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Neutralizing effects of anti-infliximab antibodies on synergistically-stimulated human coronary artery endothelial cells

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HIGHLIGHTS

- TNF-α, IL-1β and SAA synergistically elevated IL-6, IL-8, GM-CSF and GRO-α release in HCAEC supernatants.
- Infliximab was effective in lowering synergistically elevated IL-6, IL-8, GM-CSF and GRO-α release.
- IL-1Ra, IL-1α, VCAM-1, MCP-1, IL-10 and IL-17A were increased, but no synergistic responses were observed.
- An isolated fraction of polyclonal anti-infliximab antibodies was capable of neutralizing infliximab.
- Anti-infliximab antibodies in the circulation are potentially important for promoting atherosclerosis in patients.

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ABSTRACT

Background and aims: Patients with rheumatic diseases have an increased risk of atherosclerosis with up-regulated serum amyloid A (SAA), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), which were reported to activate human coronary artery endothelial cells (HCAEC). We aimed to investigate the effects of TNF-α inhibitor infliximab and anti-infliximab antibodies on the TNF-α/IL-1β/SAA activated HCAEC.

Methods: HCAEC were incubated with TNF-α, IL-1β, SAA, infliximab, anti-infliximab antibodies and their combinations. The protein levels of pro- and anti-atherogenic analytes were measured in supernatants using ELISA and multiplex assays, while mRNA expression was determined by RT-PCR. Anti-infliximab antibodies were purified from sera samples by affinity chromatography.

Results: IL-6, IL-8, GM-CSF and GRO-α were synergistically up-regulated in triple stimulation with TNF-α, IL-1β and SAA, while their levels in solely SAA- or TNF-α-stimulated HCAEC did not increase. IL-1Ra, IL-1α, VCAM-1, MCP-1, IL-10 and IL-17A were increased, but no synergistic responses were observed in triple stimulation. Infliximab was effective in lowering the synergistic effect of IL-6, IL-8, GM-CSF and GRO-α in triple stimulation, while anti-infliximab antibodies restored the levels. The changes were confirmed at the mRNA expression level for IL-6, IL-8 and GM-CSF.

Conclusions: Triple stimulation with TNF-α, IL-1β and SAA synergistically elevated IL-6, IL-8, GM-CSF and GRO-α release in supernatants of HCAEC, with infliximab substantially inhibiting their levels. An isolated, enriched fraction of polyclonal anti-infliximab antibodies was capable of neutralizing infliximab, in the presence of TNF-α/IL-1β/SAA. The long-term presence of anti-infliximab antibodies in the circulation of patients with chronic rheumatic diseases is potentially important for promoting the atherosclerotic process.

1. Introduction

Patients with systemic immune mediated inflammatory diseases, such as rheumatoid arthritis (RA), ankylosing spondylitis and psoriatic arthritis, have an increased risk of atherosclerosis and early development of cardiovascular disease [1–6]. Studies have shown that inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), are associated with impaired endothelium vasodilatation/dysfunction [7].

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When endothelial dysfunction occurs, the expression of pro-inflammatory cytokines and cellular adhesion molecules is up-regulated [8]. Inflammatory "signals" from exogenous and endogenous sources can stimulate endothelial cells, which play a critical role in the process of atherosclerosis with pro-inflammatory, activated endothelial phenotype controlling attraction, trafficking and migration of leukocytes into subendothelial space/vessel wall with expression of chemokines and adhesion molecules. The local, endothelium derived up-regulation of pro-inflammatory mediators - cytokines, can contribute to the local paracrine milieu of cytokines, perpetuating a chronic inflammatory state, and fosters atherosclerotic lesion progression, whereas endothelial derived cytokines can cause escalation of systemic inflammation [9].

All of the above mentioned chronic rheumatic diseases have uncontrolled production of TNF-α and are responsive to anti-TNF-α therapy. Monoclonal antibodies developed as TNF-α inhibitor drugs, including infliximab, are known as an effective treatment in RA patients. They bind to TNF-α and inhibit its activity and slow down clinical disease progression [10]. Moreover, treatment with TNF-α inhibitors (comprising of infliximab, adalimumab, etanercept) demonstrated a significant improvement in endothelial function (measured as flow-mediated dilatation, venous occlusion plethysmography, peripheral arterial tonometry and laser-Doppler iontophoresis) in RA patients in pooled analysis of 20 studies, which included a total of 346 patients [11], with an important note on lack of adequately powered, randomized trials and possible publication bias (due to not including negative results). Similarly, arterial stiffness (measured as pulse wave velocity) was improved after 6 months [12] and 1 year [13,14] of infliximab therapy, but carotid intima media thickness is inconsistently reported as not changed [13] or worsened [15] with the use of infliximab, and improved when different TNF-α inhibitors were used [14,16]. Meta-analyses of TNF-α inhibitors in observational and randomized control studies are also consistently reporting reduced risk of cardiovascular events (myocardial infraction, stroke) with use of these drugs [17,18]. However, it is currently unclear whether this is due to attenuated systemic inflammation or specific TNF-α inhibitor effects at plaque level [4]. While methotrexate is consistently showing similar cardiovascular event reducing risk [18,19], corticosteroid use and non-steroidal anti-inflammatory drugs data in many cases exert just the opposite, possibly due to opposing mechanisms – besides beneficial lowering of systemic inflammation also unfavorable effects on lipid levels, hypertension and other pleiotropic effects of acute and cumulative dose that increase cardiovascular risk [18,20].

