Cholesterol crystal-induced endothelial cell activation is complement-dependent and mediated by TNF

Running title: Cholesterol crystal-induced endothelial cell activation

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Abbreviations:
CC: Cholesterol crystals
C5aR: C5a Receptor
EC: Endothelial cells
HUVEC: Human umbilical vein endothelial cells
TCC: Terminal complement complex

Abstract

Cholesterol crystals are known to be a hallmark of atherosclerosis with recent studies demonstrating deposition of these crystals in early fatty streak formation as well as penetrating the intima following plaque rupture. Inflammation has also become a central focus in atheroma development and endothelial cell activation is recognized as necessary for the recruitment of inflammatory cells to the plaque. However, the extent to which cholesterol crystals can induce inflammation and activate endothelial cells is not known. To investigate this, we developed a novel model activating human umbilical vein endothelial cells using lepirudin anticoagulated human whole blood. We found that cholesterol crystals caused a marked and dose-dependent increase in the adhesion molecules E-selectin and ICAM-1 on the surface of the endothelial cells after incubation with whole blood. There was no activation of the cells when the crystals were incubated in medium alone, or in human serum, despite substantial crystal-induced complement activation in serum. Complement inhibitors at the C3 and C5 levels reduced the whole blood induced endothelial cell activation by up to 89% ($p < 0.05$) and abolished TNF release ($p < 0.01$). Finally, the TNF inhibitor infliximab reduced endothelial activation to background levels ($p < 0.05$). In conclusion, these data demonstrate that endothelial activation by cholesterol crystals is mediated by complement-dependent TNF release, and suggests that complement-inhibition might have a role in alleviating atherosclerosis-induced inflammation.

Introduction

Cardio-vascular diseases are a major cause of mortality world wide, and although survival has improved over recent years, there are many facets of these diseases not yet well understood. Atherosclerosis is frequently implicated in diseases such as stroke, myocardial infarction and aortic aneurisms. Early on, cholesterol crystals (CC) were recognized as a hallmark of the late atherosclerotic plaque, and recent studies have also demonstrated its presence already at the development of the fatty streak being one of the earliest manifestations of atherosclerosis (Duewell et al., 2010). George Abela and his group have also demonstrate that in post-mortem examination of atherosclerotic plaque rupture, there are cholesterol crystals piercing the intima thus potentially playing a role in subsequent inflammatory reactions (Abela et al., 2009; 2010).

Although first thought to be mainly a disease of lipid-deposition, today there are overwhelming data supporting a central role of inflammation both in atheroma development as well as its subsequent morbidity and mortality (Hansson and Hermansson, 2011; Hansson et al., 2002; Libby et al., 2009). The complement system appears to play a role in this response, as several studies in mice and men have indicated complement deposition in atherosclerotic plaques as well as modulating effects of complement inhibition on atheroma formation (Francescut et al., 2012; Haskard et al., 2008; Manthey et al., 2011; Niculescu and Rus, 1999; Speidl et al., 2011a; 2011b; Torzewski and Bhakdi, 2013). The complement system is known to play a role in a large number of inflammatory diseases (Klos et al., 2009; Ricklin et al., 2010), where three separate activating pathways all converge on the splitting of C3 to C3b and C3a. C3a is a known inflammatory mediator, whereas C3b is both important for opsonisation as well as amplification

and the formation of the C5-convertases (Ricklin et al., 2010; Sarma and Ward, 2011). The C5-convertases splits C5 into C5a and C5b, where C5a is a potent inflammatory mediator which effects are mainly due to its interaction with the C5a-receptor (C5aR). C5b together with C6-C9 form the terminal C5b-9 complex (TCC), which, when activated on a surface form the membrane attack complex and might lyse bacteria or cells, or in sub-lytic doses activate cells to produce inflammatory mediators. In the fluid phase, the TCC forms a soluble complex (sC5b-9) which can be detected as a complement activation product in body fluids indicating ongoing complement activation (Ricklin et al., 2010; Sarma and Ward, 2011).

Endothelial cells (EC) are known as well to play a role in inflammation, both being crucial for the recruitment of inflammatory cells and active producers of chemokines and cytokines. They are also for the same reasons central in atherosclerosis development. EC activation is a necessary prerequisite for leukocyte recruitment to the plaque (Guardamagna et al., 2009; Mai et al., 2013; Mestas and Ley, 2008; Pate et al., 2010). There are a long range of mediators that are shown to activate endothelial cells *in vitro*, among others TNF, IL-1β, sub-lytic TCC and C5a (Pate et al., 2010), which all might be relevant in EC activation in atherosclerosis.

Despite the presence of CC in the plaque, and the known role of inflammation in atherosclerosis there are limited data on the inflammatory potential of CC. Several groups have demonstrated the strong complement-activating potential of CC (Hasselbacher and Hahn, 1980; Seifert and Kazatchkine, 1987; Vogt et al., 1985). Two seminal studies also demonstrated that CC caused inflammasome activation in primed monocytes linking CC to Il-1β production, which is thought of as a central player in atherosclerosis development (Duewell et al., 2010; Rajamäki et al., 2010). In a recent study from our group, we have taken this one step further, showing that

CC-induced inflammation in whole blood is complement-dependent and that C5a combined with TNF are potent primers for CC induced inflammasome activation (Samstad et al., 2014).

In this study we aimed to examine to what extent CC could activate EC. We developed a novel EC activation assay, co-incubating whole blood with monolayers of human umbilical vein endothelial cells (HUVEC) measuring the expression of adhesion molecules on the HUVEC surface post-exposure as a marker of activation. Using this model we found that when CC were incubated with whole blood there was a potent, complement-dependent EC activation, which was mainly mediated by TNF.

