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The effects of autologous serum supplementation on cytokine release in PBMC culture: the role of complement

PhD thesis

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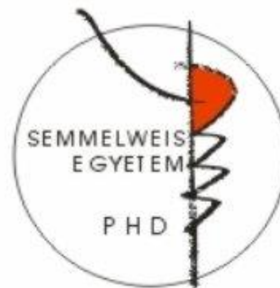

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List of Abbreviations

ARDS: acute respiratory distress syndrome

C1-INH: C1-inhibitor

CS: cytokine storm

C3aR: C3a receptor

C5aR: C5a receptor

DAMP: damage-associated molecular pattern

D-PBS: Dulbecco’s phosphate-buffered saline

EDTA: ethylenediaminetetraacetic acid

FBS: fetal bovine serum

HRP: horseradish peroxidase

IFN: interferon

IL: interleukin

JAK/STAT: Janus kinase/Signal transducer and activator of transcription

MAC: membrane attack complex

MAPK: mitogen-activated protein kinase

MASP: MBL-associated serine protease

MBL: mannose-binding lectin

NF-κB: nuclear factor-κB

NK cell: natural killer cell

PAI-1: plasminogen activator inhibitor

PAMP: pathogen-associated molecular pattern

PBMC: peripheral blood mononuclear cell

PRR: pattern recognition receptor

RBC: red blood cell

RPMI-1640: Roswell Park Memorial Institute 1640 medium

SEM: standard error of the mean

TLR: Toll-like receptor

TMB: 3,3',5,5'-tetramethylbenzidine

TNF: tumor necrosis factor

1. Introduction

1.1. General characterization of the complement system

The complement system is a highly conserved, well defined and tightly regulated network of proteins, which – as the oldest part of the innate immune system – serves as the first line of defense against invading pathogens. The name of the system comes from its ability to „complement” the antibacterial properties of antibodies in serum (1). The network consists of more than 50 proteins (2) – components, regulators, membrane proteins and receptors alike – which components activate each other by cascade-like limited proteolysis (**Fig. 1**). Mostly, but not solely these proteins are synthesized by the liver in zymogen state and exist in fluid phase or in membrane-bound form (3).

Although the complement system lacks specificity to certain targets, it plays a vital role in host defense against foreign invaders. Depending on the surface of the pathogen, the activation process could unfold on three different yet interconnected pathways (**Fig. 1**) namely, the classical, the lectin and the alternative pathways (4). The activated system is responsible for the initialization of inflammatory processes mainly through C3a and C5a anaphylatoxins, which are generated by the cleavage of the C3 and C5 complement components and act as signal molecules for the immune system. The proteins stimulate the C3aR (for C3a) and C5aR (for C5a) anaphylatoxin receptors, which are located mainly on various immune cells but also on body cells (5).

The activated complement is also responsible for the elimination of bacterial, fungal and viral pathogens as well as damaged or malignus cells through the assembly of the membrane attack complex (MAC, C5b-9), which can form a pore in the cell membrane that causes osmolysis, and/or phagocytosis by antigen-presenting or other immune cells following opsonization (6). The defense mechanism is supported by the adaptive immune system due to the connection between these antigen-presenting cells and lymphocytes (7).

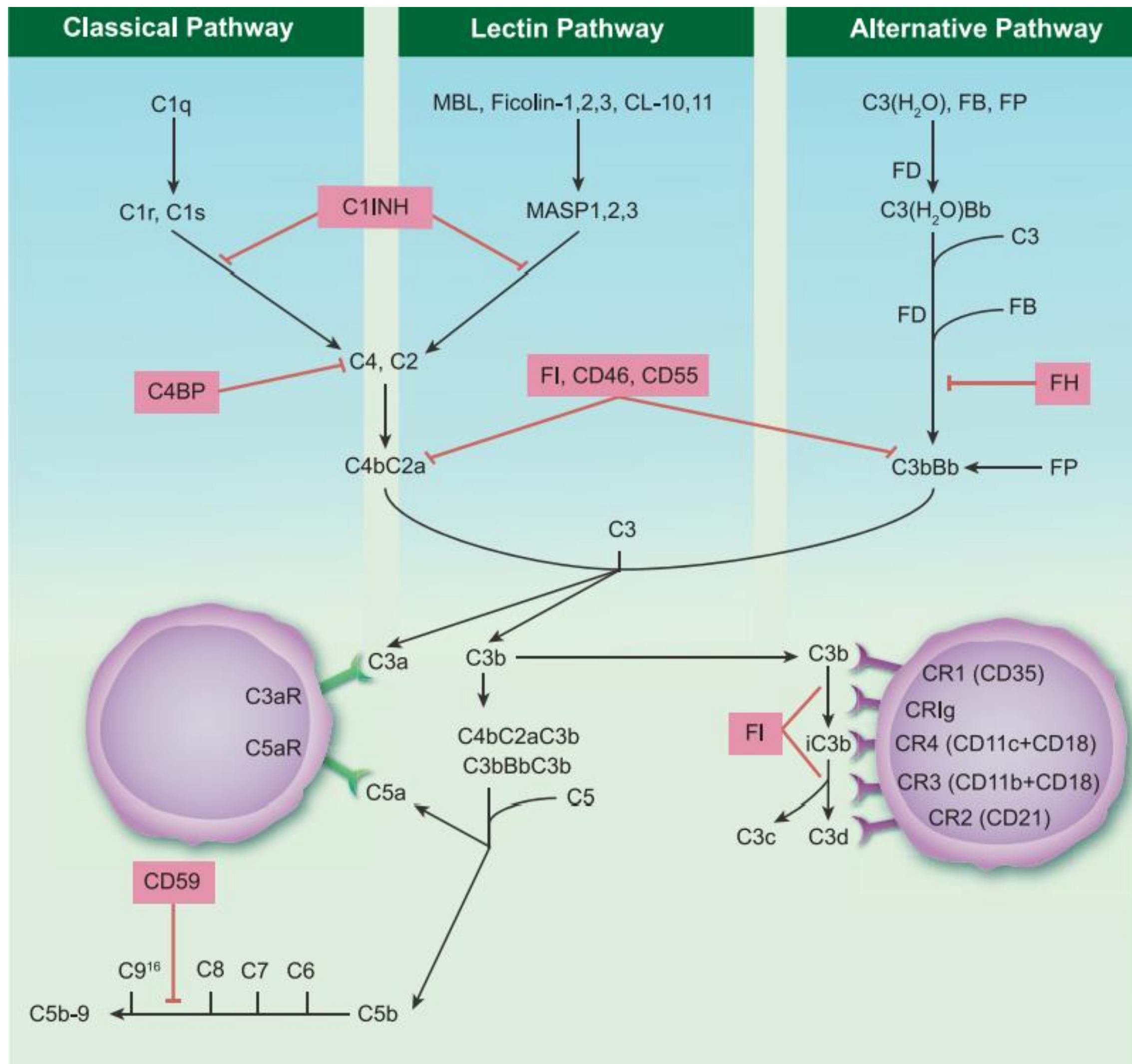

Figure 1. The activation cascade of the complement system: C1INH: *C1 inhibitor,* ***C4BP:*** *C4b binding protein,* ***FI:*** *factor I,* ***FB:*** *factor B,* ***FP:*** *factor P (properdin),* ***FD:*** *factor D,* ***FH:*** *factor H,* ***MBL:*** *mannose-binding lectin,* ***MASP:*** *MBL-associated serine protease,* ***C3aR/C5aR:*** *C3a/C5a receptor (4)*

1.1.1. Regulation of the complement activation

Tight regulation of the complement system is pivotal for the safety of the host. In the event of unregulated activation, the system can damage healthy host cells, therefore, the complement system – like every system that has such a large impact on the host – has serious regulatory measures. These mechanisms help take on the invading pathogens more effectively, while protecting the innocent bystander cells (6, 8).

There are several fluid phase and membrane-bound regulator proteins, which inhibit the activation of the complement system. The inhibition is mainly achieved by two

different ways. A group of regulators increases the rate of dissociation of the classical/lectin and the alternative pathway C3 and C5 convertases, the others are mostly cofactors of Factor I (serine protease)-mediated cleavage of covalently bound C3b and C4b, resulting in proteins, which are unable to form C3 or C5 convertases (3, 6). In most cases the purpose is to effectively inhibit C3 breakdown, since it has the central role in the functioning of the complement system, but there are regulatory mechanisms that are not revolve around C3, such as inhibition of the C1/MASP-MBL complex or inhibition of MAC formation and/or function (9, 10). Absence or malfunction of regulatory processes can result in accumulation of cell debris and various autoimmune diseases (11, 12).

1.2. The complex network of cytokine proteins

Cytokines are a large group of small proteins, which are irreplaceable elements in cell signaling and intercellular communication. Their functions include the regulation of immune cell proliferation and differentiation, the control of chemotaxis and angiogenesis and, most importantly, the regulation of innate immunity and inflammatory processes. These molecules exert various effects depending on the type of cytokine, the presence or absence of other cytokines and the target cell (pleiotropic effect), and also possess autocrine, paracrine and endocrine activity. Cytokines are also able to amplify the production and secretion of each other, resulting in a continuous and steady supply of the necessary proteins. Some cytokines have limited sequence similarity and bind to completely different receptors yet use the same intracellular signaling pathways; therefore, these proteins can be considered as complex and overlapping networks of important regulator molecules with a certain level of redundancy in each group. The majority of cytokines are produced by a wide variety of cell types but most commonly immune cells (13-16).

1.2.1. Classification of the cytokine proteins

Although the complexity of these networks has made it difficult to classify cytokines, several families (with more or less the same activity) have been identified over the years. The main groups include the interferons, the interleukins, the chemokines and the tumor necrosis factors (TNFs) (17).

1.2.1.1. Interferons

Interferons (IFNs) are known for their antiviral and anti-proliferative activity as they play a central role in the initiation and regulation of the innate immunity through the activation of macrophages, natural killer cells (NK cells) and T lymphocytes. The family contains more than 20 proteins, and have been classified into three different subgroups based on their receptor specificity, namely type-I, type-II and type-III IFNs. Type-I (IFN-α, IFN-β) and type-III (IFN-λ) interferons are responsible for the inhibition of viral infection of healthy cells, while type-II interferons (IFN-γ) are involved in immune and inflammatory responses (13, 18, 19).

1.2.1.2. Interleukins

Contrary to interferons, interleukins (ILs) are a huge group of more than 50 cytokines, which can be divided into 4 different groups, and their effects are much more diverse than the former. These cytokines play a crucial role in cell proliferation, maturation and migration as well as in the activation and differentiation of immune cell. Since many of these immunomodulatory proteins are pro-inflammatory – although there are some anti-inflammatory representatives – they also play a pivotal role in virtually all aspects of inflammation (13, 20).

Some notable interleukins are the IL-1α, IL-1β, IL-4, IL-6 and IL-10. IL-1α and IL-1β, which are both part of the IL-1 family of cytokines, are responsible for the development of inflammation and the promotion of fever and sepsis by signaling through the same receptor (IL-1R1). IL-1β is the best characterized member of the IL-1 family and is also one of the earliest mediators of inflammation (21-23). IL-6 is another early pro-inflammatory cytokine secreted during the initial stages of inflammation. IL-6 has several functions such as upregulating complement 5a receptor (C5aR) on endothelial cells, the promoting megakaryocyte maturation and T cell differentiation, increasing vascular permeability and altering acute phase protein levels (24, 25).

IL-4 is one of the anti-inflammatory cytokines responsible for the regulation/mitigation of inflammation through alternative activation of macrophages (resulting M2 anti-inflammatory macrophages), and direct downregulation of pro-inflammatory cytokine (IL-1α, IL-1β, IL-6, TNFα) synthesis and pro-inflammatory chemokine (IL-8) inhibition to mention a few (26, 27). IL-10 is also a known anti-

inflammatory cytokine as it serves as an inhibitor of pro-inflammatory interleukin and chemokine gene expression needed for a full immune response. This cytokine also interacts with many different cell types but their main targets are the macrophages as these immune cells express more IL-10 receptors (IL-10R) than any other cell. Interestingly it was reported that IL-10 also has a significant impact on mitigating neuronal damage during inflammatory processes and also regulates homeostatic mechanisms (28-30).

1.2.1.3. Chemokines

Along with interleukins, chemokines are the most important cytokines in this vast network of regulator proteins, which include more than 40 proteins divided into four different groups depending on the spacing between the first two cysteine residues. These potent chemoattractants are responsible for the control of cell migration (mainly immune cell), embryogenesis and development, and the function of the innate and adaptive immune system. The majority of chemokines are generally recognized to have pro-inflammatory properties as these cytokines are released in response to a pathogen infection or injury, resulting in the recruitment of different immune cells (neutrophil granulocytes, monocytes/macrophages and lymphocytes) to the site of the infection or injury. While most cytokines possess pleiotropic effects, the recruitment of immune cells by chemokines can exhibit a high degree of selectivity towards specific cell types. IL-8 – one of the most important chemokines – is, for example, a major chemoattractant of neutrophil and eosinophil granulocytes and monocytes and is able to induce massive cytokine secretion from them (13, 31-33).

