Final scientific report

Title: Investigating the subsets of circulating follicular T helper cells and the potential modulation of cell function in the pathogenesis of systemic autoimmune diseases

Project number: KLINO-121327

Duration: 1st October 2016 – 30th September 2020

Changes in participants compared to the application

There was a change among the researchers included in the project contract, because former participant, Prof Margit Zeher passed away in October 2018 and Tünde Tarr (EPR nr.: 410902) joined as a new participant.

Background of the study

Autoimmune diseases are multifactorial disorders which develop on the ground of genetic "predisposing" factors, epigenetic modifications, environmental and hormonal influences. In genetically susceptible individuals, tolerance that evolved against their own structures is impaired and leads to the formation of the disease.

In recent years, the most intensively studied cell type is follicular T helper (T_{FH}) cells, which have a central role in to direct antigen specific naive or memory B-cell activation in the follicles of secondary lymphoid organs. The interaction between T_{FH} and activated B cells is essential for the generation of extrafollicular short-lived plasma cells to produce low-affinity antibodies and for germinal center (GC) responses [1]. Within GCs, T_{FH} cells provide survival signals to GC B cells, which have undergone somatic hypermutation, and they direct the development of high-affinity memory B cells and long-lived plasma cells. Based on the critical role of T_{FH} cells in B-cell differentiation and antibody production, their failure to maintain self-tolerance potentially leads to the formation of autoreactive immune processes [2]. Evidences suggested that T_{FH} cells can be transported to neighboring GC as well as between adjacent follicles, moreover they can also migrate to the periphery [3]. Human blood memory T_{FH} cells form a heterogeneous group and include several subsets with different phenotypes and functions according to the presence of ICOS, PD-1, CCR7, CD62L and chemokine receptors CXCR3, CCR6. In accord with the former, memory cT_{FH} cells are distinguished not only as $CXCR5^+CCR7^-CD62L^-$ effector memory and $CXCR5^+CCR7^+CD62L^+$ central memory cells, but they are further divided into $ICOS^+PD\text{-}1^{++}CCR7^{lo}$ activated, $ICOS^-PD\text{-}1^+CCR7^{int}$ and $ICOS^-PD\text{-}1^-CCR7^{hi}$ quiescent memory cT_{FH} cells. The latter parameters define $CXCR3^+CCR6^-$

resembling Th1 cells ($cT_{FH}1$), $CXCR3^-CCR6^-$ resembling Th2 ($cT_{FH}2$) and $CXCR3^-CCR6^+$ resembling Th17 ($cT_{FH}17$) cells [4]. It is established, that T_{FH} cell subsets have distinct functional roles, $T_{FH}2$ cells and $T_{FH}17$ cells efficiently induce naïve B cells to differentiate into plasma cells and produce class-switched immunoglobulins, meanwhile the helper capacity of $T_{FH}1$ cells only occur when they are in an activated state ($ICOS^+PD\text{-}1^{++}$) and it is limited to memory B cells [5]. Recently, Foxp3 expressing $CXCR5^+$ follicular regulatory T (T_{FR}) cells, a specialized subset of regulatory T cells was identified, which can modulate GC responses through controlling the number of T_{FH} and GC B cells. Together, T_{FH} and T_{FR} cells are key regulators of the GC response: T_{FH} controls the size and output of GC while T_{FR} acts as a negative regulator [6].

Aims of the study stated at the time of the application

T_{FH} cells have a crucial role in the development and activation of B cells and subsequent antibody production, thus their failure to maintain self-tolerance potentially contributes to autoimmunity. The role of T_{FH} cell subsets as a regulatory element of humoral immune response has been in the center of interest for a while. Based on our hypothesis, changes in the number and function of T_{FH} cells alter the distribution of naïve and memory B cell subsets in primary Sjögren's syndrome (pSS) and systemic lupus erythematosus (SLE). Since their discovery, cT_{FH} cells and their subsets were investigated in several autoimmune diseases with varying results. Considering the contradictions as well as limited amount of data especially in pSS, the primary aim of the study was to clarify the distribution of blood T_{FH} subpopulations with the assessment of certain B cell subsets and routine laboratory parameters. Since co-stimulatory molecules are critical for T cell-dependent B cell activation, maturation and antibody production, we investigated the involvement of peripheral T_{FH}-like cells in B cell differentiation, immunoglobulin production and their ability to deliver B cell help during their interaction by neutralizing the interaction between these cells. We also attempted to study the potential role of specific micro (mi)RNAs on the operation of altered proportion T_{FH} and B cells in the investigated systemic autoimmune diseases.