Materials and methods

Reagents

Sterile phosphate buffered saline (PBS) with and without Ca^{2+} and Mg^{2+} and Ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma–Aldrich (St. Louis, MO). CryoTubes™ (polypropylene) was purchased from Nunc AS (Roskilde, Denmark). Lepirudin (Refludan®) was purchased from Pharmion (Copenhagen, Denmark) and used at a final concentration of 50 μg/ml in whole blood. A 4% stock solution made of paraformaldehyde (PFA) was purchased from Sigma-Aldrich (St. Louis, MN). BSA 30% was purchased from Biotest (Dreieich, Germany) and Trypsin/EDTA was from Invitrogen (Carlsbad, CA). Recombinant human C5 were purchased from Quidel (San Diego, CA). Recombinant human TNF and recombinant human IL-1β were purchased from R&D systems (Minneapolis, MN).

Antibodies used were FITC-conjugated mouse anti human ICAM-1 (CD54, Clone BBIG-I1), PerCP conjugated mouse anti-human MCAM (CD146, clone 128018) and iso-type controls (R&D Systems, Minneapolis, MN), PE conjugated mouse anti-human E-selectin (CD62E, clone 1.2B6) and iso-type control (Southern Biotech, Birminham, AL), C5 antibody (eculizumab/Soliris®, Alexion), TNF antibody (infliximab/Remicade®, Janssen Biologics), anti-IL-1β (canakinumab/Ilaris®, Novartis) and anti-CD20 (rituximab/Mabthera®, Roche). C3-inhibitor compstatin analog CP40 (Ac-Ile-[Cys-Val-Trp(Me)-Gln-Asp-Trp-Sar-Ala-His-Arg-Cys]-mIle-NH2) and its control scrambled peptide (Qu et al., 2013) were a kind gift from Professor John D. Lambris, as was the the specific C5a-receptor antagonist (AcF[OPdChaWR]), synthesized as previously described (Finch et al., 1999).

Cholesterol crystals

CC were produced as previously described (Samstad et al., 2014). Briefly, ultra-pure cholesterol (Sigma–Aldrich, St. Louis, MO) was dissolved in 1-propanol and aggregated by addition of sterile water. CC were then air-dried and tested for LPS which was below the detection limit in the limulus amebocyte lysate assay (0.3 pg/ml). CC were used at a final concentration of 2 mg/ml (3 x 10^7 particles/ml) if not otherwise specified.

Whole blood and HUVEC

A modified version of an *ex vivo* whole blood model which was described in detail previously (Mollnes et al., 2002) was used. HUVEC (ECACC, Salisbury, UK) were seeded in 48-wells plates (Costar, Corning, New York) coated with 1% gelatin (Sigma, St. Louis, MO) and grown until confluence (2-4 days) in DMEM 199 medium (Invitrogen, Carlsbad, CA) with growth supplements and 7.5% fetal calf serum. All cells were used in passage 2-5. On the day of the experiment, fresh human whole blood was obtained from healthy donors and anticoagulated with lepirudin. Confluent HUVEC layers were washed once with sterile, 37°C PBS before the addition of 100 µl whole blood, growth medium, growth medium supplemented with 50% pooled human serum (NHS) or plasma from whole blood experiments to each well. The inhibitors compstatin (20 µM), eculizumab (100 µg/ml), C5aR-antagonist (10 µM), infliximab (100 µg/ml), canakinumab (100 µg/ml), rituximab as a control antibody (100 µg/ml) or PBS were added in a total volume of 20 µl and incubated for 4 minutes at 37°C prior to stimulation with PBS, CC, recombinant TNF (0.1 - 10 ng/ml) or recombinant IL-1β (0.1 - 10 ng/ml). Samples were then incubated at 37°C with 5% CO_2 for 4 hours with gentle shaking. Thereafter, whole blood or medium was removed and EDTA (20 mM) was added before centrifugation for 15 min at 3000 x *g* at 4°C. Plasma or supernatant were stored at −70°C until analysis.

Endothelial cell activation markers

After removal of medium, plasma or whole blood, HUVEC layers were gently washed twice with ice-cold PBS, and fixed with 0.5% PFA and incubated at 4°C for 2.5 minutes, according to a modified protocol from Gräbner et al. (Gräbner et al., 2000). After gentle washing with PBS, anti-ICAM-1-FITC, anti-E-selectin-PE or their iso-type controls, and anti-MCAM-PerCP were added and plates were incubated for 30 minutes at 4°C. Cells were washed twice with PBS, briefly trypsinated and transferred to 5 ml polypropylene tubes (Sarstedt, Nuernbrecht, Germany), washed with PBS with 0.1% BSA and run on a FACSCalibur or FACS LSRII flow cytometer (BD Bioscience, San Jose, CA). HUVEC were gated as MCAM positive cells. Median values were used for fluorescense intensity values (MFI). Data were analyzed either in FacsDIVA (BD Bioscience, San Jose, CA) or FlowJo X (Tree Star Inc, Ashland, OR).

C5-deficient patient

The C5-deficient (C5D) patient used for this study is previously described in details (Lappegård et al., 2009). In experiments with the C5D patients, C5D blood, C5D blood reconstituted with recombinant C5 (50 µg/ml), and two healthy controls were all run simultaneously on the same plate.

Cytokine and complement measurements

Analysis of plasma concentrations of IL-1β, IL-6, IL-8 and TNF were done using multiplex technology. Single-plex beads were purchased from Bio-Rad (Bio-Rad Laboratories Inc., Hercules, CA) and used according to manufacturer's recommendations. The C5b-9 complex (TCC) was measured using a specific antibody aE11 which targets a C9 neo-epitope in an in-house ELISA which was previously described in detail (Bergseth et al., 2013).

Statistics and ethical approval

All data were compiled in Prism (GraphPad Inc. Version 5, San Diego, CA, USA). Groups were compared using one-way ANOVA for repeated measurements with Bonferroni's post-test for comparisons of specific groups if not otherwise specified. Informed written consent was obtained from each donor, and the local ethics committee approved the study.