Biological drugs, i.e. antibodies, might elicit an immunogenic response and anti-drug antibodies may develop. The risk of immunogenicity is associated with their structure and function (humanization, location of therapeutic target etc.), therapeutic manufacturing processes (chemical, posttranslational modifications etc.), clinical use (route of administration, dose etc.) as well as with patient characteristics (age, genetics, concomitant medication, disease status etc.) [21]. Anti-drug antibodies binding to an iditope of infliximab may neutralize drug target binding, enabling TNF-α to elicit pro-inflammatory effects. Ridker et al. reported that even chronic low grade inflammation may lead to atherosclerosis and the development of cardiovascular diseases [22]. Early anti-infliximab antibody formation increases the risk of adverse drug reactions [23] and, as reported by a recent review, was found associated with lower levels of drug, as well as higher disease activity [24]. Up to now, it remains unclear if development of anti-drug antibodies also enables the reoccurrence of pro-atherogenic effects of TNF-α on the endothelium and could increase the risk of atherosclerotic complications.

Besides TNF-α, another cytokine, critically involved in RA pathogenesis [25,26], interleukin-1β (IL-1β), is also classified as pro-atherogenic cytokine [27].Both cytokines were shown to activate NF-κB signaling in vascular smooth muscle and endothelial cells [28]. Serum amyloid A (SAA) is a major acute phase protein and inflammatory marker, also involved in RA [29] and coronary artery disease [30]. Association of serum SAA concentration with disease activity and risk of cardiovascular and renal involvement was shown in RA patients [31]. SAA stimulates pro-inflammatory cytokines, such as interleukin-1 (IL-1), TNF-α, interleukin-6 (IL-6), chemokines, such as interleukin-8 (IL-8), and initiates activation of NF-κB in endothelial cells [32]. It was shown to be an early causal agent for atherosclerosis in animal models [33,34]. Up-regulated TNF-α, IL-1β and SAA observed in the sera of RA patients were previously reported to activate HCAEC [35–37], however, their combined effects at the cellular level remain unclear. In fact, the most important sites for clinically significant atherosclerotic disease in humans are the coronary arteries and thus we used human coronary artery endothelial cells (HCAEC) as our cell culture model, since specifically RA was shown to be an independent risk factor for multi-vessel coronary artery disease [6].

Our aim was to study the individual and combined effects of TNF-α, IL-1β and SAA on HCAEC, evaluate the effects of RA therapy, namely infliximab, on cytokine-stimulated cells, and determine whether isolated anti-infliximab antibodies from RA patients block the effects of infliximab.

2. Materials and methods

2.1. Materials

Lyophilized human recombinant SAA1/2 (Peprotech, EC Ltd., London, UK), TNF-α and IL-1β (both Thermo Fisher Scientific, Massachusetts, USA) were reconstituted in cell culture sterile water according to the manufacturer's instructions to stock solutions of 1 μg/μL, 0.1 μg/μL and 0.1 μg/μL, respectively. Aliquots were stored at −80 °C until used.

Infliximab (Remsima™, Celltrion Healthcare, Incheon, South Korea), solubilized in water, was stored at a stock concentration of 10 mg/mL at 4 °C.

Polyclonal anti-infliximab antibodies were purified by affinity chromatography. Firstly, sera samples of 2 patients with chronic rheumatic diseases, who exhibited positive levels of anti-infliximab antibodies, as previously determined by an *in-house* competitive ELISA, bridging ELISA and reporter gene assay [38], were pooled. The IgG fraction was isolated using MabTrap Kit (GE Healthcare, Chicago, Illinois, USA) which consists of prepacked HiTrap protein G HP column and all suitable buffers for isolation. Later the anti-infliximab antibodies were isolated using an infliximab column. Infliximab column was prepared using cyanogen-bromide activated sepharose (GE Healthcare, Chicago, Illinois, USA) to immobilize infliximab (Remsima™) on sepharose beads through primary amines. Anti-infliximab antibodies were concentrated in phosphate buffered saline and stored at −80 °C.

This study was conducted as part of the National Research Program (#P3-0314) and has been approved by the Slovenian Ethical Committee (#99/04/15).

2.2. Cell culture and treatments

Primary HCAEC were purchased from Lonza Group (Basel, Switzerland). Cells in passage 5 were seeded to 6-well plates (TPP, Trasadingen, Switzerland) and grown to 85% confluency in endothelium growth medium (EGM-2, Lonza, Basel, Switzerland) containing 5% fetal bovine serum (FBS) (Lonza, Basel, Switzerland) at 37 °C in a humidified atmosphere at 5% CO_2. Prior to experiments, HCAEC were serum depleted for 2 h and incubated with different combinations of SAA1/2 (final concentration 500 nM), TNF-α (final concentration 2.5 ng/mL) and IL-1β (final concentration 1 ng/mL). Infliximab was used at a final concentration of 10 μg/mL in combination with the three stimulants. Anti-infliximab antibodies (final concentration 0.55 μg/mL) were used together with infliximab (final

concentration 0.1 μg/mL). All treatments were preincubated for 30 min at 37 °C, before adding to cells. After 24 h, supernatants were collected, centrifuged, aliquoted and frozen at −20 °C. Cells were lysed with Qiazol lysis reagent (Qiagen, Hilden, Germany) and stored at −80 °C.