1.2.1.4. Tumor necrosis factors

One of the best known and highly important pro-inflammatory cytokines are the tumor necrosis factors. TNFs play a vital role in initiating the cytokine response as these proteins are among the first to appear at the site of the infection; therefore, they are considered central cytokines in both acute bacterial and viral diseases. The name tumor necrosis factor refers to their ability to suppress tumor cell proliferation and induce tumor regression but they also play a role in body development and immunity. The family consists of 19 members, which can signal through 29 receptors but mainly through tumor necrosis factor receptor 1 (34, 35).

1.3. In the center of inflammation: the contribution of complement and cytokine proteins

1.3.1. Characterization of the inflammation

The release of the previously mentioned anaphylatoxin and cytokine proteins is strongly related to inflammation, a complex biological protective response of the body to harmful stimuli such as the appearance of pathogen-associated molecular patterns (PAMPs, e.g.: pathogen invasion), as well as the appearance of damage-associated molecular patterns (DAMPs, e.g.: damaged host cell) or drug administration. Inflammation can be characterized by five symptoms namely redness, pain, heat/fever, swelling or edema and loss of function. Initially a local skin and/or other tissue reaction takes place at the site of infection, injury or drug administration, resulting in increased blood flow that enables plasma proteins and immune cells to reach the site easier. Also, it increases local temperature and generates pain that alerts the host to the pathogen infection or injury. Occasionally, local organ functions suffer from the consequences of the response, particularly when edema or swelling occurs (13, 36, 37).

1.3.2. Mechanism of inflammation

At the beginning of inflammation, the harmful stimuli activate intracellular signaling (e.g.: through Toll-like receptors (TLRs)) and the production of inflammatory mediators (anaphylatoxin, cytokine), which in turn trigger/further amplify many inflammatory signaling pathways but mainly the NF-κB, MAPK, and JAK-STAT pathways (**Fig. 2**). The protective nature of this mechanism is a regulated inflammatory response (acute inflammation) that resolves within a certain amount of time (usually few days) in normal circumstances. However, an unregulated inflammatory response can cause tissue damage and can develop into chronic inflammation (that lasts for months or years), which may lead to a number of diseases like type-2 diabetes, cardiovascular disease, neurodegenerative and autoimmune diseases or even various types of cancer (38). Therefore, few hours after the onset of inflammation, repair mechanisms are initiated including decreased production of pro-inflammatory mediators, the induction of apoptosis of recruited immune cells, and the secretion of anti-inflammatory mediators, which aim to restore integrity and function of tissues and organs (36, 39).

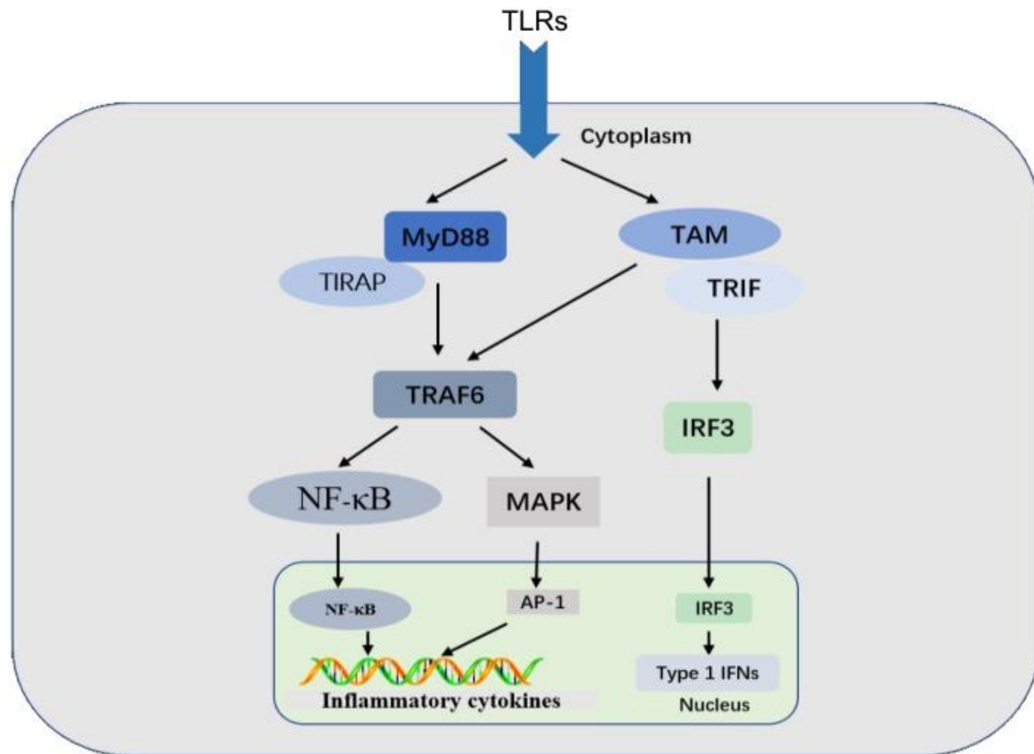

Figure 2. TLR signaling: MyD88: *Myeloid differentiation primary response 88,* ***TIRAP:*** *TIR domain containing adaptor protein,* ***TRAF6:*** *TNF receptor associated factor 6,* ***NF-κB:*** *Nuclear factor kappa-light-chain-enhancer of activated B cells,* ***MAPK:*** *Mitogen-activated protein kinase,* ***AP-1:*** *Activator protein 1,* ***TAM:*** *Tyro3, Axl, and Mer receptor,* ***TRIF:*** *TIR-domain-containing adapter-inducing interferon-β,* ***IRF3:*** *Interferon regulatory factor 3,* ***IFN:*** *Interferon (36)*

1.3.3. The role of the complement system in inflammation

The complement activation can also contribute to inflammation by the production of anaphylatoxins. C3a, but more importantly C5a are key proteins in these processes as both can initiate mast cell degranulation via C3a and C5a receptors (C3aR, C5aR) resulting in the secretion of important pro-inflammatory mediators such as histamine and several pro-inflammatory cytokines (40, 41). Furthermore, C5a is also reported to be a potent chemoattractant for neutrophil and eosinophil granulocytes, monocytes and T lymphocytes (42) and both anaphylatoxins are capable to increase vascular permeability, induce smooth muscle contraction and activate other immune cells (25, 43).

1.3.4. The threat waiting to destroy: the cytokines storm

As it was mentioned above the cytokine proteins can also greatly contribute to inflammation. There are many pro- and anti-inflammatory proteins among them, which are activated by several mechanisms (e.g.: TLR signaling). The secretion of cytokine molecules starts locally and, in most cases, does not increase further, but there are occasions, when the cause of inflammation cannot be eliminated/eradicated quickly (e.g.: resistant pathogens, serious infection and/or toxic particles/pathogen components). As a solution to the problem the reaction intensifies over time as more and more regulatory molecules (and therefore immune cells) begin to flood the affected area up to a point where the initially local response slowly begins to spill into the systemic circulation resulting in a body wide reaction. This phenomenon of uncontrol production of regulatory molecules is called cytokine storm (CS) and can cause life threatening conditions such as multiple organ failure (usually renal, hepatic or pulmonary) or even death (13, 44-46).

1.3.5. Interactions between the complement and other systems

The complement system has been reported to be related to other systems like coagulation (**Fig. 3**). There are similarities in pathophysiological roles of these separate systems since both serve as a defense mechanism, can only be initiated in the presence of a foreign or altered cell surface, both systems are activated in a cascade manner, or regulatory mechanisms and molecules shape the extent of activation (47, 48).

Plasma kallikrein is able to cleave complement C5 and is also reported to influence the activation of the C3 molecule. Thrombin is also reported to cleave C3 and C5, and the C5a generated by this form of cleavage has a chemotactic effect on neutrophil granulocytes. Several other activated components of the coagulation system (FIXa, FXa, FXIa) and plasmin (from fibrinolysis) can also cleave C3 and C5. Activated coagulation factor XIIa can activate the C1 complement complex thereby initiating the classical pathway of the complement system. Interestingly, from the complement side, C1-inhibitor (serine protease inhibitor, C1-INH) can reduce the activity of FXIa, FXIIa and kallikrein. The lectin pathway can also be initiated by the interaction of the pattern recognition receptors (PRRs) and fibrinogen/fibrin, yet from the complement side the MASPs (MBL-associated serine proteases) are also reported to play a role in the activation of thrombin, fibrinogen, and coagulation factor XIII (mainly MASP-1, but also

MASP-2). The C3a anaphylatoxin can also directly activate platelets, and C5a can induce the procoagulant phenotype of mast cells by upregulating the plasminogen activator inhibitor (PAI-1) protein, which results in a lower-level plasmin accumulation at the site of inflammation (47-50).

Based on the relationship between the two systems, it can be concluded that the complement activation can trigger the coagulation cascade and vice versa. Therefore, the inhibition of the complement system may not be sufficient to reduce/mitigate the severity of adverse effects or to slow the onset or progression of inflammation, as there appear to be many opportunities to overcome the inhibition through functional redundancies.

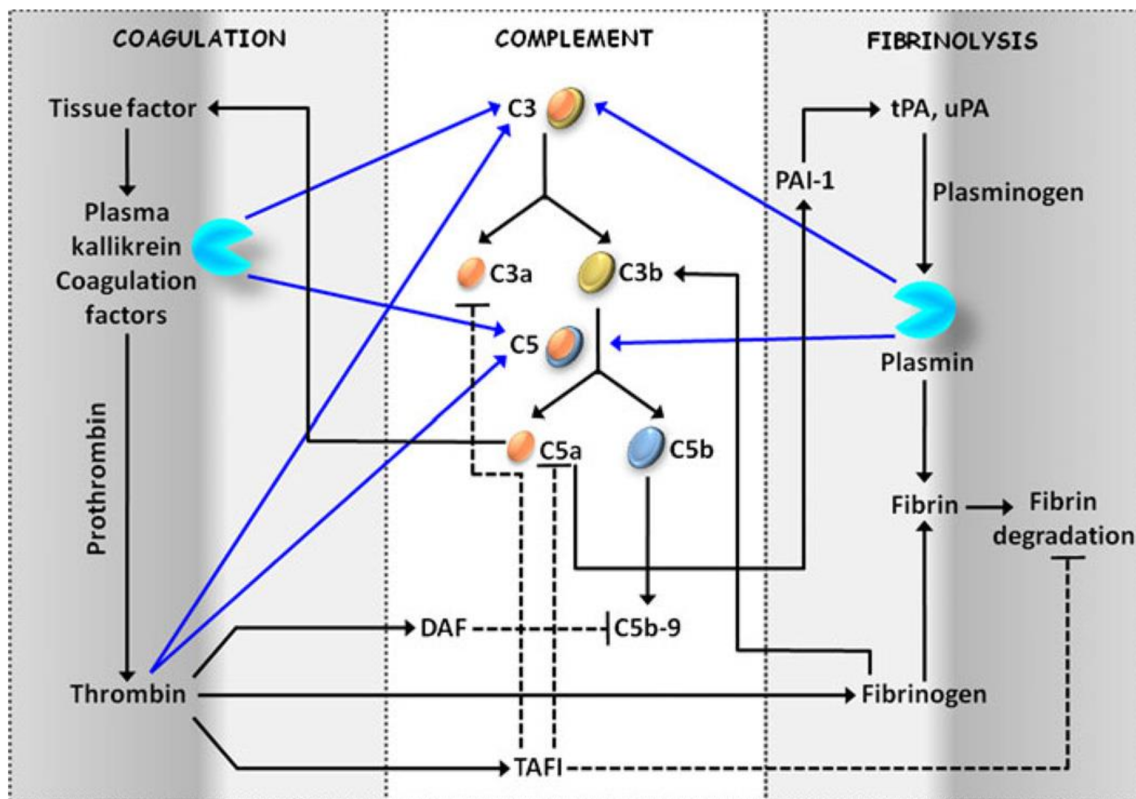

Figure 3. Interconnections of complement, coagulation and fibrinolysis pathways: tPA: *Tissue-type plasminogen activator,* ***uPA:*** *Urokinase,* ***PAI-1:*** *Plasminogen activator inhibitor-1,* ***DAF:*** *Decay-accelerating factor,* ***TAFI:*** *Thrombin activatable fibrinolysis inhibitor (47)*

1.4. The importance of the cytokine storm in the COVID-19 disease

It is well-known that infection with COVID-19 (caused by SARS-CoV-2 virus) can lead to the manifestation of symptoms such as fever, cough, myalgia, fatigue, and dyspnea. In some cases, these symptoms may progress to acute respiratory distress syndrome (ARDS) or multiple organ failure, although this outcome is rare (51-55). There is a considerable range of individual differences among patients, with some experiencing severe symptoms, while others may have only moderate, mild, or no symptoms at all. The severity of the disease is closely associated with the occurrence of a cytokine storm (CS), which is characterized by excessive release of regulatory proteins that play a major role in intercellular signaling and various other vital biological processes (52, 56, 57).