Results

HUVEC incubated with whole blood

As we already know that CC activate leukocytes and cause both the expression of leukocyte adhesion molecules and cytokine release in whole blood (Samstad et al., 2014), we here investigated to which extent CC incubated in whole blood would activate EC. In order to examine this, we developed a novel model, where we co-cultured lepirudin anti-coagulated whole blood with monolayers of HUVEC. Whole blood alone did not cause any visual alterations of the HUVEC layer as examined by light microscopy, and there were only minimal EC activation by whole blood compared to medium controls. Notably, the addition of CC to whole blood caused a dose-dependent increase in both E-selectin and ICAM-1 on the EC surface, with some donor-dependent differences. Two mg/ml was chosen as the optimal dose causing a robust activation compared to unstimulated samples (Figure 1).

Effect of complement activation by CC on EC activation

We next evaluated the role of complement inhibition on the EC surface expression of the adhesion molecules E-selectin and ICAM-1. These were significantly inhibited both at the C3 level (compstatin) and at the C5 level (eculizumab and C5aR-antagonist), where E-selectin was attenuated by 70-89% (Figure 1A) and ICAM-1 by 61-82% (Figure 1B). We confirmed these finding using a C5-deficient patient, where incubation of CC in C5-deficient whole blood did not cause EC activation, however reconstitution with recombinant C5 increased activation to the level of healthy controls (Figure 2).

HUVEC incubated with CC in human serum

To examine if the previously documented complement activation induced by CC could cause EC activation directly, we incubated CC with EC in medium containing 50% pooled human serum. Despite a robust production of TCC in the supernatant post-incubation, indicating substantial complement activation, no up-regulation of the adhesion molecules were observed (Figure 3).

The role of TNF in EC activation

Both TNF and IL-1β are regarded as early mediators in the cytokine response that are known to cause HUVEC activation *in vitro*, and we have previously demonstrated that TNF participate in the release of cytokines that are induced by CC in whole blood (Samstad et al., 2014). We therefore examined if either of these cytokines was important for CC-induced EC-activation. Using a specific inhibitor towards TNF (infliximab) or IL-1β (canakinumab) we evaluated the impact of these cytokines on HUVEC activation. Whereas IL-1β inhibition did not affect E-selectin or ICAM-1 expression, inhibiting TNF completely attenuated these responses (Figure 4), demonstrating that TNF plays a central role in CC induced EC activation.

To explore the role of TNF further, we evaluated to what extent other cytokines in CC-activated whole blood would participate in EC activation. In order to do this, we pre-activated whole blood with CC to generate all relevant cytokines, then activated EC using plasma from these experiments (Figure 5 A and B). Where indicated infliximab, canakinumab or a control antibody was added prior to stimulation of EC. As whole blood incubated without activators also caused a moderate activation of EC, we included plasma isolated from whole blood at the point of blood sampling (T0) as an additional control. The TNF inhibitor infliximab reduced EC activation to under the level of plasma from unstimulated whole blood experiments and towards background (T0) activation, demonstrating the key role of TNF (Figure 5 A and B). We also

found a dose-dependent response of HUVEC to TNF and IL-1β (Figure 5 C and D), as well as full inhibition of this activation by infliximab and canakinumab demonstrating the efficacy of the inhibitors (Figure 5 C and D).

Complement and TNF

The cytokine responses to CC are to a large extent dependent on complement activation as we have earlier demonstrated that complement inhibition attenuated these responses in whole blood (Samstad et al., 2014). Since TNF-inhibition completely attenuated the CC-induced EC activation, we addressed whether complement inhibition reduces TNF release in plasma from CC-activated whole blood and EC. Inhibition at the C5-level completely abrogated CC induced IL-1β, IL-6, IL-8 and TNF release, supporting the central role of TNF in EC-activation in this model and that TNF release occurs down-stream to complement activation (Figure 6).

Discussion

In this study we have established a novel model of HUVEC activation allowing us to study the complex interaction between EC and both cellular and humoral immune-components in fresh whole blood. Using this model, we found that CC were not able to activated EC directly, or in serum alone, but indirectly through whole blood, and that this activation was complement-dependent. Surprisingly, we also found that TNF single handedly seems to mediate this activation.

HUVEC have for a long time been used as a model system for EC activation. However, the activation of HUVEC with addition of activating substances to medium does not capture the complexity of EC activation, nor does it allow examination of the relative contribution of different mediators towards EC activation in more complex systems. To more accurately model this complexity, some have used conditioned media to activate EC (Nooteboom et al., 2006; 2005; 2004; 2002; Schildberger et al., 2011), that is medium from activated leukocytes or immortalized monocyte cell lines as well as medium mixed with plasma from heparin anti-coagulated whole blood. However there are limitations to these models as well, as molecules with short half-life, for instance C5a, the role of cell-cell interaction between EC and leukocytes, as well as the modulating role of EC on leukocyte and complement activation cannot be captured. Therefore we developed a novel model of HUVEC activation, where we co-incubated monolayers of HUVEC with lepirudin anticoagulated whole blood to examine the complex interaction of EC, leukocytes, platelets, the complement system, and other lesser known players in the inflammatory network. This allowed us not only to look at EC activation in a more physiologically complex system, but also attempt to dissect the relative importance of EC-activating mediators in this system.

The role of CC in inducing inflammation is not well elucidated, but we have previously shown that CC occur early on in atherosclerotic lesions (Duewell et al., 2010) and that CC cause inflammasome activation in LPS primed macrophages (Duewell et al., 2010). Recently, we linked complement to CC-induced inflammasome activation in a human whole blood system (Samstad et al., 2014), and found that C5a and TNF in combination act as a potent priming signal in isolated monocytes for CC induced inflammasome activation (Samstad et al., 2014). As endothelial activation is central in leukocyte recruitment to atherosclerotic plaques, we examined to what extent CC can participate in EC activation. Our data demonstrate that CC are potent activators of the whole blood system, and that this activation causes up-regulation of EC adhesion receptors thus supporting the inflammatory potential of CC in atherosclerosis.

