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CD21 LIGANDS PROVIDE BETTER ANTIBODY SPECIFIC RESPONSE THAN THE POLYCLONAL STIMULATION IN mBCs ACTIVATION

CD21 LİGANDLARI HAFIZA B HÜCRELERİN AKTİVASYONUNDA POLİKLONAL UYARIMDAN DAHA İYİ ANTİKOR SPESİFİK YANIT SAĞLAR

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ABSTRACT

Objective: In this study, we evaluated if the CD21 activation through its free ligands can improve the in vitro assessment of antigen specific antibody response of mBCs.

Methods: Amongst five healthy voluenteer, a volunteer with the highest level of anti-tetanus toxoid IgG (anti TT-IgG) antibody was detected by Enzyme-Linked Immunosorbent Assay (ELISA). The volunteer's peripheral blood mononuclear cells (PBMNCs) obtained by ficoll method were treated following conditions in vitro; in the group 1) the polyclonal stimulation and CD21 soluble ligands were applied, in the group 2) antigen-specific stimulation by tetanus toxoid (TT) and CD21 soluble ligands were applied, in the group 3) the CD21 soluble ligands C3d, iC3b and IFNα in addition to the polyclonal stimulation and antigen-specific stimulation by TT were applied. Anti-TT-IgG and total IgG antibody quantities were determined in culture supernatants at the end of the day-12 by ELISA.

Results: We found that total IgG antibody levels were higher in the 3th group where the soluble CD21 ligands added to the polyclonal stimulation and TT antigen. CD21 free ligand C3d was observed to increase specific antibody response together with TT antigen-specific stimulation. In addition, antigen-specific antibody responses were different depending on the CD21 free ligands.

Conclusion: Antigen specific stimulation in combination with C3d provided stronger specific antibody responses than polyclonal stimulation. Our data indicate that CD21 free ligands may have a potential in determining specific antibody responses.

Keywords: Antigen specific memory B cell, CD21, C3d, iC3b, IFNα.

ÖZET

Amaç: Bu çalışmada, serbest ligandları aracılığıyla CD21 aktivasyonunun, mBC'lerin antijen spesifik antikor yanıtının in vitro değerlendirmesini iyileştirip iyileştiremeyeceğini değerlendirdik.

Gereç ve Yöntem: Beş sağlıklı gönüllü arasından, Enzim Bağlantılı İmmunosorbent Testi (ELISA) ile en yüksek anti-tetanos toksoid IgG (anti TT-IgG) antikor düzeyine sahip bir gönüllü tespit edildi. Ficoll yöntemi ile elde edilen gönüllünün periferik kan mononükleer hücreleri (PKMNH) in vitro koşullarda şu işlemlere tabi tutuldu; 1. gruba poliklonal uyarım ve CD21'in serbest ligandları uygulandı, 2. gruba tetanos toksoidi (TT) ile antijen spesifik uyarım ve CD21'in serbest ligandları uygulandı, 3. gruba poliklonal stimülasyona ek olarak TT ile antijen spesifik uyarım ve alt gruplara CD21'in çözünür ligandları olan C3d, iC3b ve IFNα ayrı ayrı ve kombine olarak uygulandı. Anti-TT-IgG ve toplam IgG antikor miktarları 12. gün sonunda kültür üst sıvılarında ELISA ile belirlendi.

Bulgular: Total IgG antikor düzeyleri CD21 serbest ligandlarının poliklonal uyarım ve TT antijene ilave kullanıldığı 3. grupta en yüksek bulundu. CD21 serbest ligandı C3d'nin TT antijen-spesifik uyarımla birlikte spesifik antikor yanıtını artırdığı gözlemlendi. Ayrıca, tüm gruplarda elde edilen antijen-spesifik antikor yanıtları uygulanan CD21 serbest ligandına bağlı olarak değişkenlik gösterdi.

Sonuç: C3d ile birlikte antijen spesifik uyarım, poliklonal uyarımdan daha güçlü bir spesifik antikor yanıtı sağladı. Verilerimiz CD21 serbest ligandlarının spesifik antikor yanıtını belirlemede bir potansiyele sahip olduğunu göstermektedir.