1.5. Introduction of the Peripheral Blood Mononuclear Cell (PBMC) model

1.5.1. Overview, pros and cons of the PBMC model

PBMC culture is a well-known and frequently used versatile model system which can be used to analyze the effects of various agents and/or molecules such as antigens (e.g.: certain parts of pathogens like zymosan) and pharmaceuticals/drugs on immune cells. The culture consists of several immune cells, namely natural killer cells (NK cells) and T and B lymphocytes in 70 – 90%, monocytes in 10 – 20% and dendritic cells in 1 – 2% (58, 59). Interestingly, the advantages and disadvantages of this model are the same. The utilization of PBMC cultures is an ideal approach to evaluate the effects of antigens or drugs on lymphocytes and monocytes/macrophages, as there is no unwanted interference from other cell types, such as basophil, eosinophil and neutrophil granulocytes, erythrocytes, and thrombocytes. On the other hand, PBMC cultures cannot accurately measure the effectiveness of the full immune response, as they lack the aforementioned cell types.

1.5.2. The importance and advantages of using autologous serum in the PBMC model

In most studies where PBMC cultures are used, it is common to use heat-inactivated fetal bovine serum (between 5 – 50% in culture medium) instead of normal autologous serum. These culture conditions may unreliably reflect the response of PBMC cells to cytokine activation stimuli. The use of normal serum/autologous serum was previously reported to

harm the cells via complement activation-related lysis, so heat-inactivated fetal bovine serum (FBS) was used instead. FBS has old roots but the use of such serum is more like a force of habits nowadays, as there are reports/articles about the positive effects of using normal autologous serum on cell viability (60-62). In order to study the role of the complement system and other heat-labile components of the serum, the most suitable approach is to supplement the culture medium with autologous serum.

2. Objectives

The basic objective of our research was to study the underlying mechanisms of induced cytokine secretion/cytokine storm (CS) and if possible, mitigate or even prevent the phenomenon by using complement inhibitors/antagonist in our autologous serum-containing human PBMC model.

Our aims were to:

- Demonstrate the necessity of supplementing human PBMC cultures with autologous serum for the analysis of cytokine secretion
- Determine the appropriate amount of autologous serum that reliably models the physiological and pathophysiological mechanisms of the human body in a human PBMC model
- Determine the extent of complement autoactivation in PBMC cultures supplemented with autologous serum, and how this affects cytokine secretion
- Confirm the role of complement activation in cytokine secretion, and, if possible, determine the complement components that play a vital role in it

3. Methods

3.1. Applied reagents and chemicals

For the cultivation of the PBMC cells a special medium was prepared to ensure sufficient nutrient supply and microenvironment. Cultivation medium (named R5) components such as RPMI-1640 mammalian cell culture media supplemented with glutamine, β-mercaptoethanol (50 μM), pyruvate solution (1 mM), non-essential amino acid solution (0,1 mM) and Penicillin-Streptomycin antibiotic solution were purchased from Merck Life Science Ltd. (Budapest, Hungary). Dulbecco's phosphate-buffered saline (D-PBS), ethylenediaminetetraacetic acid (EDTA), Ficoll-Paque PLUS solution as well as red blood cell (RBC) lysis buffer, Trypan Blue dye, Zymosan A derived from Saccharomyces cerevisiae and DF2593a, a C5a receptor antagonist were also acquired for Merck Life Science Ltd. (Budapest, Hungary). Complement anaphylatoxins C3a and C5a were purchased from Bachem AG (Bubendorf, Switzerland). Eculizumab, a C5 complement component specific antibody, which inhibits the activation of the protein, was acquired from Alexion Pharmaceuticals (New Haven, United States). Berinert, a C1-esterase inhibitor was purchased from CSL Behring (Marburg, Germany).

3.2. In vitro: Peripheral Blood Mononuclear Cell (PBMC)

Throughout the course of the research many different treatments (various agents and serum pre-treatments) were used, while the general preparation protocol was the same in all experiment. The whole procedure, from processing of the whole blood to treating the PBMCs was done under a Thermo Scientific MSC-Advantage™ Class II Biological Safety Cabinet. Tubes, suspensions and samples were centrifuged in Hermle Labortechnik Z400K Refrigerated Centrifuge.

3.2.1. Donor selection and blood sampling

The experiments always started with the recruitment of healthy volunteers from whom – based on the actual experimental setup – certain amount of blood (usually between 30 and 50 ml) was collected for serum preparation and PBMC separation. Donors who suffered from acute or chronic inflammation or any inflammation related disease, had surgery in the past 3 month or were under any medical treatment were excluded from the experiments. To ensure the regularity of the experiments, all donors gave written informed

consent prior to blood collection and the Scientific and Research Ethics Committee of the Medical Research Council of Hungary granted ethical approval of this research project (TUKEB 15576/2018/EKU).

3.2.2. Separation of autologous human serum

After the donor selection and the blood sampling, one part of the drawn whole blood was used for serum separation. The blood was set aside for approximately 25 minutes at room temperature to clot in several Greiner VACUETTE Z Serum Sep Clot Activator (8 ml) blood collection tubes. After coagulation of the blood, the tubes were centrifuged at 2000 g, 4 ºC for 15 minutes. The supernatants (serum) of each donor were pooled in 50 ml Falcon tubes, and were stored at 4 ºC until use (within 2 hours).

3.2.2.1. Treatment of autologous human serum

In some experiments the treatment of the serum was necessary before use. In some cases, heat-inactivation of the serum was necessary to inactivate the complement system in it in order to measure the contribution of complement to cytokine release. In these cases, however, all other heat-labile components of the serum were also inactivated making this method of complement contribution analysis somewhat biased. After centrifugation, one part of the freshly prepared and pooled serum was transferred into 2 ml Eppendorf tubes (3 tube/donor). The tubes were then incubated at 500 rpm, 56 ºC for 30 minutes in a preheated Boeco TS-100C Thermo-shaker. Following the procedure, the tubes were cooled to room temperature and were stored at 4 ºC until use.

In other cases, the measurement of complement contribution to cytokine release was approached in a different way. After centrifugation of blood, one part of the pooled serum was distributed into 1,5 ml Eppendorf tubes (4 tube/donor). The tubes were then treated with culture medium (R5), zymosan solution (10 μg/ml), zymosan plus C5 complement inhibitor (Eculizumab) solution (10 μg/ml + 1 μM) or zymosan plus C5a complement receptor antagonist (DF2593a) solution (10 μg/ml + 1 μM), respectively. Following the treatment, the tubes were incubated at 37 ºC for 30 minutes in a Sanyo MCO-18AC(UV) CO_2 Incubator. After the incubation process, the tubes were centrifuged at 2000 g, 25 ºC for 15 minutes in order to remove the zymosan particles from the serum, which would have caused interference in the PBMC model, since zymosan can also

interact with TLR2/6 receptor, which is the starting point of a known cytokine release amplifier pathway. The supernatants (activated serum) were then transferred into new 1,5 ml Eppendorf tubes and were used immediately.

3.2.3. PBMC separation

In parallel with the serum preparation, the separation of the PBMCs were also started from the other part of the drawn whole blood, which was collected in Greiner VACUETTE K2EDTA (9 ml) blood collection tubes. The uncoagulated blood containing tubes were pooled for each donor, and were diluted in 1:1 ratio with 6 mM EDTA containing D-PBS in 50 ml Falcon tubes for easier separation. The diluted whole blood was then carefully layered onto the previously prepared Ficoll-Paque PLUS solution in 50 ml Falcon tubes for density gradient centrifugation. The tubes were centrifuged at 500 g, 25 ºC for 30 minutes. After centrifugation, the supernatant (plasma) was discarded, and the cells, which formed a thick white ring were transferred into a new 50 ml Falcon tube and washed with 6 mM EDTA containing D-PBS to dilute and remove the remaining substances from previous steps. The cells were centrifuged again at 500 g, 25 ºC for 30 minutes. After removing the supernatant, the cells were resuspended in cold, EDTA-free D-PBS to ensure the removal of thrombocytes and residual EDTA, which inhibits almost all normal biological functions, and were centrifuged at 500 g, 4 ºC for 15 minutes. Following the centrifugation, the cells were resuspended in culture medium, and if there was more than one experimental setup, the suspensions were divided accordingly. After the distribution into 15 ml Falcon tubes, the suspensions were complemented with serum free culture medium (for serum-free and pre-treated serum containing setup), medium containing normal autologous human serum, and medium containing heat-inactivated autologous human serum. Finally, the cells were centrifuged at 500 g, 4 ºC for 15 minutes, and resuspended in the same culture media as mentioned above.

3.2.4. Cell counting

Immediately after cell separation, a small portion of the cell suspension was mixed with previously prepared (10x dilution) RBC lysis buffer to ensure the removal of the remaining red blood cells, while diluting the cell suspension 10-fold. After the incubation of the samples at 25 ºC for 10 minutes, the cells were treated with Trypan blue dye at a

ratio of 1:1 in order to stain the background and the dead cells in the suspension. The cells were then transferred onto a Hirschmann Techcolor bürker counting chamber, and were counted using an Olympus CKX41 Inverted Microscope. PBMCs were round and glowing white dots.

3.2.5. Treatment of PBMC

After separation, PBMCs were placed into the inner wells of a 96-well flat-bottomed Sarstedt microtiter plate for suspension cell cultures. Every well contained **approximately 5 x 10^5 cells**. In preparation for the incubation, the appropriate wells were supplemented with R5 medium (control), zymosan (0,5 mg/ml and 10 µg/ml), C3a (550 and 5500 nM), C5a (5 and 50 nM) and/or Eculizumab (1 µM), or DF2593a (1 µM), or Berinert (1 µM) or mini factor H (1 µM) or EDTA (20 mM) depending on the experimental setup, as the treatments were different in each experiment throughout the research.

3.2.6. Cell culture and sampling

After the treatments, the plates were transferred into a Sanyo MCO-18AC(UV) CO_2 Incubator (5% CO_2, 37 ºC). Samples were collected both before (baseline or 0 min samples) the start of the experiment, and 45 minutes and 18 hours after the start of the experiment. The samples were then transferred to a V-bottomed TOMTEC microtiter plate and were centrifuged at 2500 g, 4 ºC for 10 minutes. The supernatants were carefully transferred to a new microtiter plate and – after mixing – to special TOMTEC tubes. After distribution the samples were stored at –80 ºC until complement and cytokine assay.

3.2.7. Complement ELISA

In order to assess the effects of various treatments on the complement activation, C3a, C5a and sC5b-9 complement protein concentrations were measured. Human C3a and Human C5a ELISA kits from TECOmedical AG (Sissach, Switzerland) were used for measuring C3a and C5a concentrations and Complement TCC RUO ELISA kit from Svar Life Science AB (Malmö, Sweden) was used for measuring sC5b-9 concentrations. Complement measurements were performed according to the manufacturers' protocols. In these cases, the protocols were similar to each other, only some applied reagents were

different. Briefly, the samples along with the standard and controls were appropriately diluted according to the manufacturers' instructions and distributed into a 96-well microtiter plate (part of every kit). After incubation, the wells were thoroughly washed and treated with biotinylated primary antibody solution. Following another incubation period, the plate was washed and streptavidin solution (conjugated with horseradish peroxidase (HRP) enzyme) was added to the wells. After incubation, the plate was washed again and treated with TMB (3,3',5,5'-tetramethylbenzidine) substrate solution. Following a short incubation period, STOP solution (1 M H_2SO_4) was added to the wells in order to stop the oxidization of TMB by HRP. The plate was measured using a BMG LABTECH FLUOstar® Omega Plate Reader at 450 nm wavelength, and analyzed using a Multi-user Reader Control and MARS Data Analysis Software.

3.2.8. Cytokine ELISA

The Q-Plex™ Human Cytokine Inflammation Panel 1 multiplex ELISA kit or Q-Plex™ Human Cytokine (16-Plex) ELISA kit from Quansys Biosciences Inc. (West Logan, UT, USA) was used for evaluating the effects of applied treatments on cytokine release in the PBMC model. Cytokines were measured according to manufacturer's instructions. Briefly, the samples, standards, and controls were diluted in accordance with the manufacturers' guidelines and dispensed into a 96-well microtiter plate (part of the kit). Subsequent to incubation, the wells were washed and treated with biotinylated primary antibody solution. Following an additional incubation period, the plate was washed again, and a streptavidin solution (conjugated with horseradish peroxidase (HRP) enzyme) was added to the wells. Post-incubation, the plate was washed again and treated with a previously prepared substrate solution (1:1 ratio of provided substrate A and substrate B solutions). The data were collected right after the addition of the substrate solution with an exposure time of 270 second using a Q-View™ Imager LS plate reader operated with Q-View Software.