We found earlier that in CC-induced inflammation in whole blood, both cytokine production as well as leukocyte activation, were dependent on complement activation (Samstad et al., 2014). In the present study inhibition of the complement system completely abolished CC-induced activation of EC by whole blood, strengthening our hypothesis that complement is central in CC-induced inflammation. This was the case both with inhibitors at the C3 and C5 level and as an antagonist directed at the C5 receptor had similar effects as the other inhibitors, our study indicates that C5a is the major player. Using a patient with a well-established C5-deficiency we confirmed the findings as reconstitution with recombinant C5 caused a clear increase in CC induced EC activation. A recent study found that sublytic TCC and not C3a/C5a caused IL-1β production in murine dendritic cells (Laudisi et al., 2013), but our study does not indicate that the sublytic TCC is of importance in neither human whole blood activation (Samstad et al., 2014), nor whole blood induced EC activation.

There are a some studies indicating that activation of the complement system itself can cause activation of HUVEC by increased adhesion molecule expression both through C5a and sublytic TCC (Foreman et al., 1994; Tedesco et al., 1997), as well as cytokine production, mainly IL-8 and MCP-1, and NFκB activation (Albrecht et al., 2004; Kilgore et al., 1996; 1997; Monsinjon et al., 2003; Selvan et al., 1998) . However, other studies have not been able to reproduce findings on C5a and sublytic TCC-induced increase of EC adhesion molecule expression (Jagels et al., 2000; Kilgore et al., 1995; Monsinjon et al., 2003). Our study support the latter findings, as directly stimulating HUVEC with CC in pooled human serum did not cause activation of the cells, despite potent complement activation in the serum. Although we cannot exclude the possibility that the observed increase in adhesion molecule expression is modulated by complement interaction with EC (Kilgore et al., 1995), our study indicates that complement activation products do not activate EC directly, but EC activation is caused by mediators dependent on complement activation in whole blood.

In vitro studies have found a large number of activators of HUVEC, but TNF and IL-1β are singled out as central in causing EC activation (Haraldsen et al., 1996; Mantovani et al., 1997; Nooteboom et al., 2002). Particularly, Nooteboom et al. found that upon activating EC with plasma from LPS activated whole blood, the increase of E-selectin and ICAM-1 seen were completely abolished when a combination of IL-1β and TNF inhibitors were used (Nooteboom et al., 2004). We have also established that CC cause both IL-1β and TNF production in whole blood (Samstad et al., 2014), and thus we here examined if inhibiting these cytokines also would reduce CC-induced EC activation. We used clinically available antibodies, namely infliximab (anti-TNF) and canakinumab (anti- IL-1β) to evaluate the role of these cytokines in our model. We found that both inhibitors completely inhibited activation by recombinant TNF and IL-1β

when used at 10 ng/ml demonstrating the efficacy of the inhibitors. However, when whole blood was incubated with CC and the inhibitors, infliximab completely abolished EC activation, whereas canakinumab had no effect on EC adhesion molecule expression, indicating that TNF plays the main role in EC activation caused by CC. Testing the dose-response curve of HUVEC for both TNF and IL-1β we found that both cytokines dose-dependently caused activation. Nevertheless within the time frame of our experiment, there was only a limited IL-1β production, which did not reach the activating levels found in the dose-response curves. Furthermore, we have shown earlier that CC also induce IL-1ra in whole blood, known to inhibit IL-1β activity (Samstad et al., 2014), thus a combination of these factors could explain the lack of effect of IL-1β inhibition. Therefore we cannot exclude the possibility that IL-1β can play an important role in EC activation in the long run as we know that monocytes have a robust IL-1β response to CC after 10-16 hours (Samstad et al., 2014).

We have shown earlier that TNF inhibition also reduces the overall cytokine response to CC in whole blood (Samstad et al., 2014). To examine to what extent the effect of the TNF inhibitor was due to direct interaction between TNF and HUVEC or whether TNF inhibition modulated either leukocyte activation or the general inflammatory reaction to CC, we incubated whole blood with CC without HUVEC for 4 hours, and used the plasma from these experiments to activate HUVEC. CC-induced activation could then run its course, without TNF inhibition of leukocyte activation or cytokine production. When we added the TNF inhibitor to the plasma prior to activation of HUVEC, the inhibitor still caused an almost complete attenuation of the induced EC activation, indicating that the main effect of TNF inhibition in our model is directly through reducing TNF-EC interaction.

To investigate if the effect of the complement inhibitors could be due to their role in reducing TNF release, we measured cytokine production in plasma from the whole blood and HUVEC experiments. We found that the complement inhibitors reduced the production of all four cytokines measured, reducing TNF almost to background levels. This indicates that reduced TNF production is one of the mechanisms behind the effect of complement inhibition, and again support the central role of TNF in CC-induced EC activation.

Thus, somewhat surprisingly, despite the large number of potential EC-activating factors produced in whole blood inflammation, we found that TNF singlehandedly was responsible for the activation seen in our model. Although TNF has been suggested as an important mediator in simpler EC activation models, our study confirms these findings in a more complex system, and demonstrate that other activators shown to activate *in vitro*, such as sub-lytic TCC and C5a play a minor role, if any role at all in our model. This warrants further research into the relative importance of TNF and other mediators with other sources of inflammation, as one cannot assume that factors shown to cause EC activation *in vitro* necessarily play a significant role as the complexity of the system increases.

With our novel HUVEC activation model using whole blood, we have shown that CC contribute to the inflammatory state of EC, which could be relevant in atherosclerotic diseases. Although, CC did not cause direct activation of EC, they did cause a potent activation of EC when co-incubated with whole blood, an activation which was complement-dependent. Interestingly, we also demonstrate that in our model, this complement-dependent activation is nearly completely mediated by TNF.

Disclosures

The authors have no conflict of interest.

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Figure Legends

Figure 1: CC-induced EC-activation is complement dependent

EC were incubated with whole blood and complement inhibitors or control antibody prior to the addition of 2 mg/ml CC. (A) E-selectin expression and (B) ICAM-1 expression given as mean ± SEM for 5 healthy donors. ns = non significant, * $p < 0.05$, ** $p< 0.01$, *** $p < 0.001$ as compared to CC stimulation.