2.3. Cytokines measurements

IL-6 and IL-8 levels were measured by ELISA (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) according to manufacturer's instruction. Briefly, samples were diluted with standard diluent buffer 1:50 in IL-6 and IL-8 ELISAs and incubated with biotin-labeled conjugates for 2 h. After incubation with streptavidin linked horseradish peroxidase the substrate color was measured at 450 nm with Sunrise Tecan microplate absorbance reader (Tecan, Männedorf, Switzerland). The analyte concentrations were calculated according to dilution factor and standard curve.

Other cytokines (granulocyte-macrophage colony-stimulating factor (GM-CSF), growth regulator oncogene α (GRO-α), interleukin 1 receptor antagonist (IL-1Ra), interleukin 1 alpha (IL-1α), vascular cell adhesion protein 1 (VCAM-1), monocyte chemoattractant protein 1 (MCP-1), interleukin-10 (IL-10) and interleukin-17A (IL-17A)) were measured with multiplex Magnetic LX Screening assay (R&D Systems, Minneapolis, USA) according to manufacturer's instruction. Samples were diluted 1:2 for IL-1Ra, IL-1α, VCAM-1, IL-10 and IL-17A and 1:10 for MCP-1, GM-CSF and GRO-α with diluent and incubated with magnetic beads linked to capture antibodies against measured molecules. In following steps antibodies linked to biotin and streptavidin linked to phycoerythrin were added. Fluorescence was measured with MAGPIX (Luminex, Austin, Texas, USA) and the concentrations were calculated with the system program using 5-parameter logistic standard curves.

In order to compare all the results, the data were normalized to the responses of TNF-α, IL-1β and SAA together (and set at 1) and all other responses were calculated accordingly.

2.4. RNA expression

RNA was isolated using RNeasy Plus Universal Mini kit (Qiagen, Hilden, Germany) and the purity and concentration were estimated spectrophotometrically by Nano Drop (Thermo Fisher Scientific, Massachusetts, USA). RNA was transcribed into cDNA by Reverse Transcription System (Promega, Madison, Wisconsin, USA) and stored at −20 °C. PCR was performed using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) for β-actin, IL-6, IL-8 and GM-CSF using 384-well optical plates with Light-Cycler 480 Instrument II (Roche Life Science, Basel, Switzerland). The temperature of annealing was 60 °C for all. Levels of gene expression were calculated as $2^{\Delta\Delta Ct}$ - the analyzed gene versus the β-actin reference gene values and normalized to untreated control. All reactions were run in triplicates. In Table 1, the sets of specific primers used are listed.

Table 1
PCR primers.

Name	Primer sequences (all 5′ to 3′)
β-actin	F: CCT TGC ACA TGC CGG AG R: ACA GAG CCT CGC CTT TG
IL-6	F: GTA GCC GCC CCA CAC AGA R: CAT GTC TCC TTT CTC AGG GCT G
IL-8	F: ACA CAG AGC TGC AGA AAT CAG R: TTT CAG AGA CAG CAG AGC AC
GM-CSF	F: CTA AAG TTC TCT GGA GGA TGT G R: TGT CTC TAC TCA GGT TCA GG

IL-6: interleukin-6, IL-8: interleukin-8, GM-CSF: granulocyte-macrophage colony-stimulating factor.

2.5. Statistical analysis

All experiments were repeated in biological triplicates. Data are presented as mean ± standard error of the mean (SEM). One-way ANOVA followed by Tukey's multiple comparison test of all pairs were used for statistical analysis. p values of < 0.05 were accepted as statistically significant. Statistical analyses were carried out with Graph Pad Prism 5 (CA, USA).

3. Results

3.1. The effects of infliximab and anti-infliximab antibodies on released protein levels of analytes from TNF-α/IL-1β/SAA stimulated HCAEC

3.1.1. TNF-α/IL-1β/SAA stimulated HCAEC

The inflammatory response of HCAEC stimulated with TNF-α/IL-1β/SAA separately, and in different combinations, was analyzed measuring released protein levels of IL-6, IL-8, GM-CSF, GRO-α, IL-1Ra, IL-1α, VCAM-1, MCP-1, IL-10 and IL-17A.

Our results showed that the analytes grouped into two types: those not significantly elevated with SAA or TNF-α alone, but with a synergistically elevated cytokine levels observed after triple stimulation (Fig. 1), and those elevated with all three single stimulators, but without a synergistic response to triple stimulation (Fig. 2). All combinations of double stimulation resulted in significant increases of all measured cytokines (Fig. 1 and 2) as compared to background levels.

In the first group of analytes there are IL-6, IL-8, GM-CSF and GRO-α. IL-1β alone or in combinations caused significant increase in levels of measured analytes, but also double stimulation with TNF-α/SAA led to significantly higher levels of these analytes (Fig. 1). The released levels of IL-6, IL-8 and GM-CSF in triple stimulation were statistically higher compared to all three double stimulations. The released levels of GRO-α in triple stimulation were statistically higher only compared to TNF-α/SAA double treatment.