Anahtar Kelimeler: Antijen spesifik hafıza B hücre, CD21, C3d, iC3b, IFNα .

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INTRODUCTION

Antigen-specific mBCs are of interest in various immunological fields, including organ transplantation, autoimmunity, immunodeficiencies, vaccines, and cancer immunotherapy. mBCs constitute as little as 5–15% of lymphocyte cells in peripheral blood and proliferate at a low rate. The lack of an effective method for the evaluation of mBCs in peripheral blood requires mBCs to proliferate in in vitro cell culture media and be transformed into antibody-secreting cells by polyclonal stimulation procedures (Jackson et al., 2011; Lefaucheur et al., 2010; Ojo et al., 2012;). In vitro polyclonal stimulation of mBCs is based on the use of various stimuli such as Toll-like receptor (TLR) ligands, various mitogens, Cluster of differentiation 40 Ligand (CD40L) and B-Cell Receptor (BCR) activation (Can et al., 2016; Franz et al., 2011; Jackson et al., 2011; Opelz, 2005; Phuong at al., 2020;Süsal et al., 2013) Polyclonal stimulation, which has the disadvantage of being a nonspecific method for the analysis of antigen-specific mBCs, relies on stimulation of all mBCs and subsequent selective analysis of the respective antigen-specific mBCs by a method known as Enzyme-Linked Immunospot (ELISPOT) (Franz et al., 2011; Jackson et al., 2011). ELISPOT has some difficulties in application and interpretation in the detection and quantification of mBCs. In addition, it is desired to obtain antigen-specific stimulation for antigen-specific evaluation of mBCs. In a study conducted for this purpose, it was shown that when TT antigen was added to lymphocyte cultures obtained from peripheral blood, some antibody response was obtained and when TT was added to the culture medium together with other antigens, TT-specific antibody response was increased (Tew et al., 1987). Cluster of differentiation 21 (CD21, CR2) is a BCR co-receptor that lowers the B cell activation threshold to increase antibody response. The importance of the B cell co-receptor complex in addition to the antigen-BCR complex in B cell activation is known (Delves and Roitt, 2000a). In addition to the BCR signal, the contribution of the complement-related signal is important for B cell activation. In C3d-opsonized immune complexes, C3d, one of the complement activation products, is bound to the antigen surface. B cells have CD21 receptors for C3d (Abbas et al., 2012b). The antigen C3d complex binds to BCR via antigen and to CD21 via C3d. In other words, activation of the complement system, which is the effector mechanism of the natural immune system, is a second signal for B cell activation (Abbas et al., 2012b). The B cell co-receptor complex formed by the binding of C3d to CD21 stimulates and amplifies BCR signaling pathways (Bouillie et al., 1999; Mesquita et al., 2010). Other studies have shown that CD21 activation via its ligands on other cell membranes stimulates mBCs antigen-specifically and polyclonally and increases antibody synthesis in vitro (Qin et al., 1998). Whether stimulation of the CD21 receptor via free ligands is useful for in vitro assessment of antigen-specific antibody responses of mBCs has not been investigated.

In this study, we aimed to investigate whether stimulation with TT antigen and independent ligands of CD21 would be effective in the polyclonal stimulation procedure for in vitro expansion of antigen-specific memory B cells, which are present in low numbers in peripheral blood.

MATERIALS AND METHODS

Study Design

The individual with the highest anti-TT-IgG antibody level was identified from peripheral blood samples of study participants using the ELISA method. Mononuclear cells were then isolated from this individual's peripheral blood via the Ficoll standard protocol. Peripheral mononuclear cells (PBMNCs) were counted using a Neubauer slide, and experiments were conducted with cells at a concentration of $5x10^5$ cells/ml. Three primary experimental groups were established: in the first group; polyclonal stimulation procedure as described in the literature, in the second group; TT antigen stimulation, in the third group combined polyclonal stimulation and TT antigen-specific stimulation. In addition to these three main groups, subgroups were created where CD21 free ligands—C3d, iC3b, and IFNα—were applied separately and in combination (see Table 1). Culture supernatants from all groups were collected on day 12. Anti-TT-IgG and total IgG antibody levels were measured twice for each group using the ELISA method, and differences in antibody levels were analyzed statistically.