Figure 2: CC induced EC activation is attenuated in C5-deficient patient

EC were incubated with whole blood from a confirmed C5-deficient patient with or without reconstitution with 50 µg/ml recombinant C5 or with whole blood from healthy controls. (A) E-selectin expression and (B) ICAM-1 expression on EC were measured. Four separate experiments were performed for the C5-deficient patient, with in total eight separate healthy controls. Data are given as mean ± SEM.

Figure 3: CC incubated in 50% serum induce robust complement activation but no EC activation

Expression of E-selectin and ICAM-1 on EC after incubation with 50% NHS in EC medium (both on right axis) as well as TCC production in supernatant (left axis) after stimulation with an increasing dose of CC. Data given as mean of n=3 ± SEM.

Figure 4: EC activation is mediated by TNF

EC were incubated with whole blood and the TNF antibody infliximab, the IL-1β antibody canakinumab or control antibody prior to the addition of 2 mg/ml CC. (A) E-selectin expression

and (B) ICAM-1 expression given as mean ± SEM of n=6 donors for all groups except canakinumab with n=4. ns = non significant, * $p < 0.05$, *** $p < 0.001$ as compared to CC stimulation.

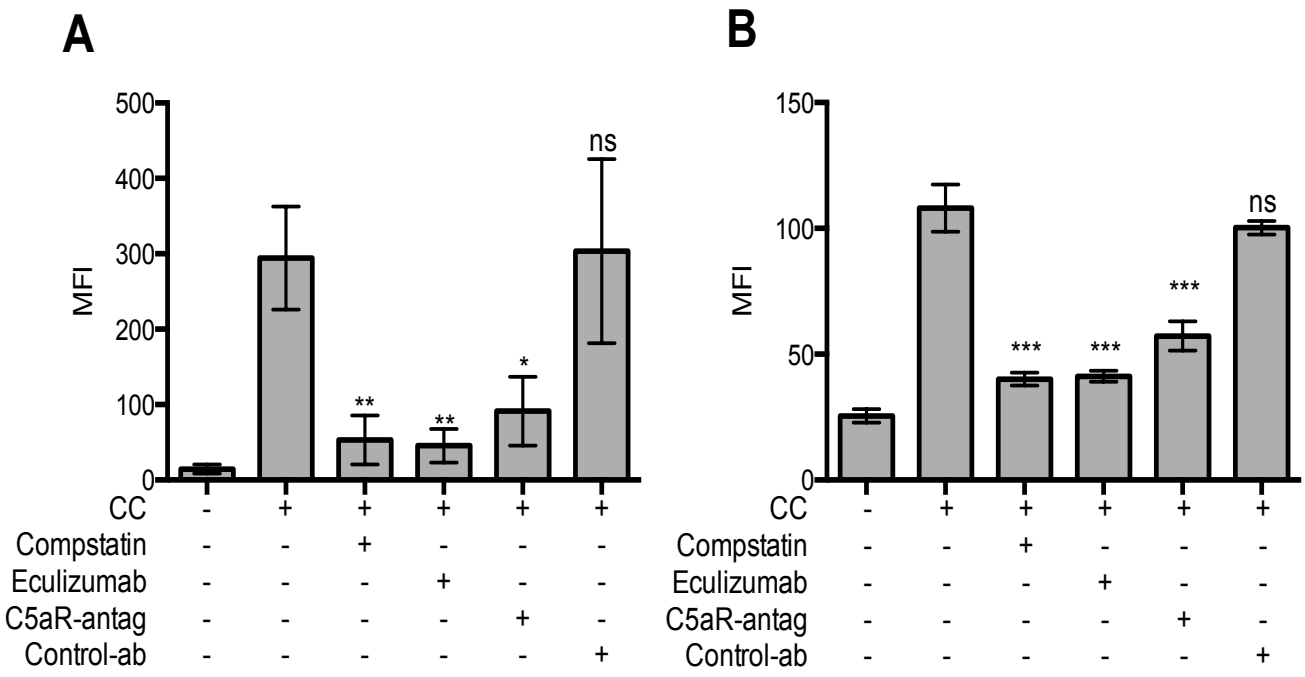
Figure 5: TNF-EC interaction is the main cause of EC activation

Whole blood was incubated with or without 2 mg/ml CC for 4 hours after which plasma was separated and frozen immediately at − 80°C. Plasma was also separated from same donor but without incubation (T0). After thawing plasma on ice, EC were incubated with plasma from T0 (white), without CC (light gray) or with CC (dark gray), and where stated, infliximab, canakinumab or a control antibody was added to the plasma. (A) E-selectin and (B) ICAM-1 were measured. HUVEC was also incubated in medium with increasing doses of TNF (C) or IL-1β (D) which dose-dependently increased expression of E-selectin and ICAM-1 which were inhibited by their respective inhibitors infliximab (anti-TNF) and canakinumab (anti-IL-1β). All data given for mean ± SEM of n=3 donors, except the lowest dose in panel C and D where n=2. ns = non significant, **** $p < 0.0001$ as compared to CC stimulated plasma

Figure 6: Complement inhibition attenuates cytokine release in whole blood

Plasma from whole blood incubated with EC, CC and inhibitors was analyzed for (A) IL-1β, (B) IL-6, (C) IL-8 and (D) TNF. Data are given as mean ± SEM for n=6 donors. ns = non significant, ** $p < 0.01$, *** $p < 0.001$ as compared to CC stimulation.