3.2.9. Statistical analysis

All data reported are means ± SEM. Statistical comparisons were performed using repeated measures ANOVA, and Dunnett's or Tukey's multiple comparisons post hoc tests

using GraphPad Prism version 8 software (GraphPad Software Inc., San Diego, CA, USA).

4. Results

4.1. The effects of mini factor H and EDTA in 50% autologous serum supplemented PBMC cultures on complement and cytokine secretion: a preliminary experiment

Both C5a (**Fig. 4A**) and sC5b-9 (**Fig. 4B**) concentrations showed a significant increase in samples treated with zymosan (0,5 mg/ml) after 45 minutes. All the applied inhibition methods caused a significant decrease in the concentration of the measured components. It should be emphasized that mini factor H, a truncated version of the complement regulator molecule factor H, was almost as effective as heat inactivation or the addition of EDTA under these conditions.

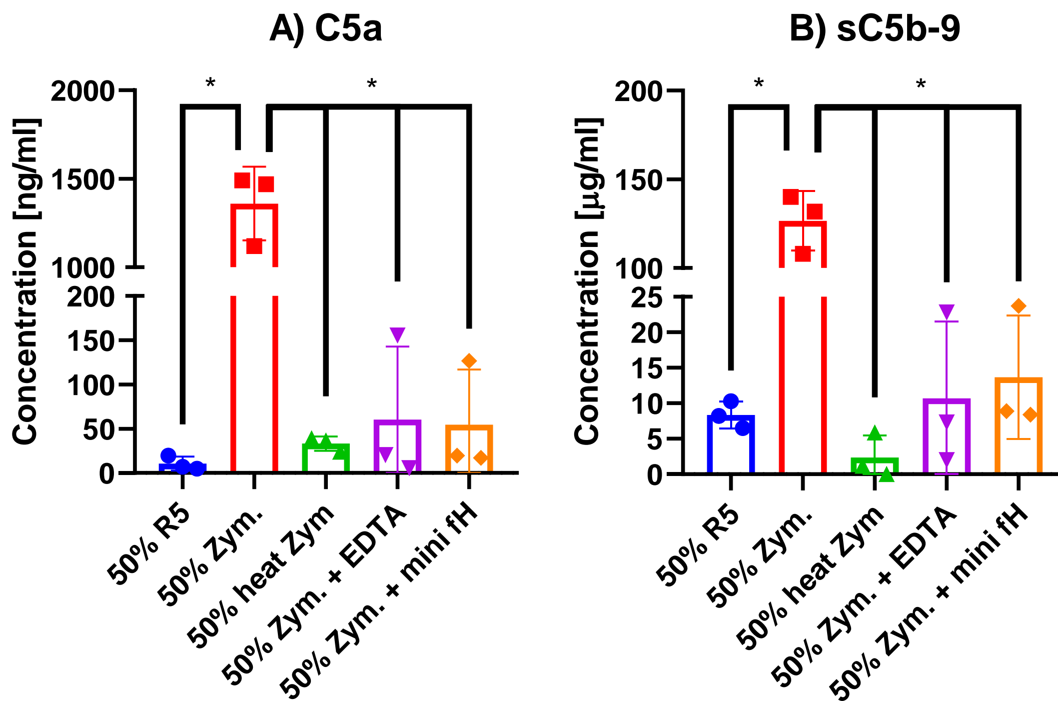

*Figure 4. C5a and sC5b-9 concentrations in 50% autologous serum containing PBMC culture 45 min after treatments. R5 means culture medium (control), Zym. means zymosan, heat means heat inactivated serum containing medium, mini fH means mini factor H. The samples collected at 45 minutes were measured with the Human C5a ELISA kit and Complement TCC RUO ELISA kit. Values are mean ± SEM. Statistical comparisons were performed using repeated measures ANOVA and Tukey's multiple comparisons post hoc test (all columns were compared to each other). * (P < 0,05) (63)*

Similarly to complement activation, zymosan treatment was able to cause a large cytokine release compared to control. Heat inactivation of serum almost completely prevented the increase in zymosan-induced cytokine release except for IL-1α (**Fig. 5A**) and IL-10 (**Fig. 5E**). Interestingly, EDTA, which inhibits most intracellular processes only partially inhibited the effect zymosan on IL-1β (**Fig. 5B**), IL-8 (**Fig. 5D**) and TNFα (**Fig. 5F**). In case of IL-1α, IL-6 (**Fig. 5C**) and IL-10 EDTA completely inhibited cytokine secretion. Mini factor H had mild (IL-1β, IL-8, TNFα) to moderate (IL-1α, IL-6, IL-10) yet not statistically significant (compared to zymosan treatment) effect on the mentioned cytokines. Taking all the results into account, we concluded that zymosan should be used at a lower concentration (10 μg/ml) in order to prevent excessive complement activation.

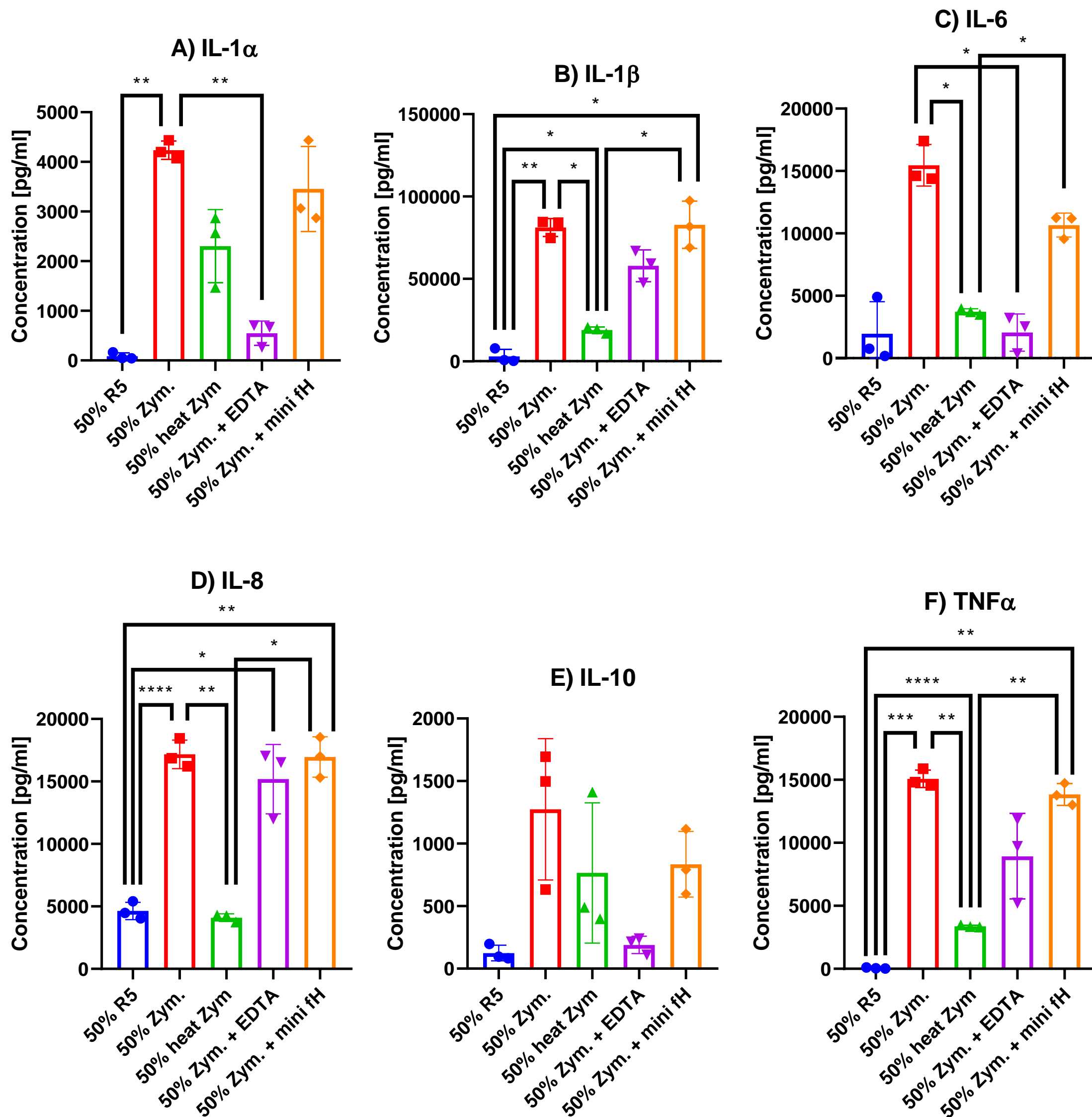

*Figure 5. Effects of 50% autologous serum and zymosan (Zym.) treatment on cytokine secretion in PBMC cultures. R5 means culture medium (control), heat means heat inactivated serum containing medium, mini fH means mini factor H. The samples collected after 18 hours were measured with the Q-Plex™ Human Cytokine (16-Plex) ELISA kit. Values are mean ± SEM. Statistical comparisons were performed using repeated measures ANOVA and Tukey's multiple comparisons post hoc test (all columns were compared to each other). * (P < 0,05) ** (P < 0,005), *** (P < 0,001), **** (P < 0,0001) (63)*

4.2. Differences in the complement activation between culture media containing 20 – 35 – 50% serum

The increase in the concentrations of C3a anaphylatoxin and sC5b-9 complement complex (**Fig. 6**) was proportional to the amount of autologous serum added to the PBMC cultures. Both complement activation products showed a small increase in 20% serum supplemented cultures compared to serum-free (therefore complement-free) conditions, and a slightly greater, although not statistically significant, increases in culture media containing 35% and 50% serum. Interestingly, the sC5b-9 concentrations of the baseline (0 min) samples were similar to those of the corresponding control samples, but C3a concentrations were not.

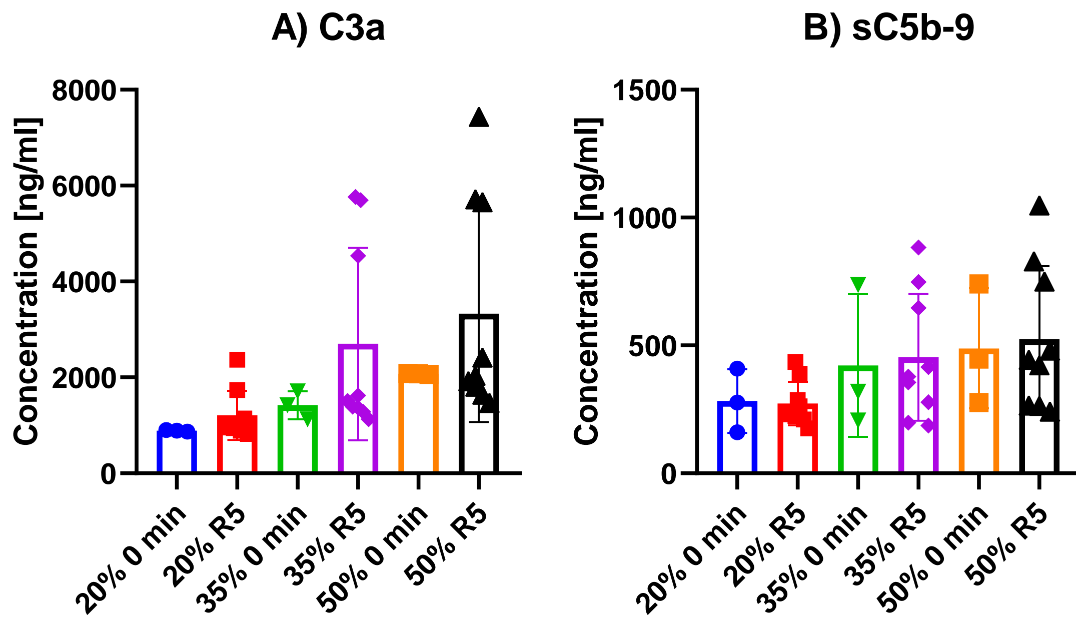

Figure 6. C3a and sC5b-9 concentrations in PBMC culture containing 20 – 35 – 50% autologous serum. R5 means culture medium (control). Samples collected at 0 (baseline) and 45 minutes were measured using Human C3a ELISA kit and Complement TCC RUO ELISA kit. Values are mean ± SEM. Statistical comparisons were performed using one-way ANOVA and Tukey's multiple comparisons post hoc test (all columns were compared to each other). The comparisons showed that the changes in C3a and C5a concentrations were not statistically significant.

4.3. Comparison of cytokine release in PBMC cultures supplemented with 20 – 35 – 50% serum

Similarly to increases in C3a and sC5b-9 concentrations, the changes in cytokine concentrations were proportional to the amount of serum added to the culture medium. It should be emphasized that the cytokine concentrations were below the detection limit in samples not supplemented with serum. The cytokine concentrations of IL-1α, IL-1β, IL-6, IL-8 and IL-10 (**Fig. 7A to 7E**) were surprisingly low in samples containing 20% autologous serum and increased severalfold in culture media with higher serum concentrations. Although the differences among samples containing 20 – 35 – 50% serum were not statistically significant, the upward trend was visible in all cases.