Cases

This methodological study was planned to evaluate whether CD21 free ligands could contribute to the increase of antigen-specific antibodies in the presence of specific TT antigen stimulation. The study was approved by the the Tekirdag Namık Kemal University Ethical Committee (Document Number: 2017/66/07/01) and informed consents were obtained from all the cases included in this study.

Obtained of the Peripheral Blood Mononuclear Cells (PBMNCs)

Anti-TT-IgG antibody levels in peripheral blood samples of volunteers who agreed to participate in the study were determined using the ELISA method. A 20 mL heparinized peripheral blood sample was collected from the individual with the highest anti-TT-IgG level. PBMNCs were isolated by gradient centrifugation using Biocoll separation solution (Merck). The buffy coat, which formed at the interface, was collected using a Pasteur pipette (reference for the method). The PBMNCs were then transferred to transport medium and diluted to achieve a concentration of approximately 20–25 cells per small square on a Neubauer slide.

Using the prepared PBMNCs, three experimental groups were formed as follows (see Table 1) Group 1: Polyclonal stimulation (as described in the literature). Group 2: Antigen-specific stimulation with TT. Group 3: Polyclonal stimulation combined with antigen-specific stimulation using TT, as well as application of CD21 soluable ligands (C3d, iC3b and IFNα) both separately and in combination.

Polyclonal Stimulation

Peripheral blood mononuclear cells (PBMNCs) at a concentration of $5x10^5$ cells per well were cultured in 24-well culture plates using DMEM medium containing low glucose, 10% serum (Sigma), 50 µM 2-mercaptoethanol (Merck), 2 mM L-glutamine (Multicell), and 100 U/mL penicillin-streptomycin (Gibco). For polyclonal stimulation, the following agents were added to the culture medium to reactivate the proliferation of all memory B cells within the total mononuclear cell population and to induce their differentiation into antibody-secreting cells (Jourdan et al., 2009; Cao et al., 2010): CD40L (50 ng/mL, Biolegend), CpG DNA (10 ng/mL, Hycultbiotech), IL-2 (20 ng/mL, Biolegend), IL-10 (50 ng/mL, Biolegend), IL-21 (100 ng/mL, Biolegend), ITS supplement (5 µg/mL insulin, 5 µg/mL tranferrin, 5 ng/mL sodium selenite, Sigma), Phorbol 12-myristate 13-acetate (PMA) (5 ng/mL, Sigma) (Jourdan et al., 2009; Cao et al., 2010) . In groups 1 and 3 of this study, polyclonal stimulation was employed to promote antibody secretion.

Co-stimulation of Memory B Cells (mBCs) by the CD21 Receptor Ligands and Tetanus Toxoid

For antigen-specific stimulation, commercially available tetanus toxin (TT, Sigma) was added to the in vitro culture medium at a dose of 5 ng/mL. In group 2, only TT was used for antigen-specific stimulation, whereas in Group 3, TT stimulation was combined with polyclonal stimulation.

To activate the CD21 receptor, soluble CD21 ligands—C3d (0.01 mM, Alfadiagnostic), iC3b (0.01 mM, Alfadiagnostic), and IFNα (0.01 mM, Stem Cell)—were applied separately and in combination across all groups. This CD21 ligand application was conducted in addition to the conditions of polyclonal stimulation, TT-specific stimulation, and cases where both stimuli were applied together to assess the impact on CD21 receptor activation.