Figure 1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Compstatin - - + - - -
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C5aR-antag - - - - + -
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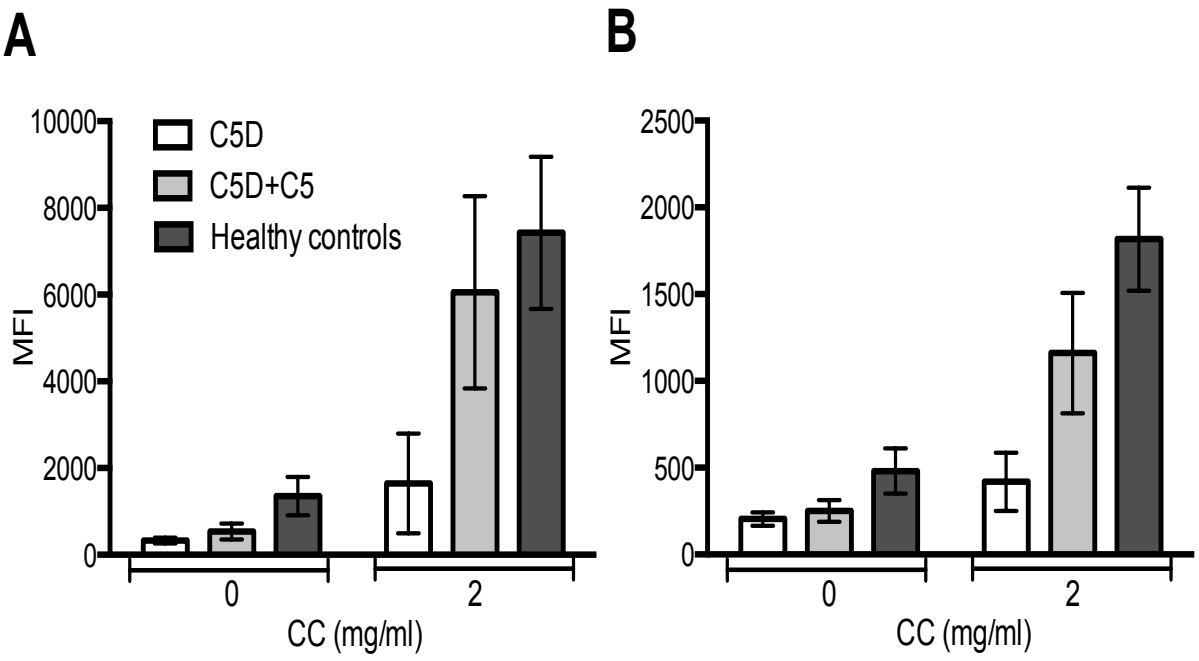
Control-ab - - - - - +

Figure 2


A
B
C5D
C5D+C5
Healthy controls
MFI
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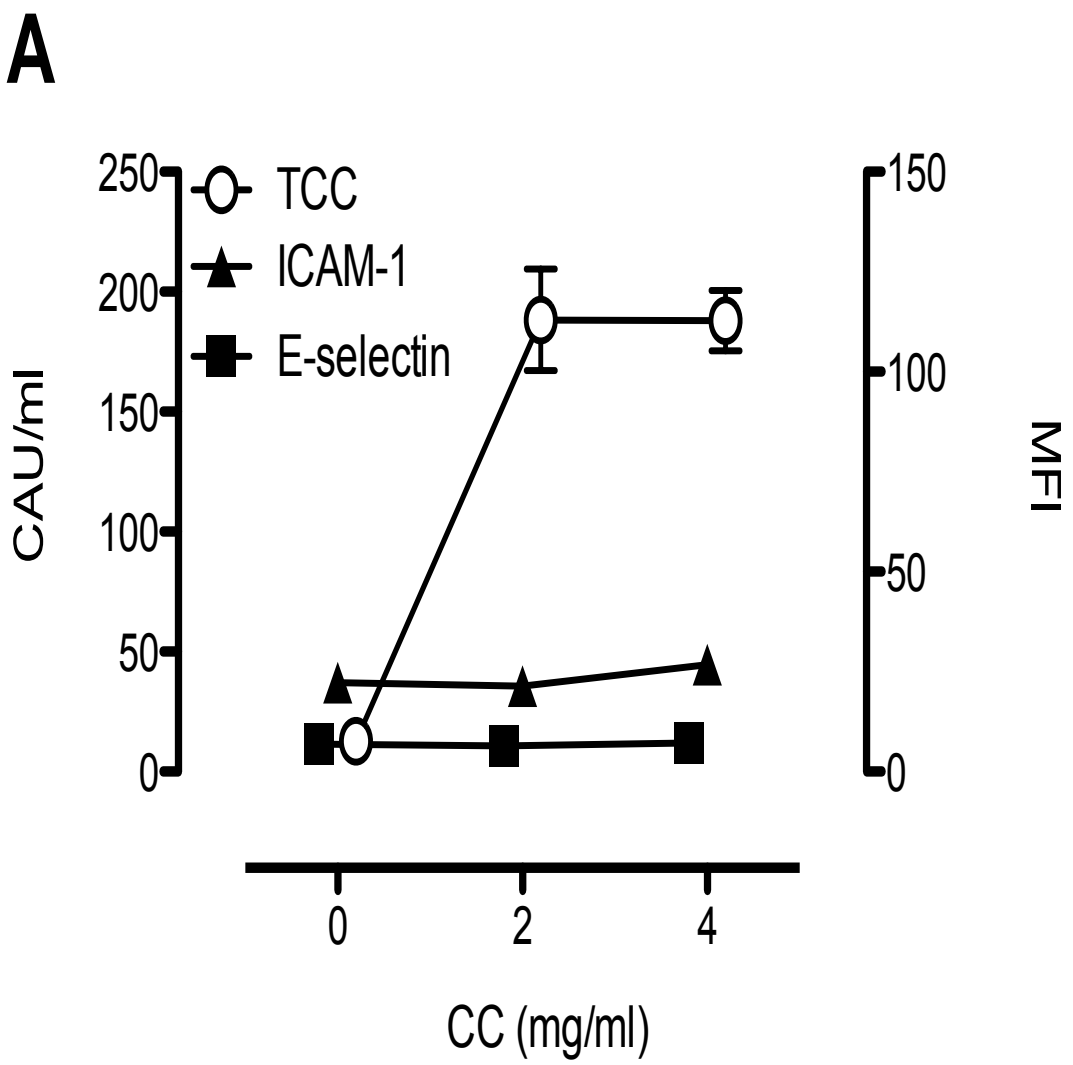
8000
6000
4000
2000
0
2500
2000
1500
1000
500
0
2
CC (mg/ml)

