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

PhD thesis

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Budapest
2023

1. Introduction

Studying the functions of the immune system is a fundamental requirement for understanding defense mechanisms against pathogens in humans. In the field of immunology, the inflammatory processes and closely associated defense mechanisms, such as the activation of the complement system and cytokine network, constitute a highly challenging and complex sector, harboring many unanswered questions. While the roles of the majority of proteins in these systems have been adequately characterized, the identification of potential relationships among individual components remains an unresolved issue. A comprehensive understanding of all protein interactions is imperative for the successful design and implementation of various drug formulations and therapies.

Nowadays, research focuses on unveiling the relationship between the complement system and the cytokine secretion with the aim of mitigating or preventing the effects of inflammation. It has been reported that C5a anaphylatoxin from the activation of the complement system has a role in cytokine secretion but to date no results have been obtained on the direct connection between the two systems. Our research offers a direct approach on the matter with reliable results.

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. 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. 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).

3.2.1. 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.

3.3. 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. Cells were counted using Trypan blue exclusion method.

3.4. 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.

3.5. 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.

3.6. 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.

3.7. 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.

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. 1A**) and sC5b-9 (**Fig. 1B**) 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.

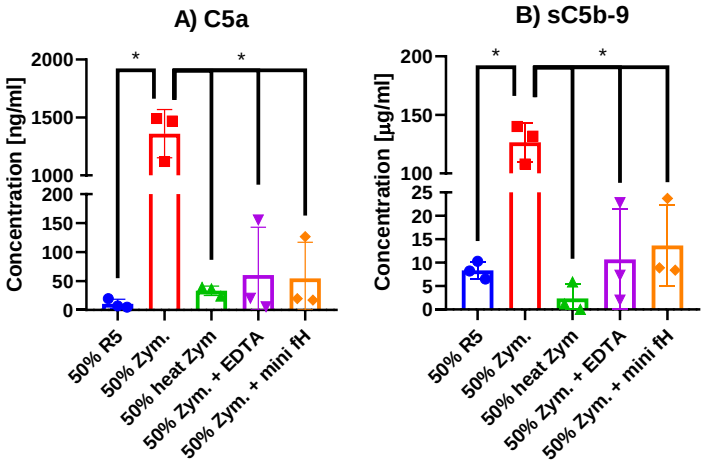


Figure 1. C5a and sC5b-9 concentrations in 50% autologous serum containing PBMC culture 45 min after treatments. R5 means culture medium (control), Zym. means zymosan, heat means heat inactivated serum containing medium, mini fH means mini factor H. The samples were collected after 45 minutes of incubation.

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. 2A**) and IL-10 (**Fig. 2E**). Interestingly, EDTA, which inhibits most intracellular processes only partially inhibited the effect zymosan on IL-1β (**Fig. 2B**), IL-8 (**Fig. 2D**) and TNFα (**Fig. 2F**). In case of IL-1α, IL-6 (**Fig. 2C**) 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.