Table 1. Experimental Groups with Polyclonal Stimulation, Antigen-Specific Stimulation with The TT Antigen and CD21 Receptor Stimulation

Group	Case No	Experimental Treatments
Group 1	**A1**	Polyclonal stimulation
	A2	Polyclonal stimulation + C3d
	A3	Polyclonal stimulation + iC3b
	A4	Polyclonal stimulation + IFNα
	A5	Polyclonal stimulation + C3d+iC3b+IFNa
Group 2	**B1**	Antigen specific stimulation with Tetanus Toxoid
	B2	Antigen specific stimulation with Tetanus Toxoid + C3d
	B3	Antigen specific stimulation with Tetanus Toxoid + iC3b
	B4	Antigen specific stimulation with Tetanus Toxoid + IFNα
	B5	Antigen specific stimulation with Tetanus Toxoid + C3d+iC3b+IFNα
Group 3	**C1**	Polyclonal stimulation + Antigen specific stimulation with Tetanus Toxoid
	C2	Polyclonal stimulation + Antigen specific stimulation with Tetanus Toxoid + C3d
	C3	Polyclonal stimulation + Antigen specific stimulation with Tetanus Toxoid + iC3b
	C4	Polyclonal stimulation + Antigen specific stimulation with Tetanus Toxoid + IFNα
	C5	Polyclonal stimulation + Antigen specific stimulation with Tetanus Toxoid + C3d+iC3b+IFNα

Anti-TT-IgG and Total IgG Antibody Analysis by ELISA Method

The amount of anti-TT-IgG and total anti-IgG antibodies in the culture supernatants collected on day 12 of the cell culture were determined using the ELISA method. This analysis assessed both the effect of CD21 receptor activation on proliferation of antigen-specific memory B lymphocytes and its impact on total IgG antibody levels. An optimized indirect ELISA method, developed in our laboratory for greater sensitivity in antibody detection, was used. To coat the high- binding ELISA plates, a 10mM Tris pH 9.0 solution was prepared, along with 3µg/mL inactivated TT toxin (Sigma) to measure anti-TT-IgG levels and 1.8 µg/mL goat-anti-human IgG (Jackson Immuno Research) to measure total IgG levels. These solutions were distributed at 100 µl per well, and the plates were covered with plastic tape and incubated overnight at +4˚C. The following day, the wells were washed twice with a 0.005% Tween-20/PBS solution. Blocking was performed by adding 300 µL of a 2%BSA/Tween-20/PBS solution to each well, covering the plates, and incubation for 30 minutes at 37˚C. The wells were then washed with 0.005%Tween-20/PBS solution. The quantitatively evaluated anti-TT-IgG and total IgG antibodies, the human reference serum was used as a standard. Serian dilutions of this reference serum were prepared in 1%BSA/Tween-20/PBS (with azide, Sigma), and distributed in duplicate into the wells as standards. For the control group, 100 µL of 1%BSA/Tween-20/PBS solution (with azide) was used per well. Supernatant samples collected on day 12 were diluted 10- and 50-fold in 1% BSA/Tween-20/PBS solution (with azide) and added to the wells at 100 µL per well. The plates were sealed and incubated at 37 °C for 1 hour. Following incubation, the wells were washed twice with 0.005%Tween-20/PBS solution. A biotin-labeled anti-human IgG antibody (Invitrogen) at a concentration of 0.11µg/mL was prepared in 1%BSA/Tween-20 solution (without azide) and added at 100 µl per well. Plates were sealed and incubated for 1 hour at 37 °C then washed twice with 0.005%Tween-20/PBS solution. Streteavidin-HRP (Thermo) prepared at concentration of 9,6.10-2u/mL in 1%BSA/Tween-20/PBS solution (without azide) was added at 100 µL per well. Plates were sealed and incubated for another hour at 37 °C, followed by two washes with 0.005%Tween-20/PBS solution. For detection, an ABST solution (Sigma) prepared in citrate phosphate buffer (pH 4.2, Sigma) and filtered through a 0.2 µm filter, was diluated to 0.01% in 30% H_2O_2 and added at 100 µL per well. Plates were incubated at room temperature for 5 minutes. The reaction was stopped with 250mM oxalic acid (Sigma), and optical densities were read at a 450 nm wavelength using a Thermo ELISA reader at. Optical densities of anti-TT-IgG and total IgG antibodies obtained by ELISA were converted to quantitative values using standards.