Figure 3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250
200
150
100
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CAU/ml
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E-selectin
150
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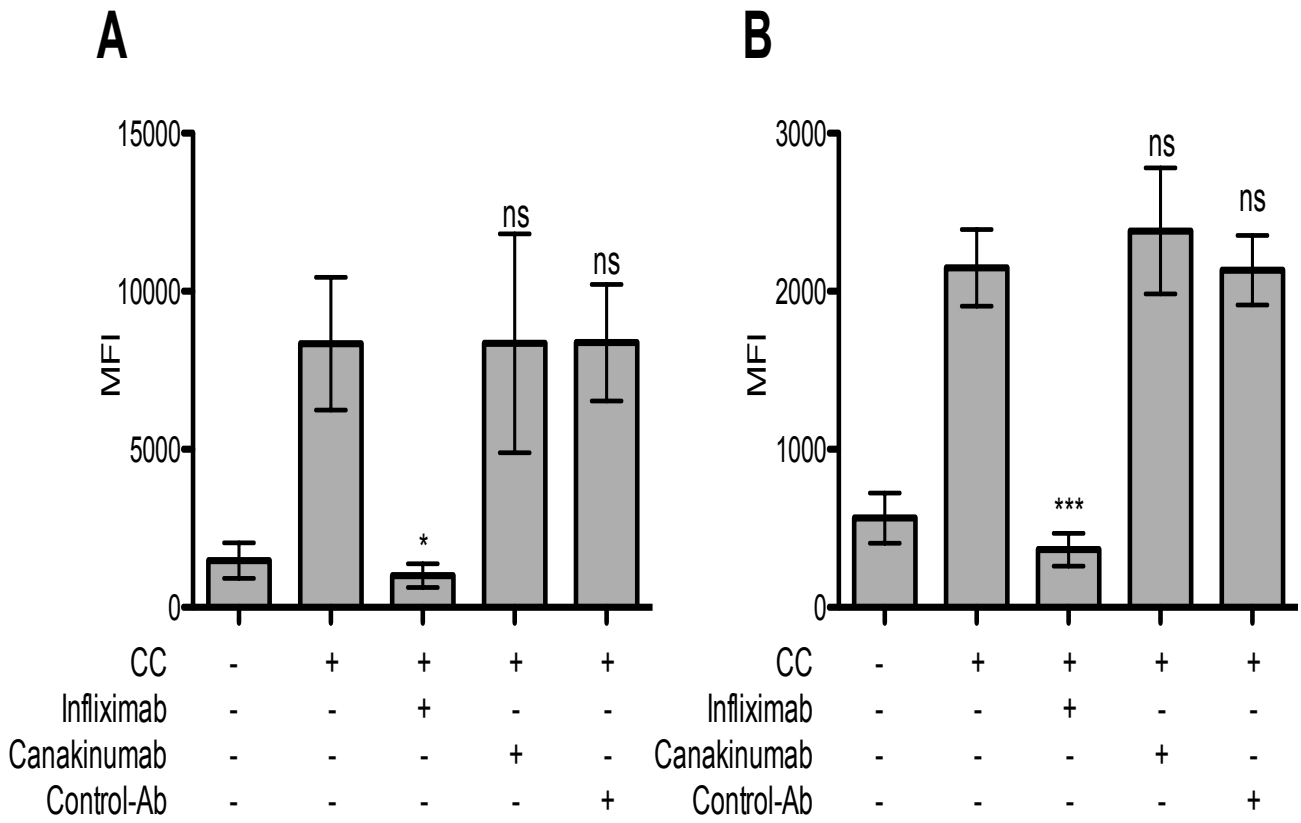
2
4
CC (mg/ml)

Figure 4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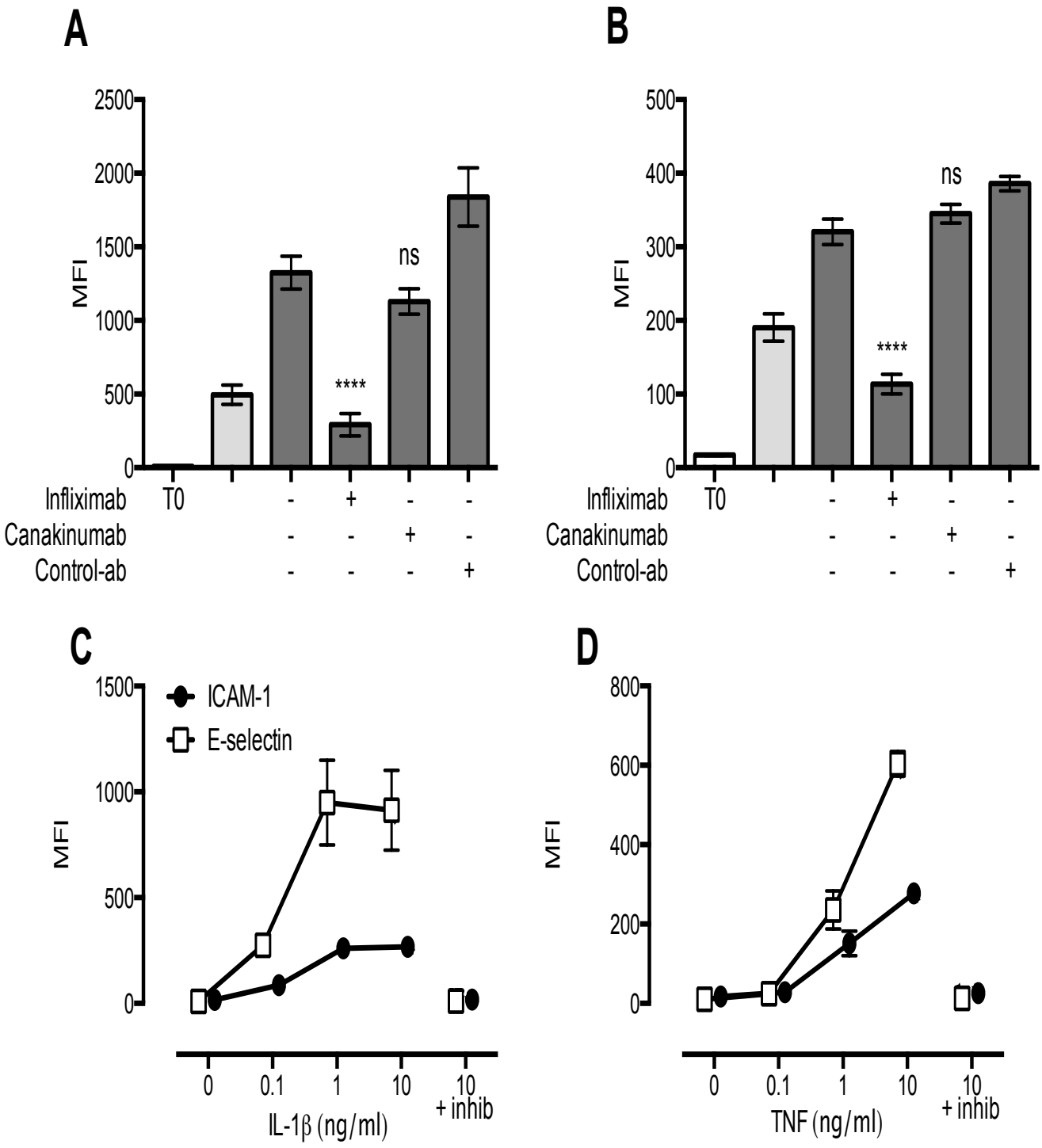
Control-Ab - - - - +