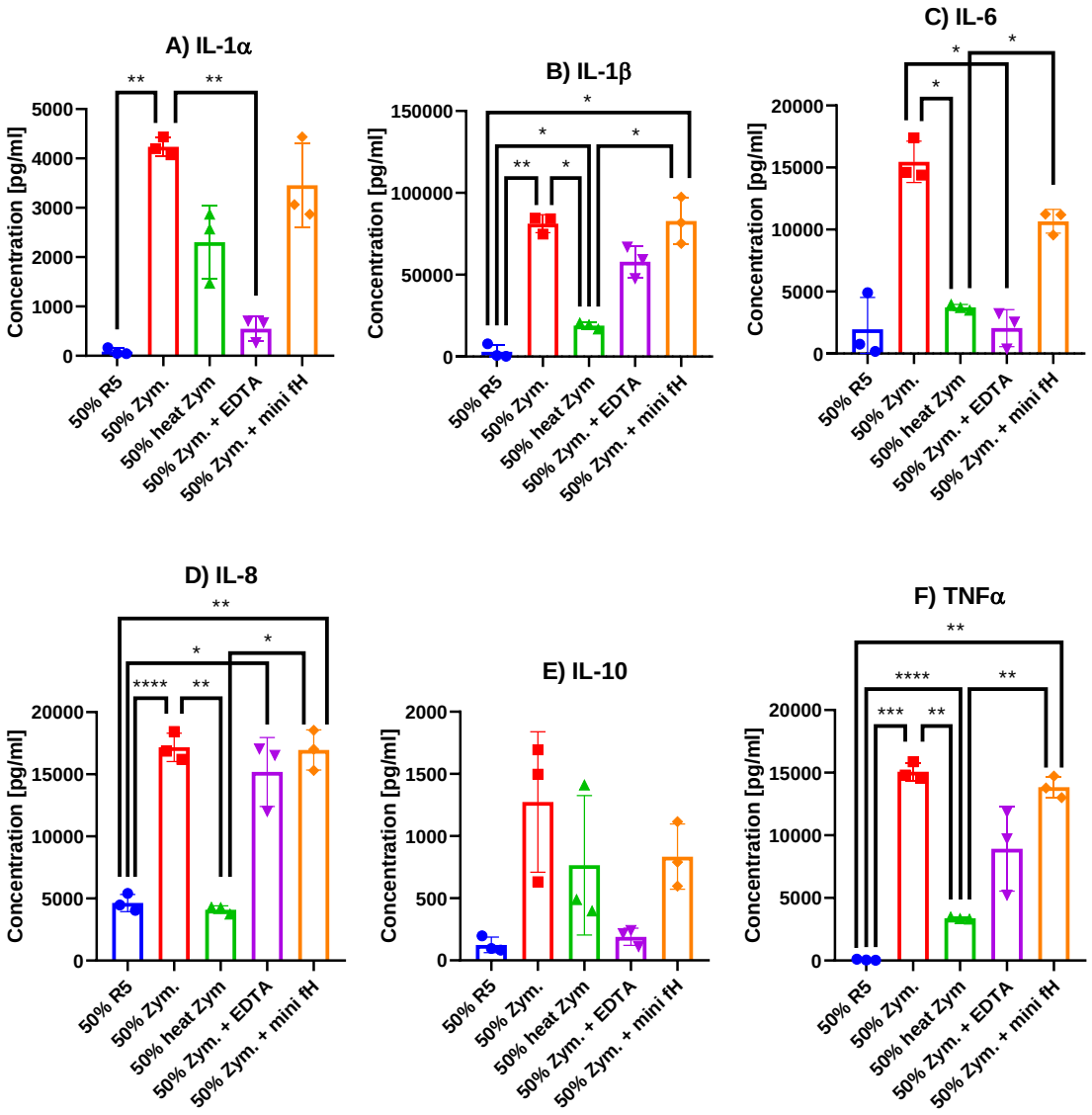


Figure 2. Effects of 50% autologous serum and zymosan (Zym.) treatment on cytokine secretion in PBMC cultures. R5 means culture medium (control), heat means heat inactivated serum containing medium, mini fH means mini factor H. The samples were collected after 18 hours of incubation.

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. 3**) 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.

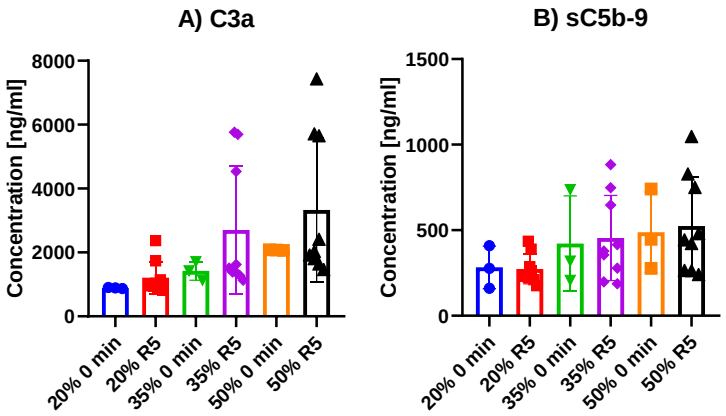
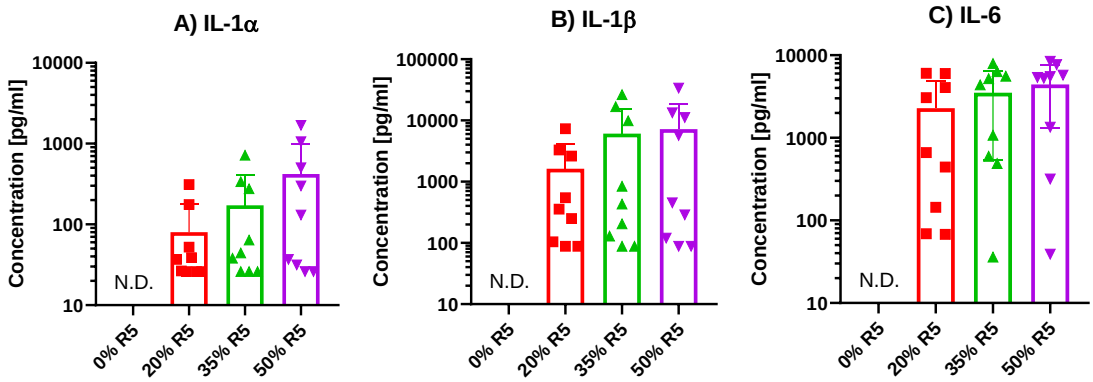