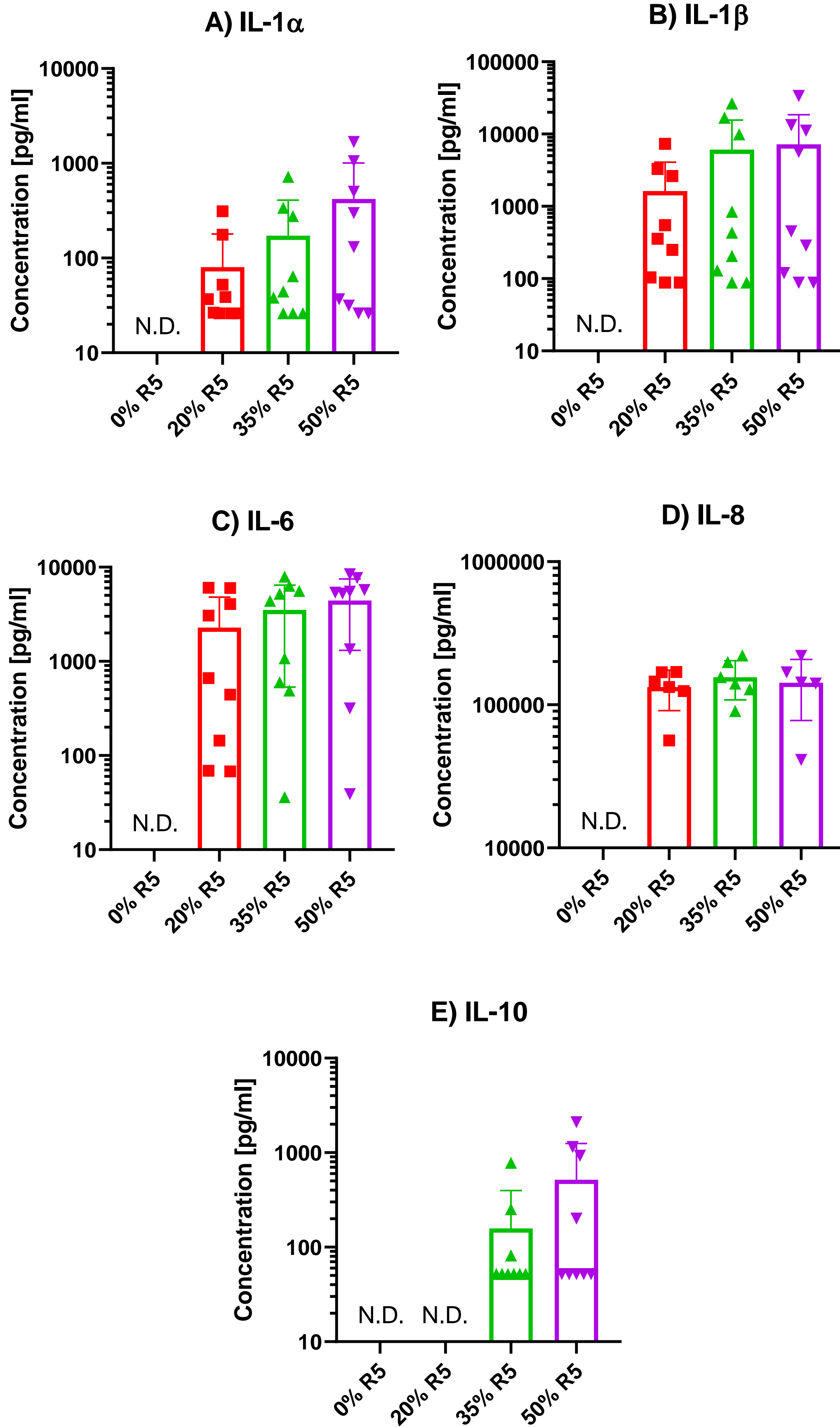
A) IL-1α
B) IL-1β
C) IL-6
D) IL-8
E) IL-10
Concentration [pg/ml]
N.D.
0% R5
20% R5
35% R5
50% R5
10
100
1000
10000
100000
1000000

*Figure 7. Cytokine secretion in PBMC cultures supplemented with 20 – 35 – 50% autologous serum. R5 means culture medium (control). The samples collected after 18 hours were measured using Q-Plex™ Human Cytokine Inflammation Panel 1 multiplex ELISA kit. Values are mean ± SEM. Statistical comparisons were performed using repeated measures ANOVA and Tukey's multiple comparisons post hoc test (all columns were compared to each other). * (P < 0,05) ** (P < 0,005)*

4.4. Autoactivation of the complement system in PBMC cultures supplemented with 20% autologous serum

After the addition of 20% autologous serum to the culture medium, C3a was present in baseline (0 min) and R5 samples, as a result of a weak, spontaneous complement activation, which is considered a normal phenomenon in this model. C3a concentration was similar in baseline (0 min) and R5 (45 min) samples, meaning that complement activation did not change over time (**Fig. 8A**). Previous experiments showed (data not shown) that this small autoactivation has no deleterious effect on the cells cycle or any secretory processes. In the heat-treated serum samples the C3a concentration increased only slightly (**Fig. 8A**) since initial activation caused by the heating could not proceed due to denaturation of proteins, interrupting the activation cycle. Naturally the C5 inhibitor Eculizumab had no effect on C3a secretion, but, surprisingly, the C1-esterase inhibitor Berinert did not have an adequate blocking effect either (**Fig. 8B**).

Similar to C3a, a sC5b-9 concentration was also measable in the samples containing 20% autologous serum. In contrast to the concentration of C3a, the concentration of sC5b-9 was about 1,2-fold higher in the R5 (45 min) samples than baseline (0 min) samples (**Fig. 8C**). This is also part of the autoactivation associated with the use of this model and can be considered normal based on our previous findings (data not shown). Heat-treatment of serum caused a 2-fold rise in the concentration of sC5b-9 at baseline (0 min), but a smaller increase was observed in the R5 (45 min) samples (**Fig. 8C**). Interestingly the concentrations of C3a and sC5b-9 were similar in all heat-treated samples, suggesting that heat-treatment of the serum inactivated not only the complement cascade but also the degradation of the complement peptides. As expected, Eculizumab completely inhibited the assembly of the sC5b-9 complex in the control samples to the

extent that it pushed back the activation to the level of baseline (0 min) samples. Berinert had no quantifiable effect on the complex formation (**Fig. 8D**).

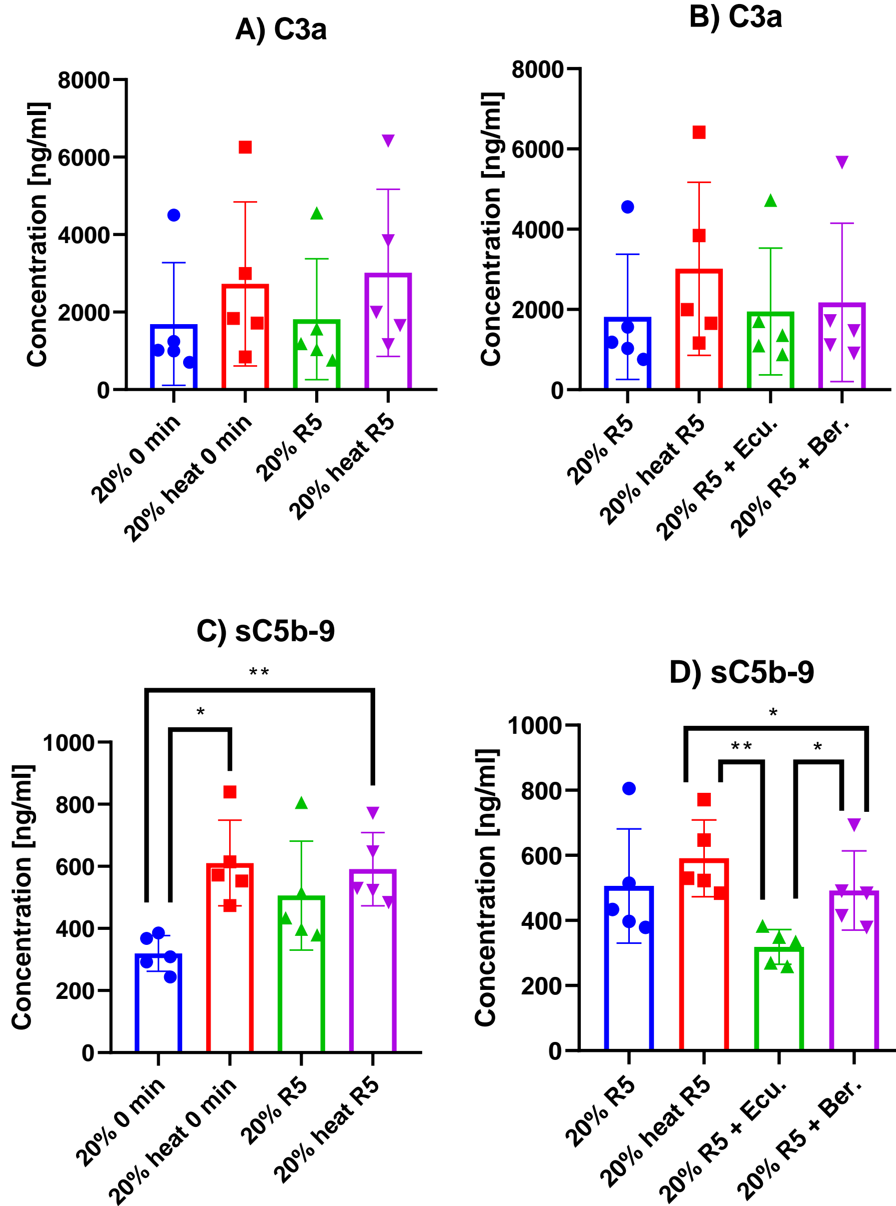

Figure 8. C3a and sC5b-9 concentrations in PBMC cultures containing 20% autologous serum. Eculizumab (Ecu.) and Berinert (Ber.) were used to inhibit complement activation. R5 means culture medium (control), heat means medium containing heat inactivated serum. Samples collected at 0 (baseline) and 45 minutes were measured using Human C3a ELISA kit and Complement TCC RUO ELISA kit. Values are mean ± SEM. Statistical comparisons were performed using repeated measures ANOVA and Tukey's multiple

*comparisons post hoc test (all columns were compared to each other). * (P < 0,05) ** (P < 0,005)*

4.5. Effects of complement autoactivation on cytokine release

Supplementation of the culture medium with 20% serum induced a basal level of cytokine secretion (**Fig. 9**) compared to the serum-free condition, when the cytokine concentrations were below the detection limit (**Fig. 7**). As mentioned above, autoactivation of the complement system is present in this model, which in turn seemed to have a negligible effect on cytokine secretion (**Fig. 9**). Heat inactivation of the serum had visible effect only in the case of IL-8 compared to untreated control (**Fig. 9B**). The presence of Eculizumab resulted in a small reduction in IL-6 and IL-8 concentrations (**Fig. 9A and 9B**), which did not reach the level of statistical significance. However, the C5 inhibitor did not induce any changes in IFNγ and TNFα conncetrations (**Fig. 9C and 9D**). Berinert had no effect on cytokine concentrations. The results indicate that the negligible autoactivation of complement contributed only a small extent to the cytokine secretion in the culture medium containing 20% autologous serum.