Statistical Analyses

In this study, the data whose results were evaluated statistically were obtained with one experimental repetition and two technical repetitions for all application groups. An Independent-Samples T test was used to compare the antibody levels detected in each application group. A P value of <0.05 was considered statistically significant, indicating that the respective application had a measurable effect.

RESULTS

Detection of Anti-TT-IgG and Total IgG Antibodies in Culture Supernatants by Indirect ELISA

The highest level of anti-TT IgG antibodies was observed in the application where specific antigenic stimulation with TT and CD21 receptor stimulation with C3d were combined (Table 1, B2; Fig 1). In this application (Table 1, B2), anti-TT IgG antibody levels were 10.69 times higher than in the application with specific TT antigenic stimulation alone (Table 1, B1), where neither polyclonal stimulation nor CD21 receptor stimulation with C3d was performed. This difference was statistically significant (Independent-Samples T test, p=0.001).

Total IgG levels were also measured in the same samples, and anti-TT-IgG levels were calculated as a proportion of total IgG. With this adjusted analysis, the increase in anti-TT IgG was 13.01-fold, a statistically significant difference (p=0.001) (Fig 2, Fig 4). Group 2 subgroups showed a significant decrease in total IgG levels compared to all other groups (Fig 2, Fig 3).

When comparing the condition where TT-specific antigenic stimulation and CD21 receptor stimulation with C3d were applied (Table 1, B2) to conditions without polyclonal stimulation and with polyclonal stimulation combined with C3d (Table 1, A2), anti-TT-IgG levels were 5.02-fold higher (p=0.018) in the absence of polyclonal stimulation (Table 1, B2) and showed a 9.84-fold increase (p=0.005) when the anti-TT-IgG to total IgG ratio was evaluated.

These results suggest that combining TT antigen-specific stimulation with CD21 receptor stimulation using C3d generates a significantly higher anti-TT-IgG response than polyclonal stimulation combined with C3d alone. Specifically, CD21 receptor stimulation with C3d in the presence of antigen increased the antigen-specific antibody response of memory B cells by 10.69-fold, and by 5.02-fold compared to polyclonal stimulation plus C3d alone. This approximately two-fold difference indicates that antigen-specific stimulation has a more substantial impact on the specific antibody response than polyclonal stimulation alone.

In the presence of specific TT antigen and polyclonal stimulation, IFNα (C4) and the combined condition of C3d+IFNα+iC3b (Table 1, C5) increased anti-TT-IgG levels by 4.31-fold (p=0.040) and 6.44-fold (p=0.011), respectively, compared to TT-specific stimulation alone (Table 1, B1) and polyclonal stimulation (Table 1, C1). When evaluating the ratio of anti-TT-IgG to total IgG, increases of 7.01-fold (p=0.033) and 9.73-fold (p=0.009) were observed, respectively. It is suggested that this increase may be attributable to the presence of IFNα.

In the C1 group, the total IgG antibody secretion amount was shown to be 27% higher than in polyclonal stimulation (A1) (Fig 2, Fig 3). It was shown that the change in the amount of specific antigen (anti TT-IgG antibody) in total IgG antibody was at the same rate in groups B1 and C1, and that they had a greater increase than polyclonal stimulation (A1) (Fig 4).