Figure 3. C3a and sC5b-9 concentrations in PBMC culture containing 20 – 35 – 50% autologous serum. R5 means culture medium (control). Samples were collected at 0 (baseline) and 45 minutes.

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. 4A to 4E**) 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.



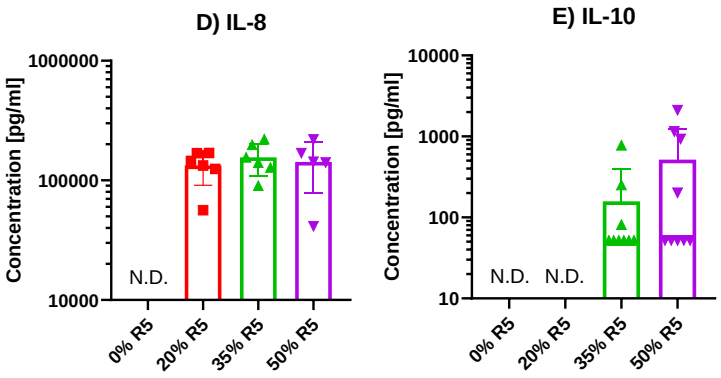


Figure 4. Cytokine secretion in PBMC cultures supplemented with 20 – 35 – 50% autologous serum. R5 means culture medium (control). The samples were collected after 18 hours.

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. 5A**). 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. 5A**) 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. 5B**).

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. 5C**). 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. 5C**). 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. 5D**).