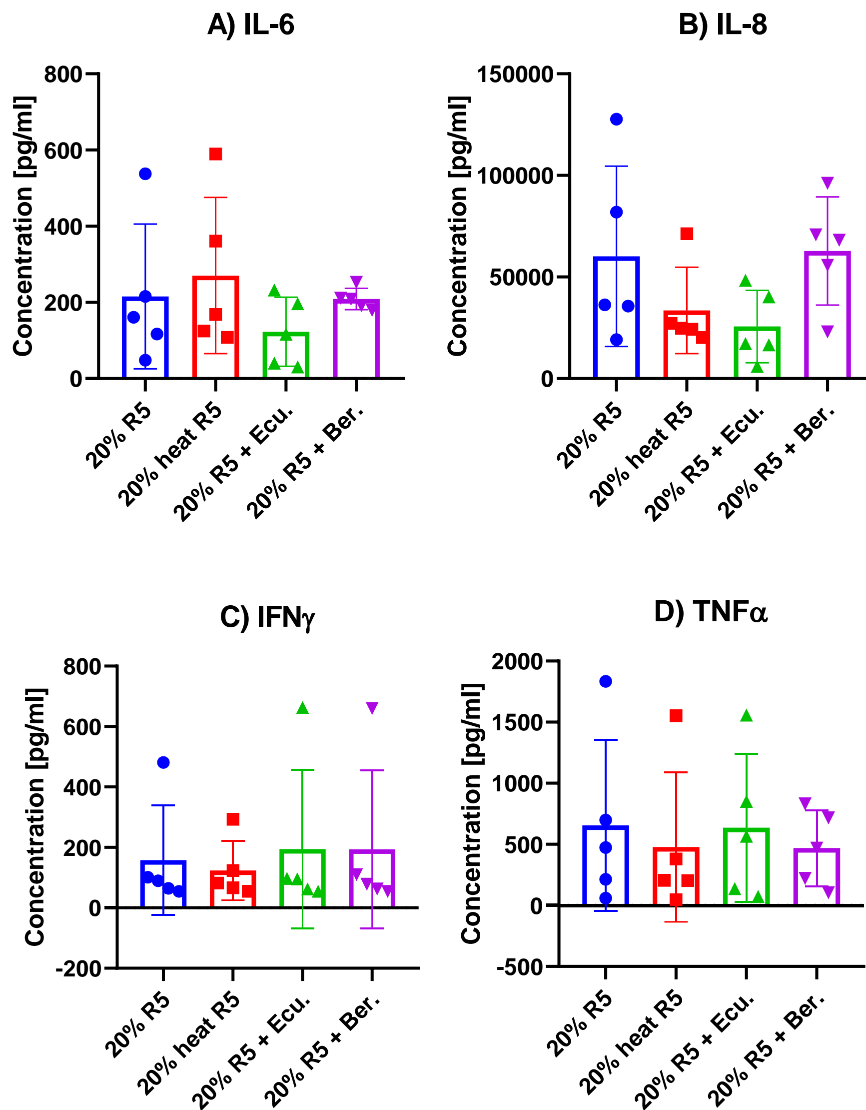

Figure 9. Effects of complement/serum autoactivation on cytokine concentrations in control samples (R5) of 20% autologous serum containing PBMC culture. Eculizumab (Ecu.) and Berinert (Ber.) were used to block complement activation. R5 means culture medium (control), heat means medium containing heat inactivated serum. The samples collected at 18 hours were measured using Q-Plex™ Human Cytokine Inflammation Panel 1 multiplex ELISA kit. Values are mean ± SEM. Statistical comparisons were performed using repeated measures ANOVA and Tukey's multiple comparisons post hoc test (all columns were compared to each other). The comparisons showed that the changes in cytokine concentrations were not statistically significant (64).

4.6. Effects of zymosan-pretreated serum and zymosan on sC5b-9 concentration

Serum pretreated with zymosan caused an approximately 3-fold increase in sC5b-9 concentration at 45 min (**Fig. 10A**) compared to R5, but it was prevented by the C5 complement inhibitor, eculizumab. The concentration of sC5b-9 was not affected by DF2593A. On the other hand, addition of zymosan to the culture medium caused a huge increase in the concentration of sC5b-9. Eculizumab and DF2593A had the same effects as zymosan-pretreated serum (**Fig. 10B**).

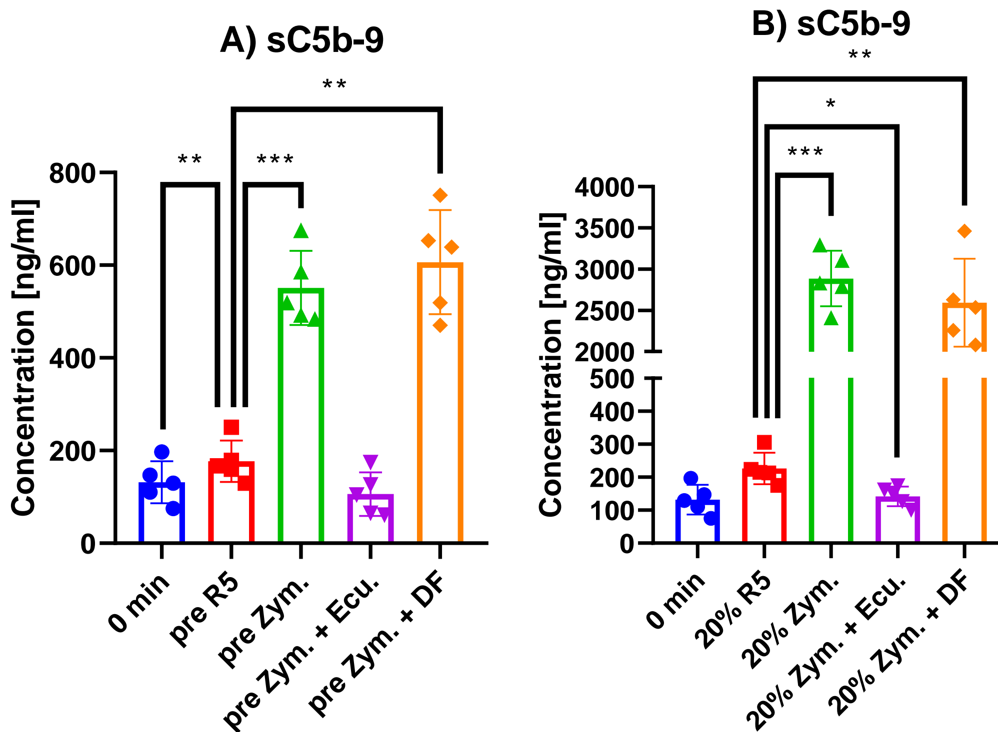

*Figure 10. Effects of zymosan-pretreated serum (pre Zym., left) and zymosan (Zym., right) on sC5b-9 concentration in PBMC cultures. The effects of eculizumab (Ecu.) and DF2593a (DF) were tested to see if they antagonize the effects of zymosan. R5 means culture medium (control). Samples collected at 0 (baseline) and 45 minutes were measured using a Complement TCC RUO ELISA kit. Values are mean ± SEM. Statistical comparisons were performed using repeated measures ANOVA and Dunnett's multiple comparisons post hoc test (all columns were compared to R5). * (P < 0,05), ** (P < 0,005), *** (P < 0,001) (64)*

4.7. Effects of anaphylatoxins on cytokine secretions

C5a anaphylatoxin affected only IL-8 release out of the nine cytokines measured in the serum-free culture medium (no other cytokine concentrations were detected in the control and treated samples). As displayed in **Figure 11**, C3a had a very limited effect on cytokine secretions at both concentrations applied, but C5a caused a significant and similar increase in IL-8 concentration at both concentrations. The combined use of anaphylatoxins resulted in almost the same effect as C5a alone. IL-8 chemokine is one of the most important chemo-attractants; therefore, its activation by C5a is worth noting.

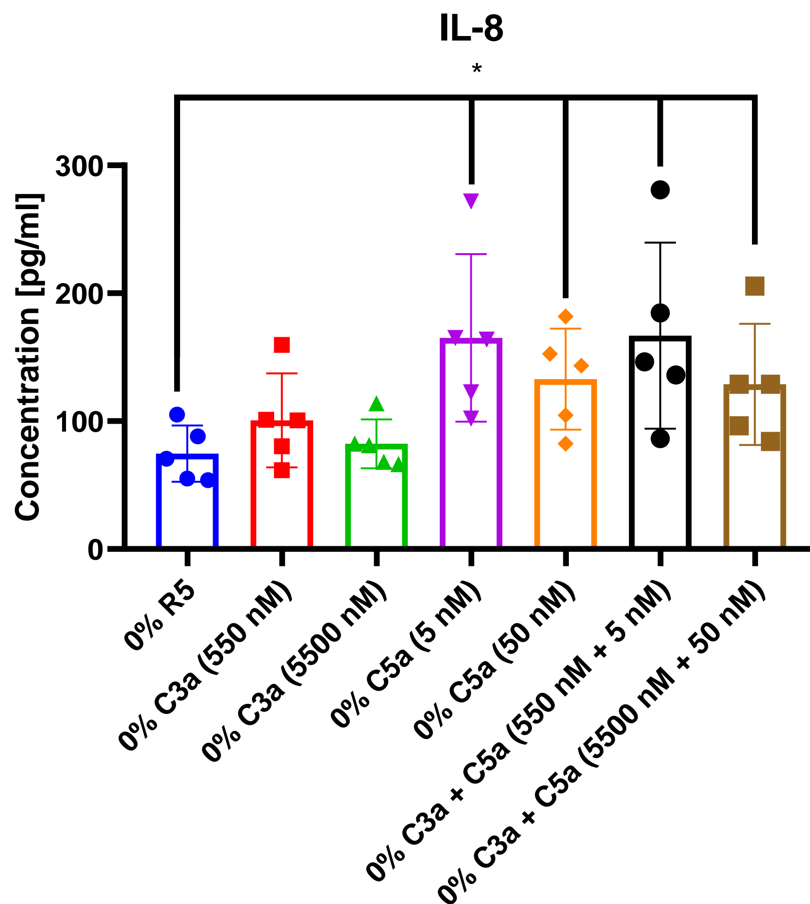

Figure 11. Effects of C3a and C5a anaphylatoxins on IL-8 concentrations in serum-free (0% serum) PBMC culture. R5 means culture medium (control). Samples were collected after 18 hours of incubation and measured using a Q-Plex™ Human Cytokine Inflammation Panel 1 multiplex ELISA kit. Values are mean ± SEM. Statistical

*comparisons were performed using repeated measures ANOVA and Tukey's multiple comparisons post hoc test (all columns were compared to R5). * (P < 0,05) (64)*

4.8. Effects of C5a and zymosan on cytokine release in serum free conditions

Serum-free conditions were used to test the role of zymosan on cytokine secretion through TLR2/6 receptor activation, and C5a was also used to analyze a possible contribution to zymosan-induced cytokine release. Zymosan induced small increases in IL-1β, IL-2 and IL-10 concentrations (**Fig. 12A, 12B and 12E**), moderate increases in IL-6 and TNFα concentrations (**Fig. 12C and 12F**), and a massive increase in IL-8 concentrations (**Fig. 12D**) in serum free conditions. Co-administration of zymosan and C5a caused negligible increase in IL-2 (**Fig. 12B**) and IL-6 (**Fig. 12C**) concentrations and only a moderate increase in IL-8 concentration (**Fig. 12D**) compared to zymosan alone. Interestingly, C5a attenuated the effect of zymosan on IL-1β, IL-10, and TNFα release to an extent that was statistically significant only for the anti-inflammatory IL-10 secretion. **Figure 13** shows how the donors responded individually to these treatments.

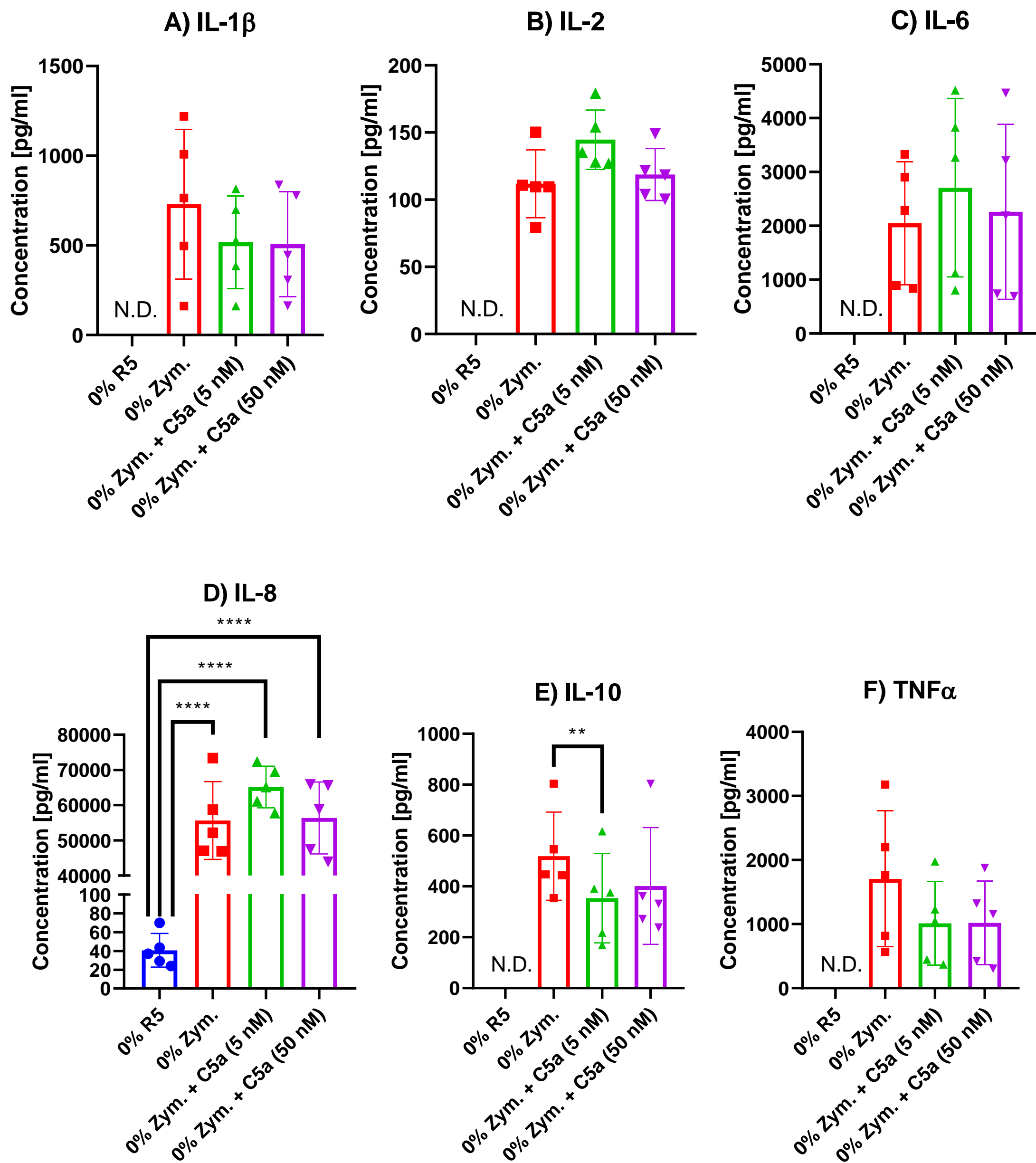

*Figure 12. Effects of zymosan (Zym.) and zymosan + C5a anaphylatoxin on cytokine release in serum-free (0% serum) PBMC cultures (most cytokine concentrations were undetectable without treatments). R5 means culture medium (control). Samples were collected after 18 hours of incubation and measured using a Q-Plex™ Human Cytokine Inflammation Panel 1 multiplex ELISA kit. Values are mean ± SEM. Statistical comparisons were performed using repeated measures ANOVA and Tukey's multiple comparisons post hoc test (every column was compared to each other). ** (P < 0,01), **** (P < 0,0001) (64)*