The progress of the investigation

The research was progressed roughly according to the estimated work schedule. The death of Prof Margit Zeher and the subsequent uncertainty caused a 5 month decline in our research organisation and acquisition. For reasons beyond our control, miRNA analysis from isolated peripheral B and T_{FH} cells is not yet completed. There was a delay in collecting the needed

number of samples for our investigation due to an error committed by our collaborating partner and the SARS-CoV2 pandemic.

Data under publication are in text, data not yet published are demonstrated as figures in the final report.

Result obtained in the project

1. The role of circulating T_{FH} cell subsets in the pathogenesis of primary Sjögren's syndrome

Primary Sjögren's syndrome (pSS) is a chronic, systemic autoimmune disease that predominantly affects middle-aged women. Similarly to other systemic autoimmune diseases, increased lymphocyte activation, disproportional programmed cell death, in parallel with faulty autoantigen scavenging, are important in the pathogenesis of pSS. Furthermore, B-cell hyperactivation, inadequate elimination of autoreactive B cells and autoantibody production are key hallmarks of pSS. Our aim was to investigate the expansion of cT_{FH} subsets and T_{FR} cells and assess their correlations with various circulating B cell subpopulations and routine serological parameters in patients with pSS and compare them to values measured in healthy controls. Moreover, we evaluated the role of cT_{FH} cells in helping B cells in an *in vitro* functional assay. Peripheral blood from 38 pSS patients and 27 healthy controls was collected and assessed for the frequencies of T_{FR}, cT_{FH} and B cell subsets by flow cytometry.

We have previously shown the important role of T_{FH} cells and their IL-21 cytokine secretion in autoreactive B cell activation and autoantibody production in pSS [7]. In the present study, we confirmed an increased frequency of activated cT_{FH} cells in an independent cohort of pSS patients, moreover we investigated the distribution of recently described subtypes of cT_{FH} cells. Although the ratio of the latter did not differ significantly in pSS patients compared to healthy controls, a slight decrease of $T_{FH}2$ cells was observed in patients, while the percentages of $T_{FH}1$ cells tended to increase within the $CD4^+CXCR5^+$ cT_{FH} pool. Our data are in support of no specific abnormality within T_{FH} subsets, but rather an enhanced activation status. We correlated the expression of activation markers ICOS and PD-1 with the percentages of cT_{FH} subsets and discovered that $cT_{FH}1$ cells were in an activated state while $cT_{FH}17$ and $cT_{FH}2$ cells were probably quiescent in the peripheral blood of patients with pSS. Correlation analysis with serological markers reinforced our previous findings that activated cT_{FH} cells are in a strong relation with serum IgA and anti-La/SSB but regarding the cT_{FH} subsets, only $cT_{FH}1$ and partially $cT_{FH}1/17$ cells seem to play role in the pathogenesis of pSS. Results of the correlation with B cells were restricted only to activated cT_{FH} and $cT_{FH}2$ cells. Activated cT_{FH} showed negative correlation with un-switched memory B cells and correlated positively with

transitional B cells and plasmablasts, while, $cT_{FH}2$ cells correlated positively with memory B cell subtypes and demonstrated negative association with naïve and mature-naive B cells. Inevitably, these data raise the question of how the counterintuitive decrease in memory B cell and $cT_{FH}2$ cell frequencies could contribute to humoral autoimmunity in pSS. Since long-lived plasma cells differentiate and accumulate in the target tissue of pSS patients, the observed tendency in decrease of $cT_{FH}2$ cells at the periphery may be a consequence of their migration towards the affected organs. Our study also explored the possible importance of T_{FR} cells in pSS and revealed that both the ratio and number of cT_{FR} cells were increased in pSS patients with seropositivity for anti-Ro/SSA autoantibody compared to seronegative patients. It is hypothesized, that blood T_{FR} cells are originated from thymic Treg cells after their interaction with activated dendritic cells in the T-cell zone of secondary lymphoid organs, but they join the circulation prior to complete differentiation into tissue resident T_{FR} cells, thus they are mostly immature cells with high CD25 expression which are deficient in humoral suppression. Since CD40L signaling and the production of IL-21 cytokine is necessary for T_{FH}-cell-dependent B-cell maturation and antibody production, blockade of CD40L-CD40 signaling pathway or the neutralization of IL-21 could be beneficial for reduction of autoantibody production and inhibition of B cell abnormality. Functional grade purified anti-human IL-21 or/and anti-human CD40/TNFRSF5 were added to disrupt the interaction between the cells in a $CXCR5^+$ T_{FH}-B cell and $CXCR5^-$ T_H-B cell co-culture system. Blockage of CD40 and IL-21 decreased both IgM and IgG antibody production in the co-culture system, but simultaneous treatment with both substances proved to be more effective. When we analysed the fold-decrease of immunoglobulin production, we were able to demonstrate a difference between patients with pSS and healthy individuals, since the neutralization effect was more pronounced in pSS.