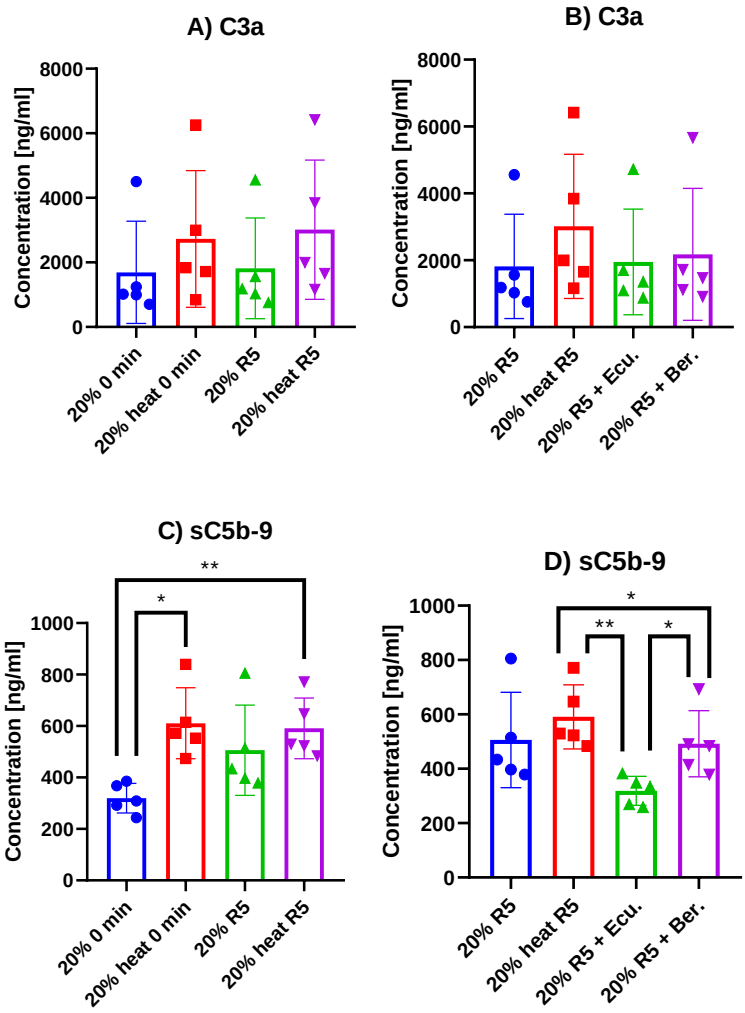


Figure 5. C3a and sC5b-9 concentrations in PBMC cultures containing 20% autologous serum. Eculizumab (Ecu.) and Berinert (Ber.) were used to inhibit complement activation. R5 means culture medium (control), heat means medium containing heat inactivated serum. Samples were collected at 0 (baseline) and 45 minutes.

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. 6**) compared to the serum-free condition, when the cytokine concentrations were below the detection limit (**Fig. 4**). 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. 6**). Heat inactivation of the serum had visible effect only in the case of IL-8 compared to untreated control (**Fig. 6B**). The presence of Eculizumab resulted in a small reduction in IL-6 and IL-8 concentrations (**Fig. 6A and 6B**), which did not reach the level of statistical significance. However, the C5 inhibitor did not induce any changes in TNFα conncetrations (**Fig. 6C**). 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.

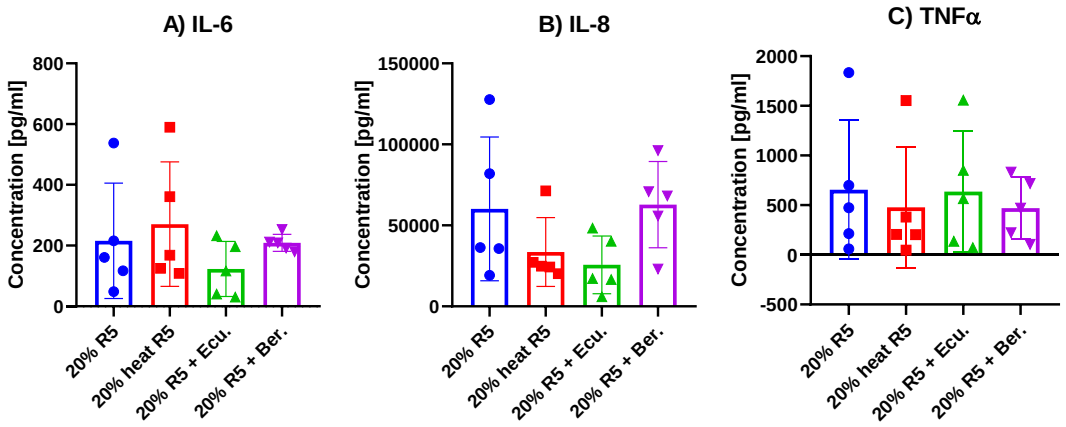


Figure 6. Effects of complement/serum autoactivation on cytokine concentrations in control samples (R5) of 20% autologous serum containing PBMC culture. Eculizumab (Ecu.) and Berinert (Ber.) were used to block complement activation. R5 means culture medium (control), heat means medium containing heat inactivated serum. The samples collected after 18 hours of incubation.

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. 7A**) 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. 7B**).