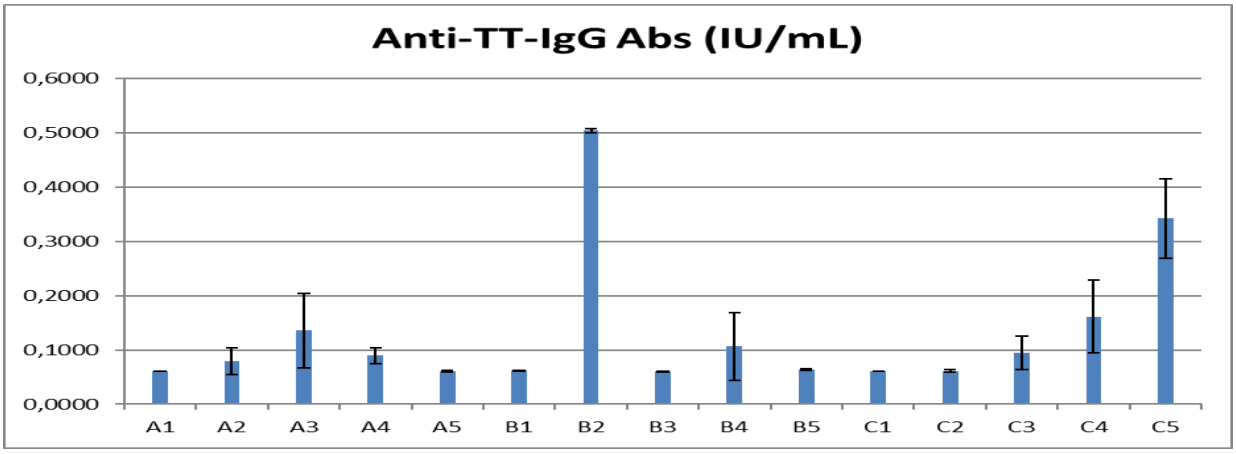

Figure 1. Anti-TT-IgG Ab Quantities In All Groups. B2 And C5 Show Statistically Significant Increase.

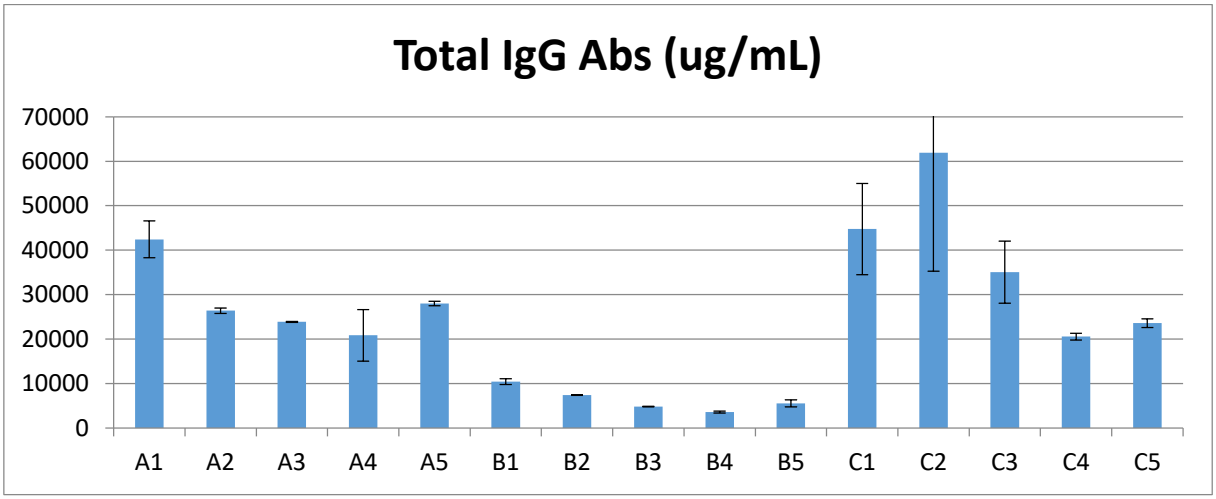

Figure 2. Total IgG Ab Quantities In All Groups.