In the second group, there are analytes which did not synergistically increase in supernatants after triple stimulation. Those are IL-1Ra, IL-1α, VCAM-1, MCP-1, IL-10 and IL-17A. These analytes were up-regulated with all our treatments, including TNF-α and SAA single treatments, with two exceptions. IL-17A was not up-regulated in TNF-α and SAA single treatments, while MCP-1 was not up-regulated in SAA single treatment. Importantly, TNF-α stimulation induced levels of the secreted analytes similar to IL-1β stimulation (no significant differences observed, except for IL-17A), while SAA stimulation induced a significantly lower response, as compared to IL-1β for all analytes, except for MCP-1. TNF-α stimulation was significantly different to SAA stimulation for released IL-1α and IL-10. All combinations of double stimulation did not further increase the protein levels of IL-1Ra, IL-1α, VCAM-1, MCP-1, IL-10 and IL-17A compared to IL-β alone stimulation. The levels of IL-1Ra, IL-1α, MCP-1, IL-10 and IL-17A in triple stimulation were not significantly higher than the levels in double stimulations, with the exception of VCAM-1 triple stimulation compared to double stimulation with IL-1β/SAA (Fig. 2).

It needs to be pointed out that the released levels of the measured molecules were different, which cannot be observed due to normalization. Specifically, in triple stimulation, we observed the highest IL-6 around 40,000 pg/mL, IL-8 around 80,000 pg/mL, GM-CSF around 6000 pg/mL, GRO-α around 120,000 pg/mL, IL-1Ra around 400 pg/mL, IL-1α around 300 pg/mL, VCAM-1 around 40,000 pg/mL, MCP-1 around 60,000 pg/mL while IL-10 and IL-17A levels were considerable lower (the highest IL-10 release was 26 pg/mL and IL-17A release 122 pg/mL).

3.1.2. The effects of infliximab on TNF-α/IL-1β/SAA stimulated-HCAEC

Infliximab at a concentration of 10 μg/mL was effective in lowering released molecules of both groups of analytes after stimulation with TNF-α alone. Significant changes were observed in the second group

