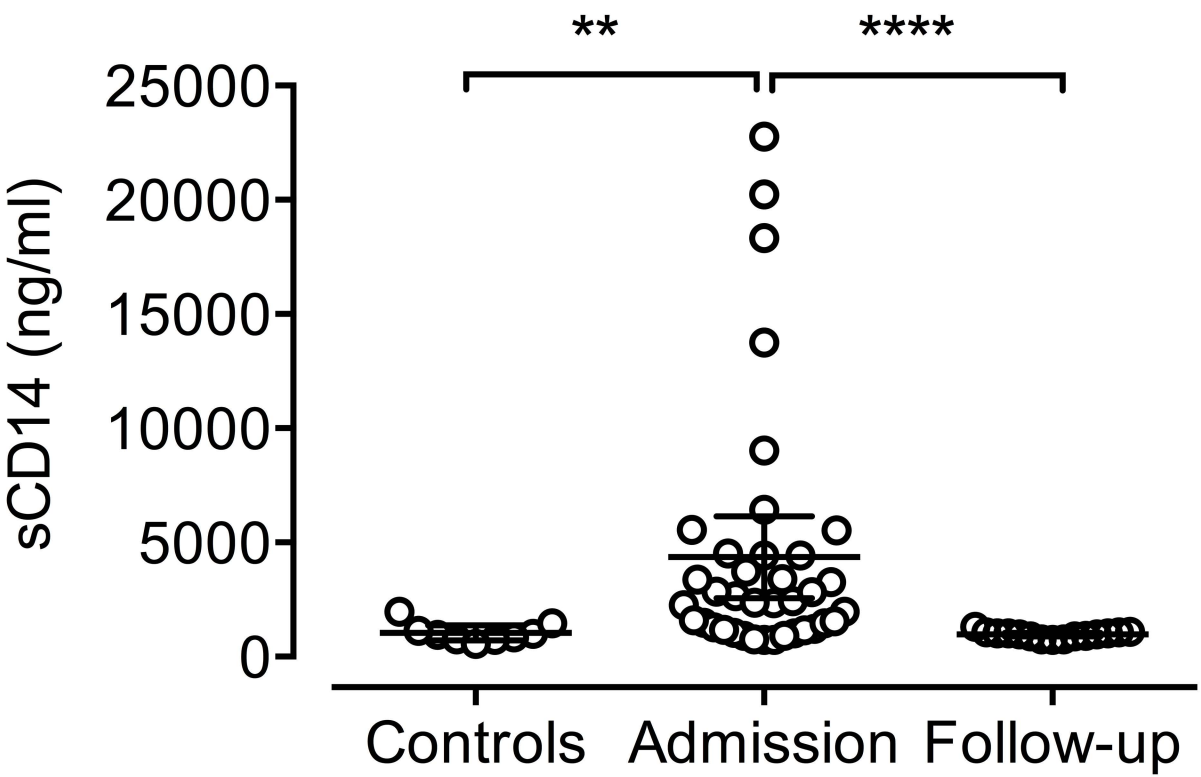
40
20
0
Controls
Admission
Follow-up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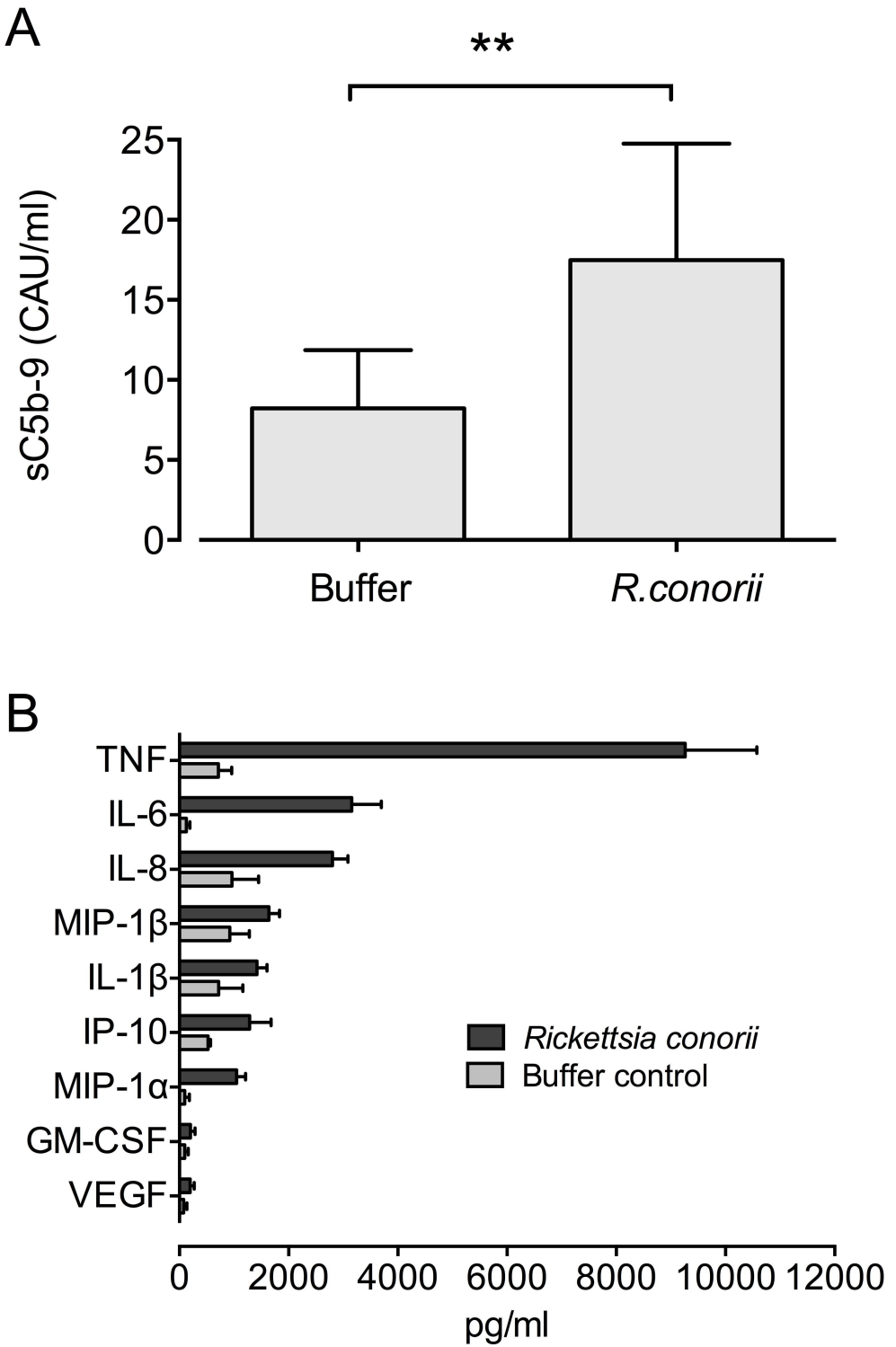
sCD14 (ng/ml)
Controls
Admission
Follow-up


A
**
25
20
15
10
5
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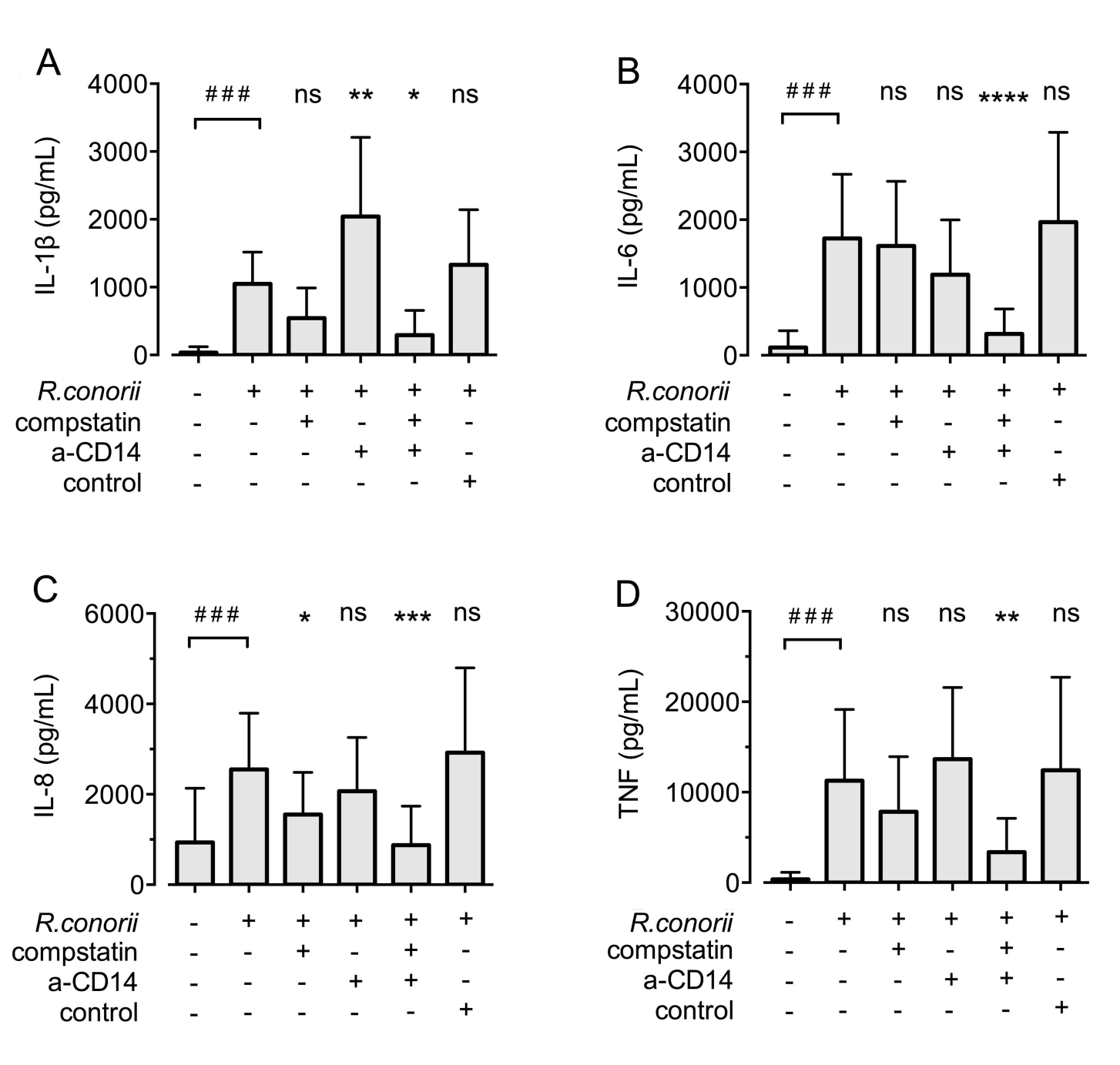
sC5b-9 (CAU/ml)
Buffer
R.conorii
B
TNF
IL-6
IL-8
MIP-1β
IL-1β
IP-10
MIP-1α
GM-CSF
VEGF
Rickettsia conorii
Buffer control
0
2000
4000
6000
8000
10000
12000
pg/ml


A
IL-1β (pg/mL)
4000
3000
2000
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0
ns ** * ns
R.conorii - + + + + +
compstatin - - + - + -
a-CD14 - - - + + -
control - - - - - +
B
IL-6 (pg/mL)
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3000
2000
1000
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R.conorii - + + + + +
compstatin - - + - + -
a-CD14 - - - + + -
control - - - - - +
C
IL-8 (pg/mL)
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4000
2000
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compstatin - - + - + -
a-CD14 - - - + + -
control - - - - - +
D
TNF (pg/mL)
30000
20000
10000
0
ns ns ** ns
R.conorii - + + + + +
compstatin - - + - + -
a-CD14 - - - + + -
control - - - - - +