Figure 5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300
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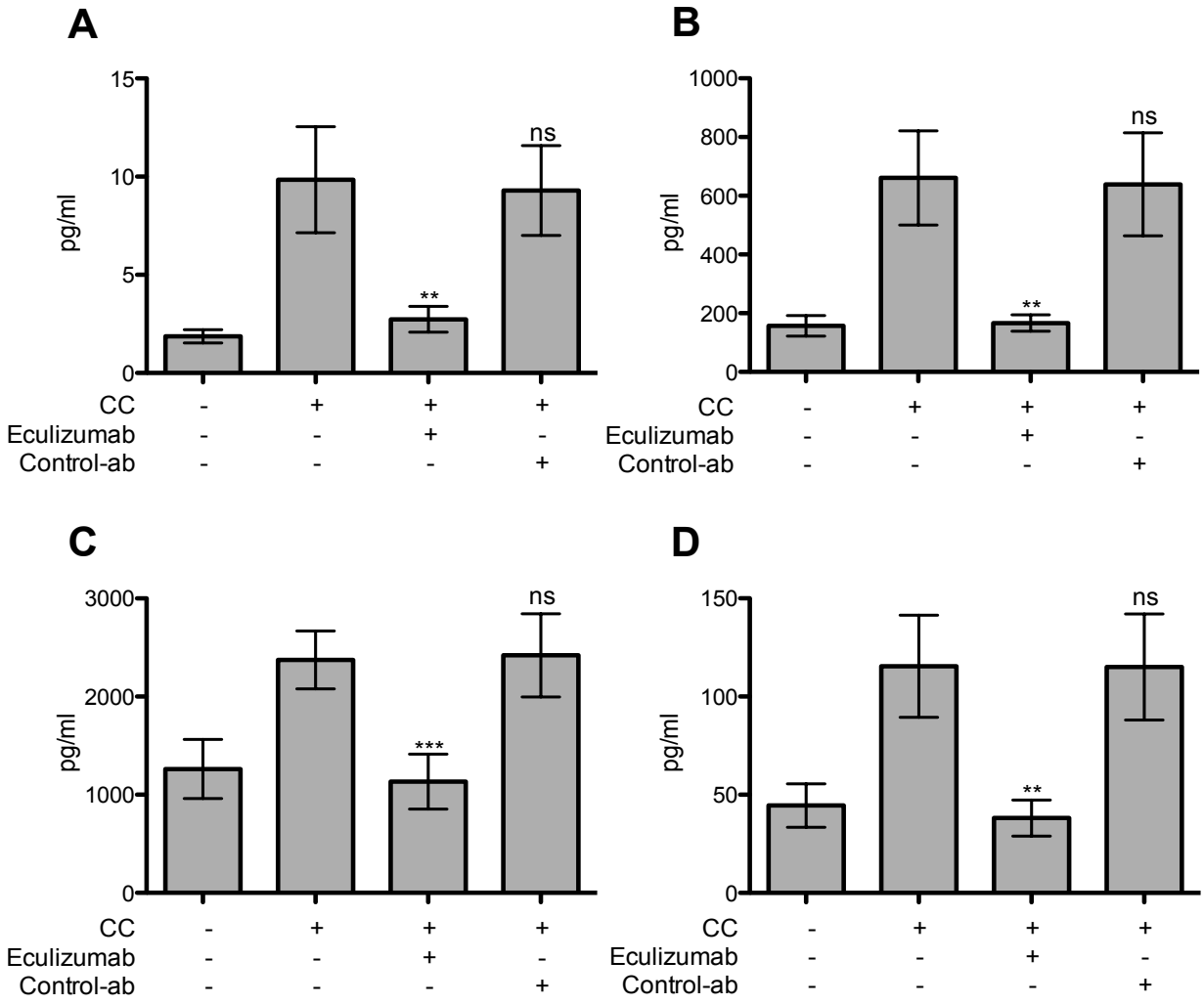
Infliximab T0 - + - -
Canakinumab - - + -
Control-ab - - - +
C
1500
1000
500
0
MFI
ICAM-1
E-selectin
0 0.1 1 10 10 + inhib
IL-1β (ng/ml)
D
800
600
400
200
0
MFI
0 0.1 1 10 10 + inhib
TNF (ng/ml)

Figure 6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CC
Eculizumab
Control-ab