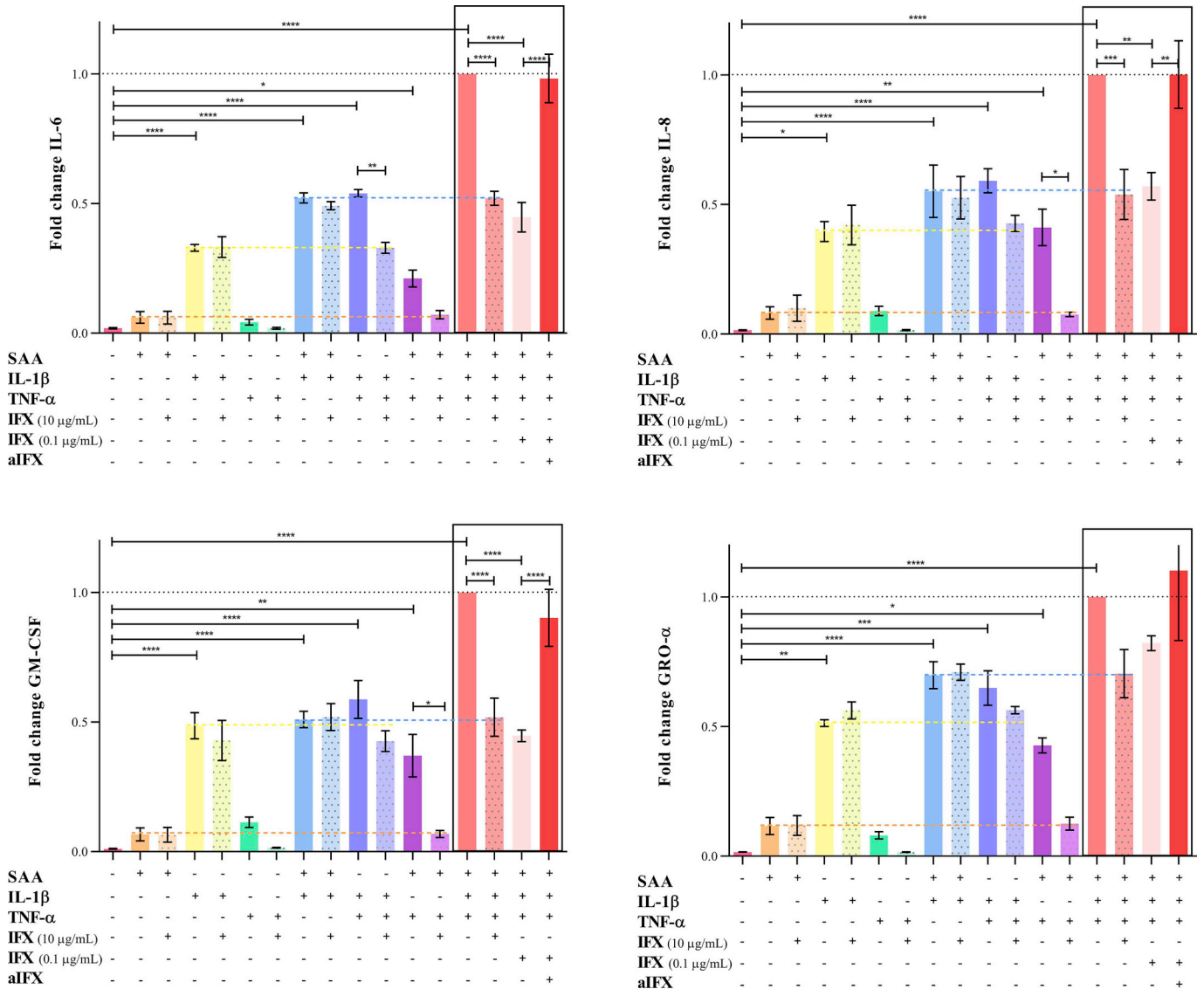

Fig. 1. The effect of infliximab and anti-infliximab antibodies on released protein levels of IL-6, IL-8, GM-CSF and GRO-α from cytokine-treated HCAEC. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Dotted lines represent the infliximab successful inhibition of the TNF-α impact, black squares represent triple stimulation together with infliximab and anti-infliximab effects. SAA: serum amyloid A, TNF-: tumor necrosis factor alpha, IL-1β: interleukin 1β, IFX: infliximab, aIFX: anti-infliximab antibodies (0.55 μg/mL).

(IL-1Ra, IL-1α, VCAM-1, MCP-1 and IL-10, while IL-17A was without any significant change), since the induction with TNF-α alone was more pronounced in that group (Fig. 2). As expected, infliximab did not affect the levels of released analytes after SAA or IL-1β stimulation of HCAEC alone or in combination of SAA and IL-1β (Fig. 1 and 2). When TNF-α was present with a second stimulator (SAA or IL-1β), successful inhibition/neutralization of TNF-α with infliximab was observed. The levels of released molecules returned to the level of the second stimulator alone, some with statistically significant changes (dotted lines in Fig. 1). Similarly, in triple stimulated cells (TNF-α/IL-1β/SAA), addition of infliximab decreased the released analytes to the level of IL-1β/SAA double stimulation, again showing neutralization of the TNF-α effect. The strongest effects were observed in IL-6, IL-8 and GM-CSF levels, which levels in the presence of infliximab decreased by around 50% compared to triple stimulation (Fig. 1). Infliximab alone had no effect on the levels of measured analytes,the responses were statistically not different from background (data not shown). Interestingly, we also tested neutralization of TNF-α with 100x lower concentration of infliximab, using only 0.1 μg/mL of infliximab, and observed similar inhibition of released analytes (Fig. 1).

3.1.3. The effects of anti-infliximab antibodies isolated from patient sera on TNF-α/IL-1β/SAA stimulated HCAEC

The effects of anti-infliximab antibodies were observed in combination with triple stimulation (Fig. 1 and 2). Isolated anti-infliximab antibodies alone had no effect on the levels of measured cytokines (data not shown), however, anti-infliximab antibodies (0.55 μg/mL) did restore the protein levels of IL-6, IL-8, GM-CSF and GRO-α released from HCAEC treated with infliximab and TNF-α/IL-1β/SAA to the same levels present when cells were treated with triple cytokines. This indicates a complete neutralization of infliximab TNF-α inhibition achieved by anti-infliximab antibodies (Fig. 1).

3.2. The effects of infliximab and anti-infliximab antibodies on mRNA expression levels of analytes from TNF-α/IL-1β/SAA stimulated HCAEC

The inflammatory response of stimulated HCAEC was analyzed also for mRNA expression levels of *IL-6*, *IL-8* and *GM-CSF*.

An increase in IL-6, IL-8 and GM-CSF was confirmed at the mRNA expression level following single or double stimulation with IL-1β or triple cytokine stimulation, which is similar to the trend observed in