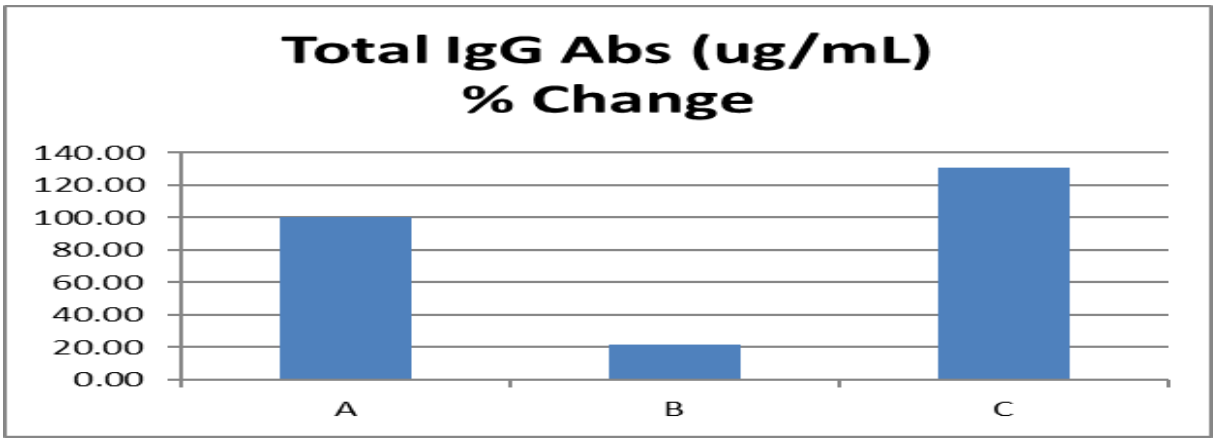

Figure 3. Total IgG Antibodies Amongst The Main Groups

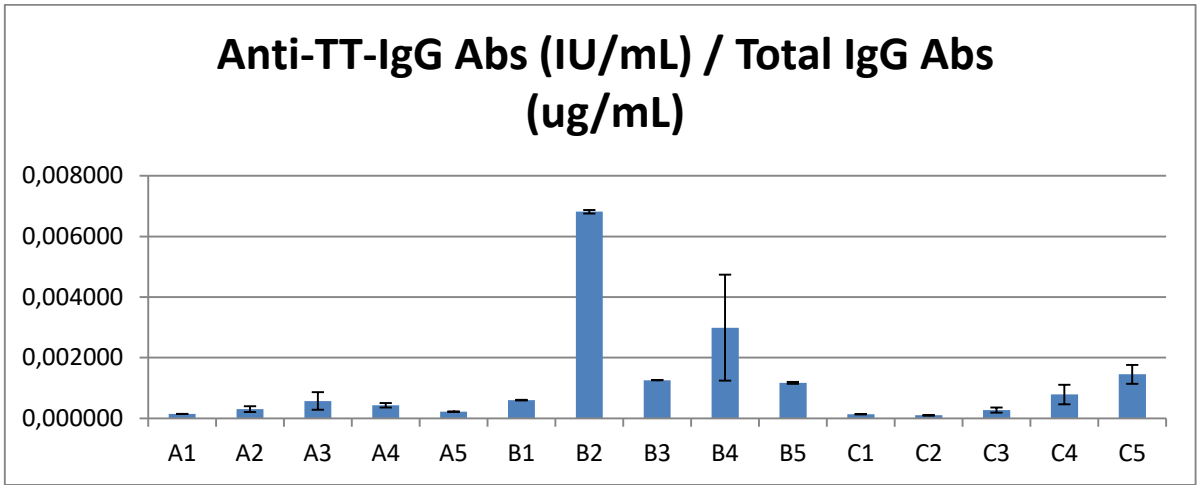

Figure 4. Comparison of the Anti-TT-IgG Ab Within the Total IgG Ab in All Groups.