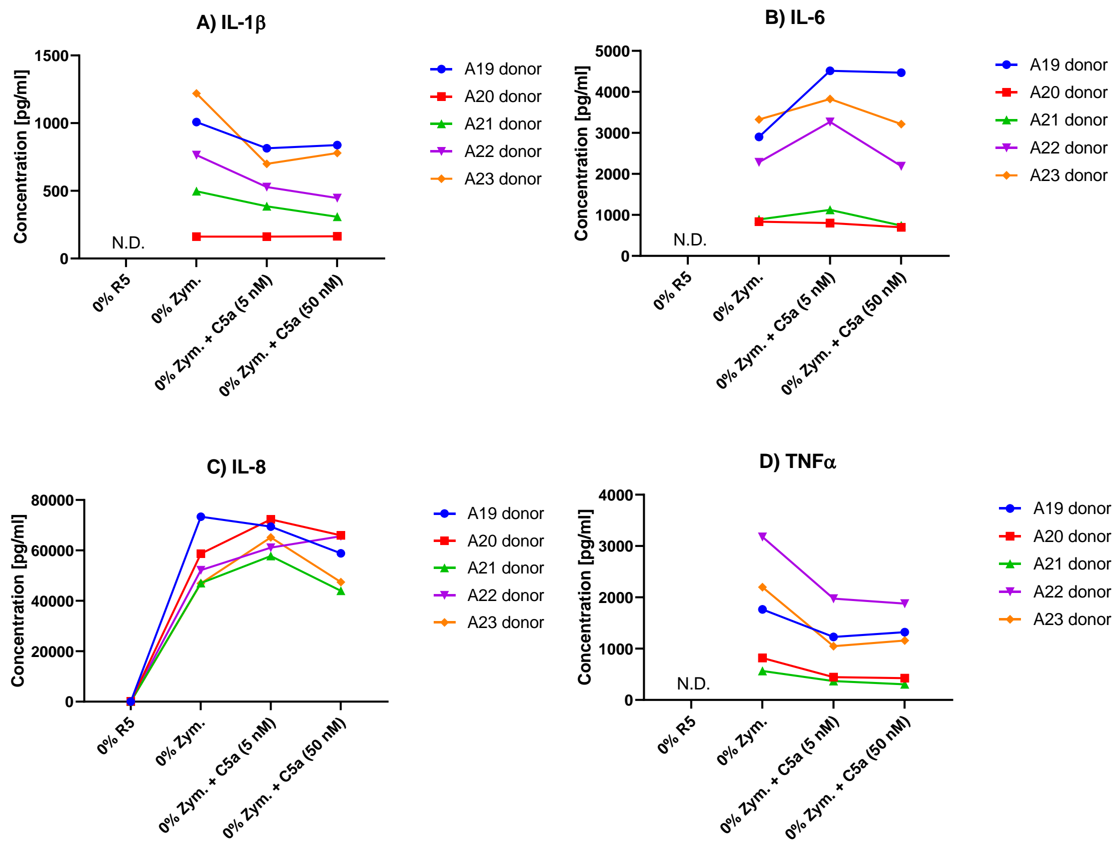

Figure 13. Representation of individual variation of data shown in ***Figure 12.*** *R5 means culture medium (control), Zym. means zymosan.*

4.9. Effect of zymosan-pretreated serum on cytokine release

As shown in **Figure 10A**, zymosan induced a marked complement activation. Serum was pretreated with zymosan and then removed by centrifugation to measure cytokine release after addition of serum in which the complement system was activated. Such an approach can reveal the effects of complement components at increased concentration without the modifying effect of TLR2/6 receptor activation by zymosan. Zymosan-pretreated serum markedly increased the secretion of IL-8, which effect was abolished by the C5 inhibitor (**Fig. 14B**). The release of other cytokines was hardly influenced by zymosan-pretreated serum (data not shown). Only a small tendency for an increase in IL-2 secretion was detected, which was reversed by eculizumab (**Fig. 14A**). The complement receptor C5a antagonist (DF2593a) had no effect on IL-8 or IL-2 concentrations. These results suggest that the effects of zymosan-pretreated, i.e., complement-activated serum on IL-8 secretion are mediated by other C5-derived complement products too, not just C5a alone.

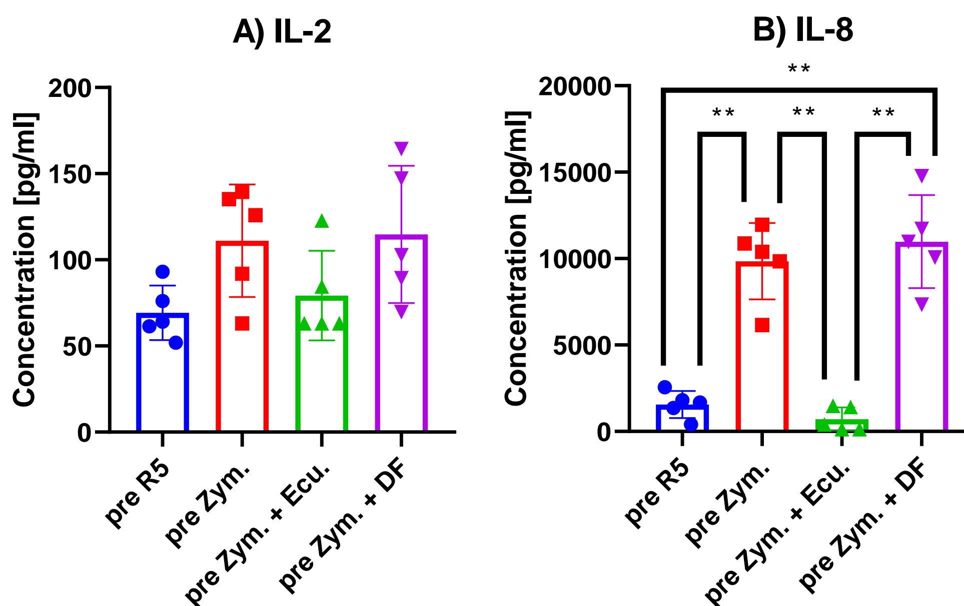

*Figure 14. Effect of serum pretreated with zymosan (pre Zym.), and the influence of C5 complement inhibitor eculizumab (Ecu.) or complement C5a receptor antagonist DF2593A (DF) on the effect of serum pretreated with zymosan. R5 means culture medium (control). Samples were collected after 18 hours of incubation and measured using a Q-Plex™ Human Cytokine Inflammation Panel 1 multiplex ELISA kit. Values are mean ± SEM. Statistical comparisons were performed using repeated measures ANOVA and Tukey's multiple comparisons post hoc test (every column was compared to each other). ** (P < 0,01) (64)*

4.10. Effects zymosan on cytokine release in serum-supplemented conditions

Treatment of PBMC cultures containing 20% serum with zymosan increased cytokine secretions in complement-dependent and TLR2/6 pathway-dependent manners, which effects were approximately 10- to 20-fold greater than the effects of zymosan in the serum-free medium (**Fig. 15**). Zymosan induced marked elevations in the concentration of the main pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNFα) but caused smaller increases in the secretion of IL-1α, IL-2, IL-10 and IFNγ. Eculizumab moderately inhibited the zymosan-induced secretion of IL-1α, IL-1β and TNFα, with limited effect on IL-2, IL-6, and IFNγ, and no effect on IL-8. DF2593a had no marked effect on zymosan-induced cytokine secretion, although a slight tendency for a decrease in IL-1α

and IL-1β secretion was observed. **Figure 16** shows how the donors responded individually to these treatments.

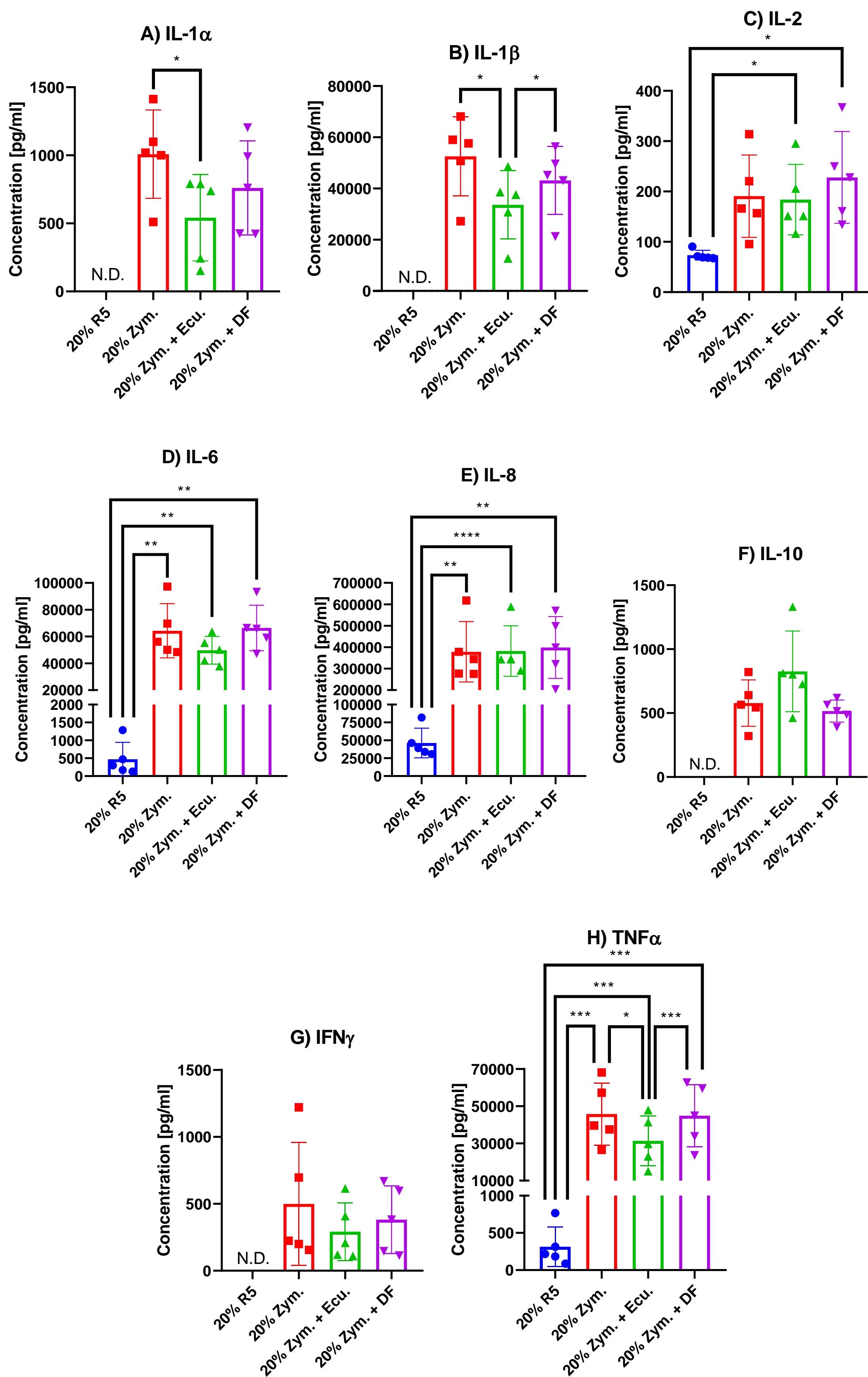
A) IL-1α
B) IL-1β
C) IL-2
D) IL-6
E) IL-8
F) IL-10
G) IFNγ
H) TNFα
Concentration [pg/ml]
N.D.
20% R5
20% Zym.
20% Zym. + Ecu.
20% Zym. + DF