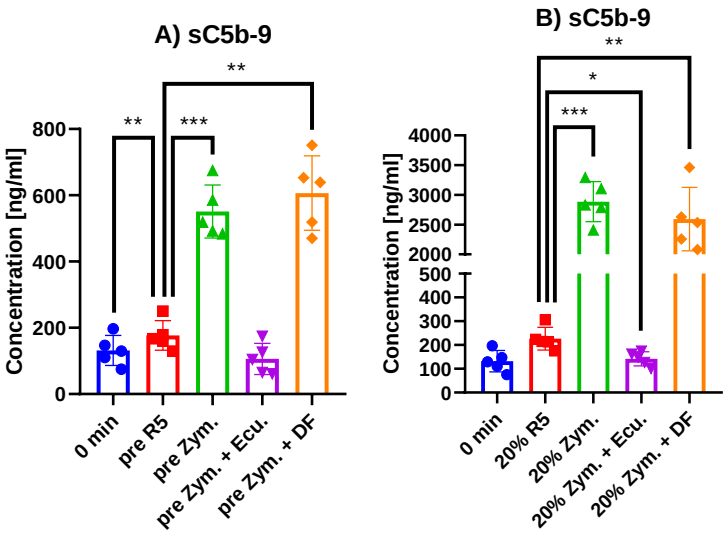


Figure 7. Effects of zymosan-pretreated serum (pre Zym., left) and zymosan (Zym., right) on sC5b-9 concentration in PBMC cultures. The effects of eculizumab (Ecu.) and DF2593a (DF) were tested to see if they antagonize the effects of zymosan. R5 means culture medium (control). Samples were collected at 0 (baseline) and 45 minutes.

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 8**, 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.

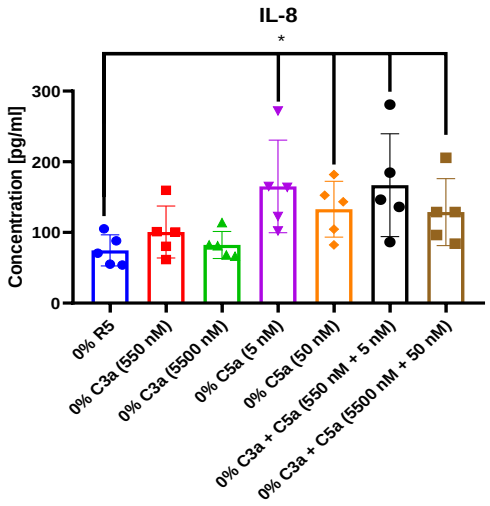


Figure 8. Effects of C3a and C5a anaphylatoxins on IL-8 concentrations in serum-free (0% serum) PBMC culture. R5 means culture medium (control). Samples were collected after 18 hours of incubation.

4.8. Effect of zymosan-pretreated serum on cytokine release

As shown in **Figure 7A**, 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. 9B**). 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. 9A**). 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.

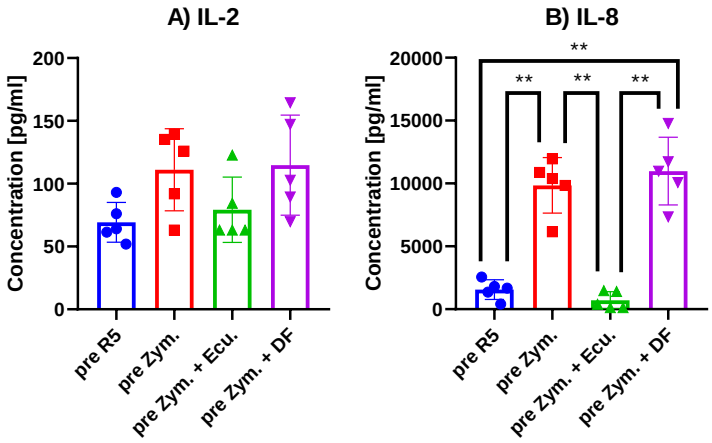


Figure 9. Effect of serum pretreated with zymosan (pre Zym.), and the influence of C5 complement inhibitor eculizumab (Ecu.) or complement C5a receptor antagonist DF2593A (DF) on the effect of serum pretreated with zymosan. R5 means culture medium (control). Samples were collected after 18 hours of incubation.

4.9. 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 (**Fig. 10**).

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 and IL-2. Eculizumab moderately inhibited the zymosan-induced secretion of IL-1α, IL-1β and TNFα, with limited effect on IL-2 and IL-6 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.