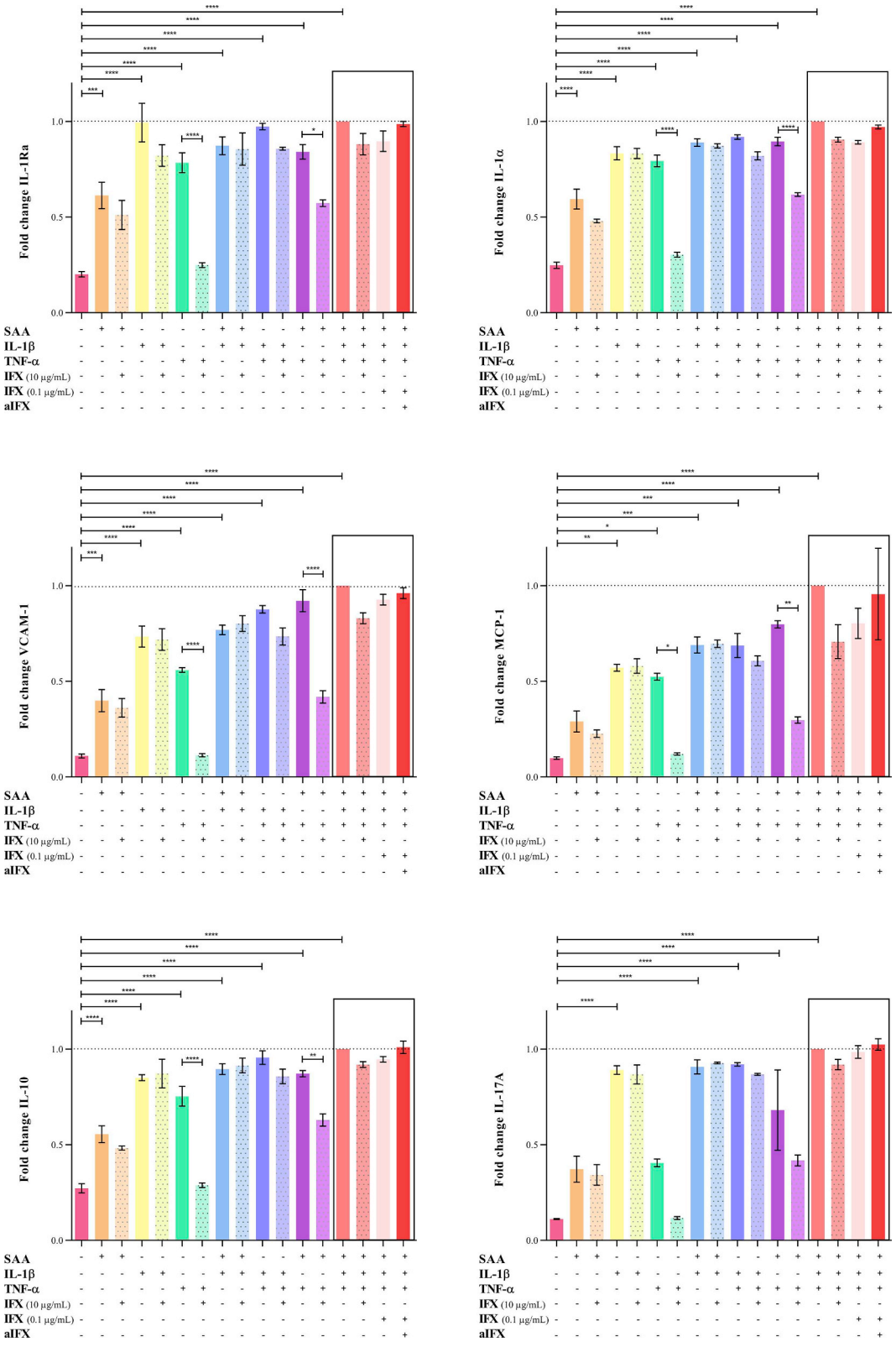

Fig. 2. The effect of infliximab and anti-infliximab antibodies on released protein levels of IL-1Ra, IL-1α, VCAM-1, MCP-1, IL-10 and IL-17A from cytokine-treated HCAEC.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Black squares represent triple stimulation together with infliximab and anti-infliximab effects. SAA: serum amyloid A, TNF-α: tumor necrosis factor alpha, IL-1: interleukin 1β, IFX: infliximab, aIFX: anti-infliximab antibodies (0.55 μg/mL).

released protein levels of analytes. However, at the mRNA expression level at 24 h, the synergistic effect after triple stimulation was not observed.

In addition, infliximab elicited a ~50% inhibition compared to triple stimulated cells, detected as mRNA levels of IL-6, IL-8 and GM-CSF. In addition, anti-infliximab antibodies were capable of