DISCUSSION

Antigen-specific mBCs play a crucial role in assessing humoral immune capacity, especially in areas like organ transplantation, immunodeficiencies, autoimmune diseases, monitoring of vaccine efficacy and cancer immunotherapy. Currently, humoral immune response capacity is often measured through antibody levels, but this approach lacks sufficient precision, necessitating ex vivo analysis of antigen specific mBCs (Franz et al., 2011). Developing cell culture techniques that support the in vitro antibody-secreting cells is therefore essential (Lefaucheur et al., 2010; Ojo et al., 2012). Although recent efforts have been made to create labeled antigen tetramers targeting the BCR for use in the direct detection of antigen-specific mBCs by flow cytometry, except for a few examples, almost all studies conducted with mBCs are based on the ELISPOT method, which allows polyclonal stimulation, a nonspecific stimulation method, and antigen-specific analysis (Phuong et al., 2020). In vitro Polyclonal stimulation of mBCs is based on the use of various stimuli such as Toll-like receptor (TLR) ligands, various mitogens, cytokine cocktails, CD40L and BCR activation of the resulting mononuclear blood cells in vitro (Can et al., 2016; Jackson et al., 2011; Opelz, 2005; Susal et al., 2013). After in vitro polyclonal stimulation, the number of antibody-secreting cells is usually determined by the ELISPOT method to perform antigen-specific mBC detection and quantification. Although there are concerns that differences in vitro stimulation and conditions may affect the frequency of antibody-secreting cells and the functionality of subpopulations, making it difficult to compare studies using different stimulations, today's quantification of mBCs is based on their in vitro proliferation and conversion into antibody-secreting cells (Franz et al., 2011; Jackson et al., 2011). In the in vitro quantification of mBCs specific to a particular antigen type, the idea of stimulating them with sensitized antigen alone instead of polyclonal stimulation did not provide as strong an antibody response as expected. In a study conducted

for this purpose, it was shown that while some antibody response was obtained when TT antigen was added to lymphocyte culture obtained from peripheral blood, TT-specific antibody response increased when TT was added to the culture medium together with other antigens. The increase in the secondary antibody response in vitro was explained by the immunoadjuvant effect of molecules that stimulate natural immunity (Tew et al., 1987). Studies in mouse models have shown that suppression or knockout of CD21 reduces the immune response (Ahearn et al., 1996; Hebell et al., 1991; Molina et al., 1996; Thyphronitis et al., 1991). In humans, CD21 gene defects are known to cause hypogammaglobulinemia and the CD21 receptor enhances antigen-specific B cell responses under normal conditions (Thiel et al., 2012). In addition to the antigen-BCR complex, the B cell co-receptor complex is known to be important in B cell activation (Delves and Roitt, 2000b). In addition to the BCR signal, the contribution of the complement-related signal is important for B cell activation. In C3d-opsonized immune complexes, C3d, one of the complement activation products, is bound to the antigen surface. B cells have CD21 receptors for C3d (Abbas and Pillai, 2012a). The antigen-C3d complex binds to the BCR via antigen and to CD21 via C3d. In other words, the activation of the complement system, which is the effector mechanism of the innate immune system, is a second signal for B cell activation (Abbas and Pillai, 2012a). The B cell co-receptor complex formed by the binding of C3d to CD21 stimulates and amplifies the BCR signaling pathways (Bouillie et al., 1999; Mesquita et al., 2010). These mechanisms explain why the complement system elements, which are free ligands of CD21 added to the medium in the presence of specific TT antigen, should increase the secondary humoral response of mBCs in vitro.

In our study, we aimed to determine the effects of stimulation with TT antigen and free ligands of CD21 (C3d, iC3b and IFNα) in addition to the polyclonal stimulation procedure, which has been reported to be useful in the in vitro multiplication of antigen-specific mBCs, which are few in number in peripheral blood, and their transformation into antibody-secreting cells. To the best of our knowledge, this is the first study to investigate the effect of CD21 receptor activation by its free ligands on the proliferation and transformation of antigen-specific memory B lymphocytes into antibody-secreting cells in vitro.

CONCLUSION

As a conclusion, we suggest that in vitro CD21 receptor stimulation via its soluble ligands either solely in the presence of a specific antigen or in addition to the polyclonal stimulation protocol and a specific antigen would provide better specific antibody secretion from the cultured mBC that is the main procedure in the antigen specific antibody evaluation today.

Conflict of Interest

We declare that there is no conflict of interest in this study.

Author Contributions

Plan, design: TB, DYS and IMT; Material, methods and data collection: TB, DYS and HA; Data analysis and comments: TB, DYS and HA; Writing and corrections: HA, TB, DYS.

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