The manuscript, entitled "*The imbalance of circulating follicular T-helper cell subsets in primary Sjögren's syndrome associates with serological alterations and abnormal B-cell distribution*" by Szabó K, Jámbor I, Szántó A, Horváth IF, Tarr T, Nakken B, Szodoray P and Papp G has been submitted to the journal of Immunology & Cell Biology in July 2020, and after the first review round, we had to perform several experiments for the revision and it is presently being under the second review round (manuscript ID ICB-20-OA-0194-R1).

2. The role of circulating T_{FH} cell subsets in the pathogenesis of systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease with heterogeneous symptoms. The disease is characterized by the presence of autoantibodies directed against nuclear antigens and the inflammatory process can affect numerous organs,

including the skin, joints, kidneys and the central nervous system. Lupus is a relapsing and remitting disease, involving mild, moderate as well as severe forms. The most important manifestation in SLE is the deposition of immune complexes (ICs) in the kidneys. The existence of abnormal B cell responses is supported by those studies in which B-cell activating factor (BAFF) and ectopic GC have been shown in renal biopsies in patients with lupus nephritis. Previously, our workgroup reported elevated percentages of $ICOS^+PD\text{-}1^+$ cT_{FH} cells in patients with lupus and these cell proportions showed associations with SLE disease activity index [8]. Similarly to pSS, our aim was to study the alteration in the distribution cT_{FH} subsets and T_{FR} cells and investigate their correlations with B cell subsets in the peripheral blood as well as with routine laboratory data in patients with lupus. We also intended to study the role of cT_{FH} cells in directing B cell response with *in vitro* functional assay, but relying on our previous observations, we modified our protocol which caused a short decline in the workplan. Peripheral blood from 67 patients with lupus and 43 healthy individuals was collected and immunophenotyping of lymphocytes was performed by multicolor flow cytometry. PBMCs were isolated from heparinised venous blood samples by Ficoll-Histopaque density-gradient centrifugation. The distribution of cT_{FH} cells, cT_{FH} subsets, cT_{FR} cells and different B cell subsets were quantified with 4-Color analysis by BD FACS Calibur flow cytometer. Blood T_{FH} cells, their subsets and cT_{FR} cells were determined within $CD4^+$ lymphocytes and B cell subpopulations were assessed within $CD19^+$ cells.

The frequency of activated $ICOS^+PD\text{-}1^+$ cT_{FH} (Fig.1A) cells and T_{FR} cells (Fig.1B) was significantly increased compared to healthy individuals.

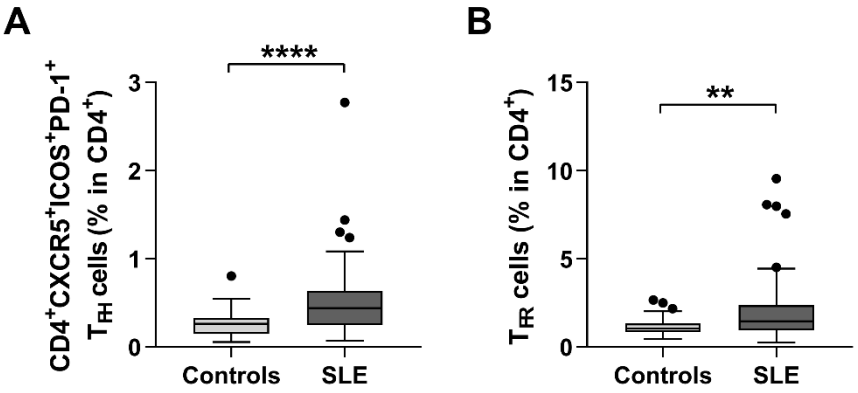

Figure 1.

There were no detectable difference in the percentages of $T_{FH}1$, $T_{FH}1/17$ and $T_{FH}2$ cells in patients with SLE compared to controls, however, only a mild increase was showed in $T_{FH}1$ cells compared to controls but it was not significant (Fig.2A). Interestingly, the proportions of

$T_{FH}17$ cells were significantly decreased in lupus compared to healthy donors and the difference was more evident in patients with anti-dsDNA autoantibody seropositivity (Fig.2B).