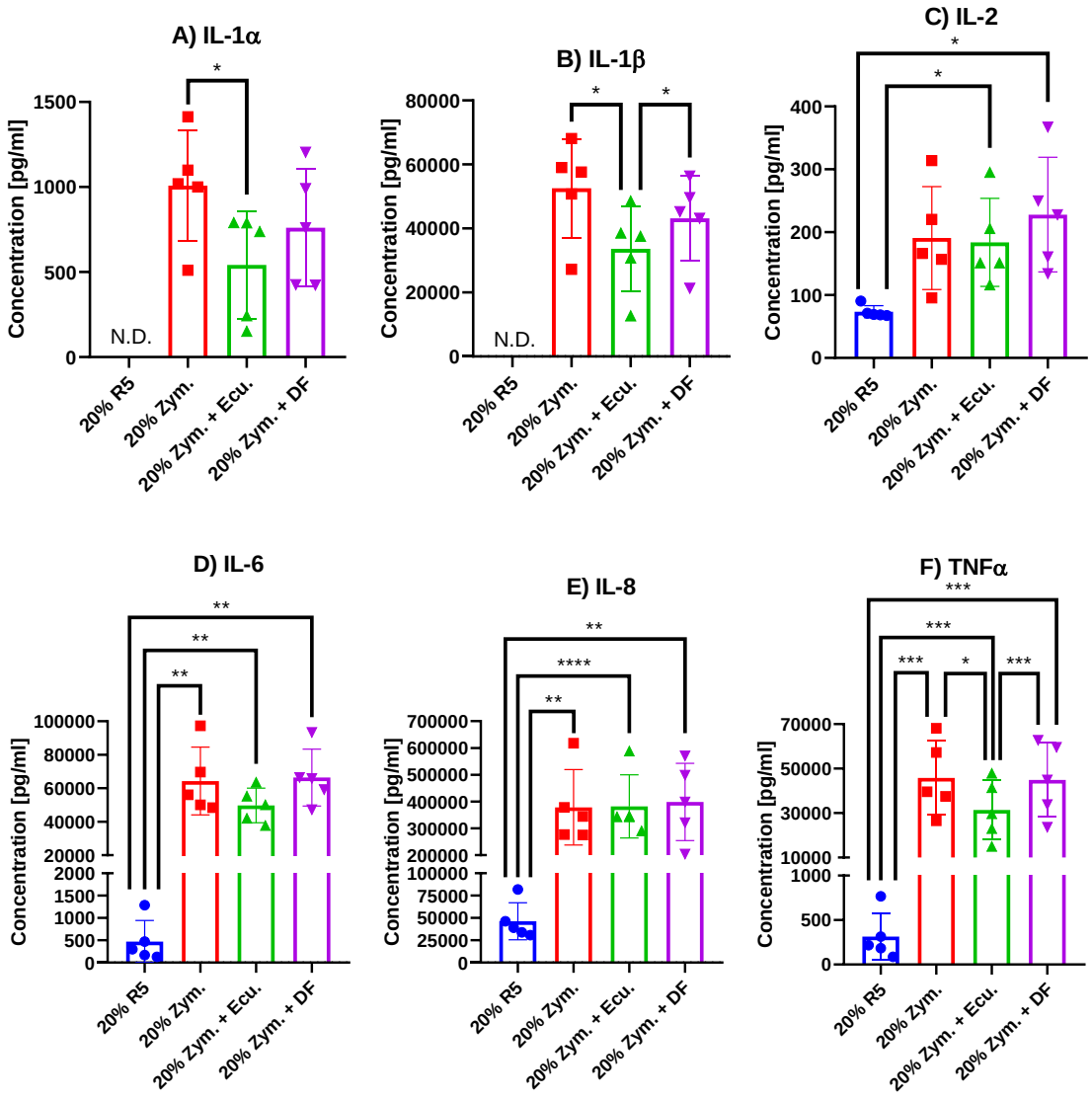


Figure 10. The effect of zymosan (Zym.) on cytokine release in PBMC cultures containing 20% autologous serum, and the effect of the complement C5 inhibitor, eculizumab (Ecu.), or the complement C5a receptor antagonist, DF2593A (DF), on zymosan-induced cytokine releases. R5 means culture medium (control). Samples were collected after 18 hours of incubation.

5. 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.

6. Bibliography of the candidate's publications

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