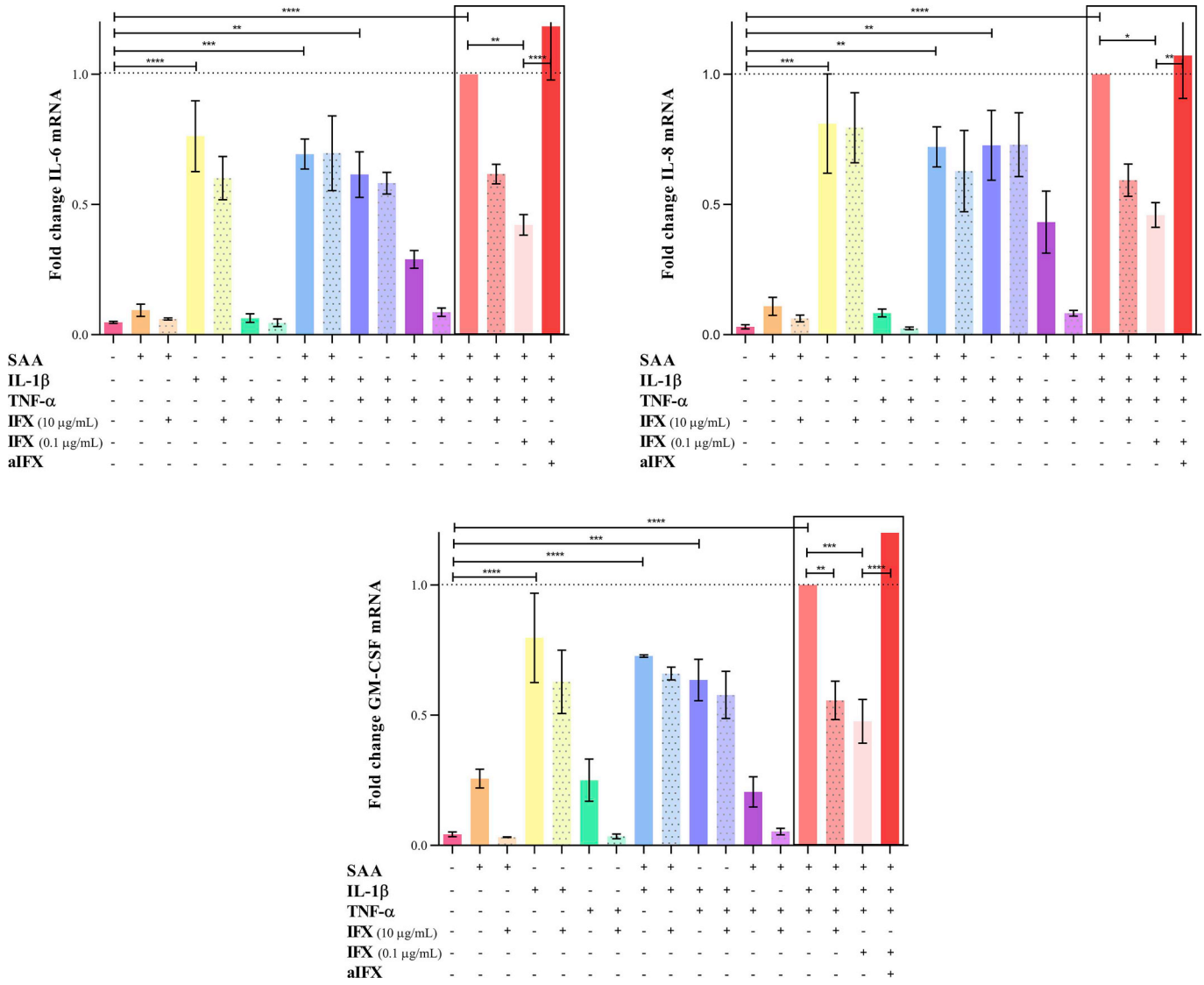

Fig. 3. The effects of infliximab and anti-infliximab antibodies on mRNA expression levels of IL-6, IL-8 and GM-CSF following stimulation of HCAEC. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Black squares represent triple stimulation together with infliximab and anti-infliximab effects. SAA: serum amyloid A, TNF-: tumor necrosis factor alpha, IL-1: interleukin 1β, IFX: infliximab, aIFX: anti-infliximab antibodies (0.55 μg/mL).

significantly inhibiting infliximab effects at the mRNA level, as shown for IL-6, IL-8 and GM-CSF (Fig. 3).

4. Discussion

This is the first report utilizing isolated enriched polyclonal anti-infliximab antibodies from human sera in cultures of endothelial cells, such as HCAEC. The sera comprising anti-infliximab antibodies were previously tested positive with a competitive ELISA, bridging ELISA and reporter gene assay, meaning they are functional and directed against epitopes on infliximab, preventing them to bind TNF-α. Therefore, they are potentially able to neutralize the anti-TNF-α drug *in vivo*, in the patient body [38]. The current study showed that anti-infliximab antibodies from patient sera are capable of functional inhibition of infliximab, resulting in reversal of the full HCAEC activation in a cell culture model. This is in line with Weisshof et al. where TNF-α induced IL-8 secretion from HT-29 colon cancer cells, which decreased in presence of infliximab, and was reestablished in presence of its neutralizing antibodies from sera of patients with inflammatory bowel disease [39].

The emergence of anti-drug antibodies might represent a critical point in the treatment of patients suffering from chronic inflammatory diseases, as they might impact pharmacokinetics of drug enhancing drug clearance, resulting in suboptimal levels of active drug and loss of treatment efficacy [40]. Anti-drug antibody development may also cause hypersensitivity reactions and immune-complex mediated diseases thus decreasing bioavailability with formation of immune complexes [41]. In addition, another effect will influence the anti-drug antibodies binding site - binding idiotope of the drug that may neutralize drug target binding and directly affect clinical efficacy, as also shown for infliximab [24].

The studies determining clinical meaning/impact of anti-drug antibodies are inconsistent with opinion that [42] low anti-drug antibody levels do not impair the efficacy of TNF-α inhibitor therapy, since adequate drug levels remain, but high anti-drug antibody levels impair treatment efficacy by considerably reducing unbound drug levels. In regard to the clinical impact of serum anti-drug antibody levels, there is a need to carefully distinguish between whether the used assays determine clinically important neutralizing anti-drug antibody or not. Some of them report only the presence, and binding (thus forming immune complex and changing pharmacokinetics, which might be clinically corrected with increasing drug dose) while others report

neutralizing antibodies as discussed in our previous paper [38]. Additionally, some studies determined immune complex bound antibodies or others which utilize drug-tolerant assays [43]. Murdaca et al. [24] showed that neutralizing antibodies against infliximab decrease the possibility to achieve minimal disease activity or clinical remission and increase the need for higher dosage. Furthermore, the topic of therapeutic drug monitoring of infliximab in association with anti-drug antibodies for promotion/prevention of atherosclerosis has not yet been adequately addressed. TNF-α inhibitors can induce favorable changes in lipid profiles with alteration of HDL composition, while TNF-α is associated with insulin resistance, another traditional risk factor for atherosclerosis [44]. A meta-analysis confirmed their long term effect to increased HDL but not to LDL or atherogenic index [45]. Thus, the clinically observed reduction in cardiovascular disease does not seem to be explained by the changes in traditional risk factors, but rather by the improvement of systemic inflammation [45,46]. Furthermore, HDL levels were found to be a predictor of clinical response to infliximab [47].