*Figure 15. The effect of zymosan (Zym.) on cytokine release in PBMC cultures containing 20% autologous serum, and the effect of the complement C5 inhibitor, eculizumab (Ecu.), or the complement C5a receptor antagonist, DF2593A (DF), on zymosan-induced cytokine releases. R5 means culture medium (control). Samples were collected after 18 hours of incubation, and measured using a Q-Plex™ Human Cytokine Inflammation Panel 1 multiplex ELISA kit. Values are mean ± SEM. Statistical comparisons were performed using repeated measures ANOVA and Tukey's multiple comparisons post hoc test (every column was compared to each other). * (P < 0,05), ** (P < 0,005), *** (P < 0,001), **** (P < 0,0001) (64)*

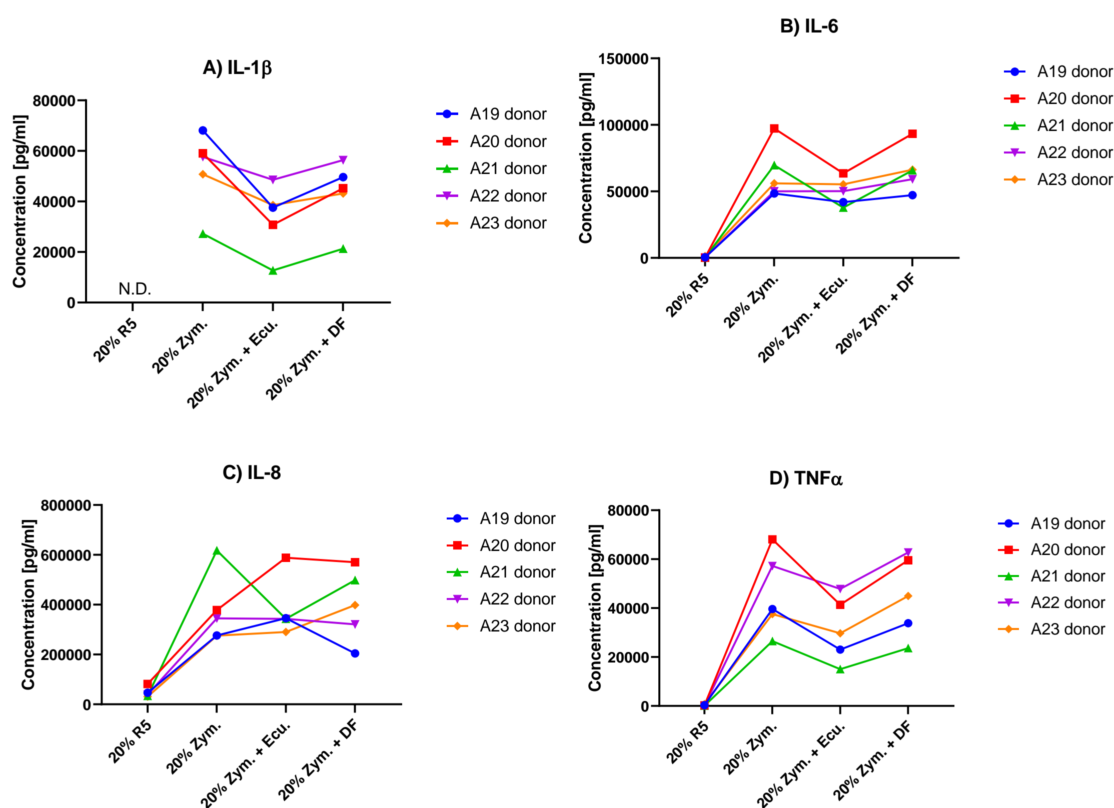

Figure 16. Representation of individual variation of data shown in ***Figure 15.*** *R5 means culture medium (control), Zym. means zymosan, Ecu. means Eculizumab and DF means complement C5a receptor antagonist, DF2593A.*

5. Discussion

5.1. The neccesity of using autologous serum

Supplementation of the PBMC culture medium with autologous serum caused huge increases in the release of most cytokines (IL-1β, IL-2, IL-6, IL-8, TNFα) and enhanced the effects of zymosan which is a widely known complement activator and TLR2/6 agonist (65, 66). These results emphasize that addition of autologous serum to the medium in PBMC culture is essential to obtain reliable results when analyzing cytokine release, and the mechanisms of various interventions for cytokine release. Serum is an essential part of the system as serum proteins (e.g., complement components) could influence the process and act as factors that modify cytokine production. The addition of autologous serum to the incubation medium appeared to have no discernible impact on cell viability or any adverse effects on the PBMC culture. This deduction is supported by the observation that cell death would have substantially affected cytokine concentrations, and notably, the cell mass after centrifugation in the 18-hour samples visually exceeded that of in the baseline (0 min) or 45 min samples. Our results are supported by some previous findings (60, 61), which clearly indicated that addition of autologous serum had a beneficial effect on PBMC cultures.

5.2. The optimal amount of autologous serum in PBMC culture

Both complement C3a and sC5b-9 concentrations as well as IL-1α, IL-1β, IL-6, IL-8, IL-10 cytokine concentrations increased in parallel with the amount of autologous serum used in the PBMC culture medium. Compared to baseline (0 min) samples, control samples containing only 20% serum showed no difference in either C3a or sC5b-9 concentrations at 45 min. In the same control samples 20% autologous serum only minimally influenced cytokine secretion. Results obtained from other experiments showed the efficacy of 20% serum in the culture medium, which, based on the complement activation results, implies a lower risk of autoactivation and subsequent cell death. The effects of zymosan were already maximal in media containing 20% serum, and the use of 20% serum also proved to be sufficient to support massive cytokine secretion compared to the serum-free conditions. Based on these results the use of 20% serum was the optimal choice for the following experiments.

5.3. Effects of complement autoactivation

The use of autologous serum in the PBMC model always comes with a certain level of complement autoactivation, which depends on various factors, but most importantly the amount of serum used in the cell culture medium. We found that the use of 20% autologous serum had the smallest effect on the parameters measured in the contol samples, therefore we investigated the possible amplifying effect of complement autoactivation on the cytokine secretion using this experimental setup.

The minor autoactivation of the complement system (C3a, C5a, sC5b-9), which occurred in the untreated samples had only a negligible influence on the results. The increase in cytokine secretion upon supplementation with autologous serum is not necessarily related to harmful effects of the serum, due to activation of the complement system, since its autoactivation had minimal effects on cytokine secretions. The results of heat-treated serum also support this assumption since the residual activated complement from the heat-inactivation process also had little effect on cytokine secretion. Furthermore, in zymosan pre-treated serum, complement activation only slightly affected the release of IL-8, but no other cytokines, making it unlikely that complement activation is harmful to the cells in this model. Based on compiled data from several experiments, the phenomenon of complement autoactivation can be taken as a normal process in this model.

5.4. Relationships between the complement system and cytokine release

There were large individual differences in cytokine release among the donors, a phenomenon not unprecedented in these experiments (67, 68). Blood was collected from healthy donors, so these results indicate individual variations in both complement concentration and cytokine release rather than mild to severe inflammatory disease of some donors. Furthermore, the response of PBMC cultures to various stimulations also varied markedly. These observations are consistent with the observations that there are huge variations among the responses of patients to infections such as SARS-CoV-2, as some patients respond with a cytokine storm, while others do not (52, 57). Based on these data, drawing a fully comprehensive conclusion that authentically reflects the whole population is at least challenging and must be handled carefully.

Although the pilot study of our test system (63) showed promising results about the contribution of complement to the release of certain cytokines, these results were not comprehensive. Therefore, we decided to pursue this approach further.

Although the role of C5a in the stimulation of cytokine release has already been suggested using a serum-free, whole-blood cell model (69), a serum-free and pooled-human-serum PBMC model (70), a heat-inactivated FBS PBMC model (71), an adult-bovine-serum PBMC model (72), and various *in vivo* models (73-75), to mention a few, our results offer a more direct approach to explore the role of complement in cytokine secretion (64). Addition of complement C5a anaphylatoxin to serum-free PBMC cultures increased IL-8 concentrations in the culture medium, while C3a was ineffective. The effect of C5a was already maximal at the lower concentration of 5 nM, so it could not be further increased by higher C5a concentrations. Based on these results we conclude that there is a connection between the complement system and the cytokine secretion through C5a anaphylatoxin receptors in our model. However, as C5a had a limited effect (minor IL-8 increase) in a serum-free medium in the presence of the TLR2/6 activator, zymosan, the effect of C5a receptor activation can play a role in IL-8 secretion only under special conditions. Interestingly, the zymosan-pretreated serum had a much greater effect on IL-8 secretion (and had a small effect on IL-2 secretion) than C5a itself, and the effect of the zymosan-pretreated serum was completely negated by the addition of C5 antibody. These results suggest that not only C5a receptor activation, but also other C5-derived proteins can increase IL-8 release. In fact, the effect of zymosan on IL-1α, IL-1β, IL-2, IL-6 and TNFα release was inhibited by eculizumab in cultures containing autologous serum. Surprisingly, DF2593A did not decrease the effects of zymosan-pretreated serum or the effects of zymosan in cultures containing autologous serum, which can only be explained if we assume that C5a receptor activation only plays a smaller role under these conditions. Another explanation could be that the effect was mediated by C5aR2 and not C5aR1, and inhibition of the former by DF2593A is not yet supported by literature data. Indeed, zymosan caused a large increase in sC5b-9 concentration, whether it was used to pretreat the serum, or added directly to the culture medium supplemented with autologous serum. These results therefore raise the possibility that although the C5a anaphylatoxin is the likely mediator of complement-mediated cytokine production, activation byproducts and other complement mediators could also enhance the production of certain cytokines.

6. Conclusions

After a thorough discussion of the experimental results of several measurements, we drew the following conclusions:

- The use of autologous serum in a human Peripheral Blood Mononuclear Cell (PBMC) model is essential for a reliable analysis of the pathophysiological mechanisms of cytokine release after various treatments. Data from the scientific literature and our own experiments support that the use of autologous serum is beneficial to the PBMC culture.
- Choosing the right quantity of autologous serum to be used in the PBMC model is important to obtain reliable results and to better understand the underlying mechanisms. The use of 20% autologous serum instead of 35% or 50% ensures a lower level of complement autoactivation (less risk of cell lysis).
- Minor autoactivation of the complement system is always present in the PBMC model. It is known that there is a low level of alternative pathway activation in normal physiological conditions, therefore the *in vitro* model must contain (from the start) some active complement components that persist but are not amplified further. The results show that this autoactivation has little or no additional effect on the results obtained from PBMC cultures (minimal cell death, or cytokine release amplification).
- Huge individual variations in cytokine release in both the control and treated samples indicate different intensity of response from the selected donors (similar to the COVID-19 infection), therefore, these data should be interpreted with caution.
- There is a definite relationship between the complement activation and the induction and/or amplification of the cytokine release. Complement C5 products (most likely C5a) appear to mediate IL-8 chemokine release, presumably partially without C5aR activation, although other complement byproducts (upward from C5 activation) or complement mediators may also influence the process.
- The created model is more than suitable for testing the safety of various drugs or other pharmaceutical products, which are still in the development phase.

7. Summary

Preventing or at least mitigating the effects of sudden and uncontrolled release of cytokine proteins (cytokine storm) in response to harmful stimuli (pathogen invasion, drug/vaccine administration) is pivotal to prevent life threatening conditions or even death. The focus of our research is to understand the underlying mechanisms of induced cytokine secretion using human PBMC culture supplemented with autologous serum.

Our aim was to demonstrate the importance of using autologous human serum in the PBMC model, and also to determine the amount of the serum that can reliably model pathophysiological mechanisms without the risk of possible negative effects such as cell lysis caused by complement overactivation. Our goals were also to quantify the level of complement autoactivation in the model system used, and also to determine which complement components participate and what role they play in the induced cytokine secretion.

The methods included the use of our self-developed PBMC model, which contains autologous human serum. The cells and the serum were separated from freshly drawn blood of healthy volunteers and treated when needed. The separated cells (PBMC) were cultured in a medium supplemented with autologous serum, and were treated with various agents (inducers and inhibitors). Samples were taken at the start and 45 minutes and 18 hours after the beginning of the experiment. The complement and cytokine concentrations of these samples were measured with complement and multiplex cytokine ELISA methods.

The results showed that autologous human serum is essential for analyzing the pathophysiological mechanisms behind the induced cytokine release, and has positive effects on cell viability. The right amount of serum is also important to achieve reliable results. The results also indicated that the autoactivation of the complement system has a minimal effect on the measured parameters. Huge individual variations were also present in the results but the measured data showed a definite and direct relationship between the complement system (most likely C5a) and the cytokine release (especially IL-8 release). Complement byproducts and mediators may also have influenced the process.

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9. Bibliography of the candidate's publications

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