The responsiveness of HCAEC to individual cytokines was previously reported [32,35,36,48,49], however, in the present study, we investigated their combined effects on HCAEC for the first time. The concentrations of TNF-α and IL-1β we used corresponded to the levels observed in RA patients at baseline by Danis et al. [50]. In our study, the cytokines released from stimulated HCAEC have been grouped and analyzed into 2 types - those which are synergistically elevated in TNF-α/IL-1β/SAA stimulation and those which are not. In the first group there are IL-6, IL-8, GM-CSF and GRO-α, which are only slightly responsive to single SAA or TNF-α stimulation, but IL-1β induces them more potently. IL-6, IL-8, GM-CSF and GRO-α were found to be associated with atherosclerotic development and cardiovascular disease in epidemiologic studies [27,51–53]. The second group of measured analytes also consisted of molecules importantly implicated in development of atherosclerosis, but were not synergistically increased in triple inflammatory stimulation and consisted of those inhibiting atherosclerotic development (IL-1Ra, IL-10) and those shown to “activate” it (IL-1α, VCAM-1, MCP-1, IL-17A). Only low levels of released atheroprotective IL-10 [54] and IL-1Ra [51] were observed. Furthermore, the levels of IL-1α, as well as IL-17A, were low in all stimulations in our study, even in combinations. However, adhesion molecule VCAM-1 and chemotactic MCP-1 were expressed at higher levels and showed significantly increased expression in double and triple stimulations.

We used two concentrations of infliximab, whereby 0.1 μg/mL corresponds to the dose determined in serum of non-responding patients, while 10 μg/mL represents the concentration present in responders, as it was reported by Wolbink et al., where the median (interquartile range) 0.5 (0.2–2.2) μg/ml of infliximab is reported in RA non-responders and 3.6 (1.4–8.2) μg/ml in RA responders [55]. Following only TNF-α stimulation, we observed that infliximab inhibits HCAEC activation to background levels and in combinations of stimulants infliximab decreased the levels of measured analytes to only SAA and/or IL-1β stimulation. This reduction of TNF-α effect was present with both concentrations of infliximab to a similar level, showing that we reach successful inhibition of 2.5 ng/mL of TNF-α already with 0.1 μg/mL of the infliximab. It is hard to compare this stimulation directly with *in vivo* data, as studies report up to 1000x different levels of TNF-α in sera of RA patients [50,56]. Calculating from molecular weight of infliximab, which is 150 kDa while TNF-α is 17 KDa, we had 453x more infliximab molecules than TNF-α molecules in the cell culture medium when cells were stimulated with 10 μg/mL of infliximab, and 4.5x more, when stimulated with 0.1 μg/mL of infliximab.

The concentration of anti-infliximab antibodies used to block the effects of infliximab on TNF-α stimulated cells was determined from competitive ELISA, back-calculating concentration from the titer where 100% inhibition of binding of HRP-labeled infliximab to TNF-α was observed. In our sample, the inhibition was reached with 0.55 μg/mL of anti-infliximab antibodies and thus this concentration was used for *in vitro* experiments. The concentration of isolated anti-IFX antibodies was measured with *in-house* bridging ELISA using the MA-IFX10F9 standard developed at KU Leuven [57,61]. Competitive ELISA is our *in-house* assay and its results highly correlate with functional reporter gene assay results, enabling the determination of only neutralizing anti-infliximab antibodies [38].

Our results demonstrate that clinically important concentrations of infliximab [58] prevent TNF-α- stimulated effects in our cell model. When HCAEC are stimulated with combinations of cytokines, infliximab is able to neutralize the extent that is contributed by TNF-α stimulation, even more in IL-6, IL-8, GM-CSF, GRO-α, where infliximab excludes a synergistic response. In a similar study, Rios-Navarro et al. evaluated the impact of adalimumab, infliximab and etanercept on interactions between human leukocytes and HUVEC activated with TNF-α. They showed a decrease of leukocyte adhesion to the level of background [59]. Using mouse anti-human TNFR1 and TNFR2, Qiu et al. showed the inhibition of TNF-α-induced adhesion molecules, IL-6 and GM-CSF, in HCAEC [48].

To conclude, TNF-α, IL-1β and SAA synergistically elevated IL-6, IL-8, GRO-α and GM-CSF release in supernatants of HCAEC. Infliximab was capable of functionally inactivating the TNF-α effect on HCAEC, used alone or in combination with SAA and/or IL-1β. According to our results, it will be interesting to investigate if IL-1β inhibitors are also capable of inhibiting the synergistic triple effects (TNF-α/SAA/IL-β) described in the current report, as the CANTOS study recently described anti-IL-1β antibody reduces cardiovascular event rates in patients with history of myocardial infarction [60]. An isolated fraction of polyclonal anti-infliximab antibodies was capable of neutralizing the infliximab effects in HCAEC, allowing for the reversal of the TNF-α stimulating effect to increase cytokine levels back to triple stimulated (TNF-α/IL-1β/SAA) HCAEC levels. This suggests that long-term presence of neutralizing anti-infliximab antibodies in the circulation of patients with chronic rheumatic diseases could promote chronic inflammation, not only in primary disease, but also in atherosclerosis *in vivo*, which would need to be explored further in the future.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

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