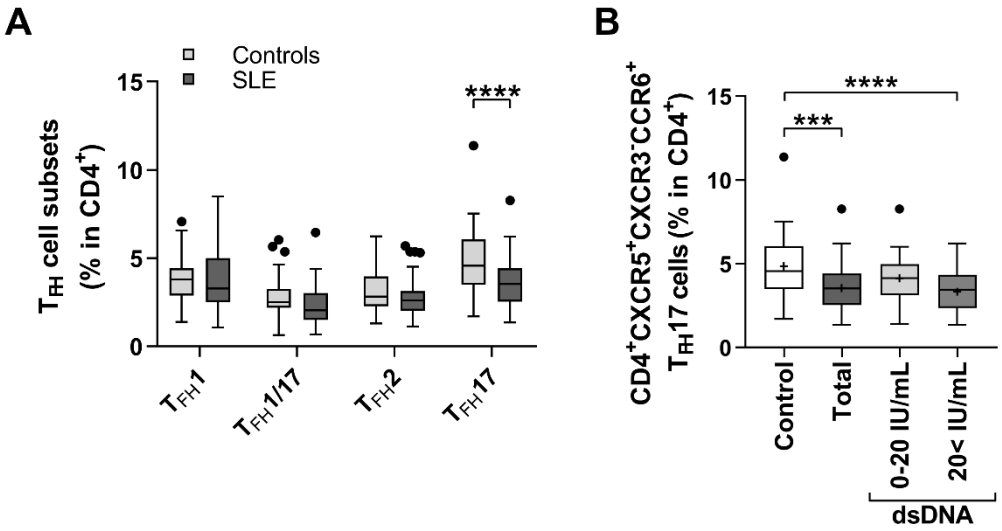

Figure 2.

In parallel with the investigation of cT_{FH} subsets in lupus, we also measured the proportion of different B cell subpopulation to properly describe condition of the representative patient population. The percentages of naive B cells were significantly increased in SLE patients compared to values in healthy individuals, while the ratio of un-switched memory B cells was significantly diminished in lupus compared to control values (Fig.3).

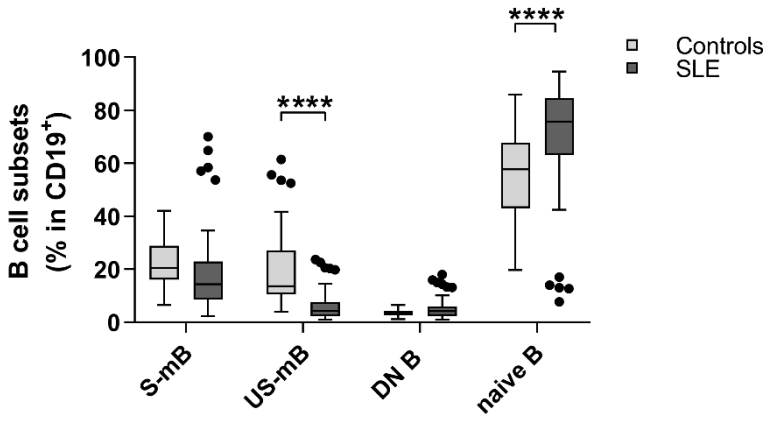

Figure 3.

The proportion of plasmablasts was significantly elevated compared to controls (Fig.4A). The ratios of mature-naïve B cells and transitional B cells were significantly increased, while the percentages of primarily-memory B cells were significantly increased in lupus compared to controls (Fig.4B).

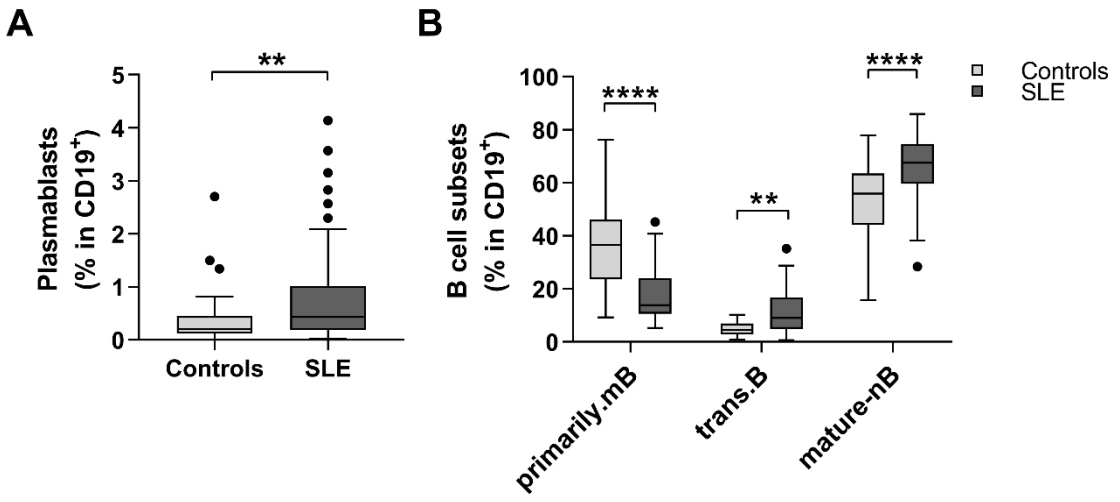

Figure 4.

We also investigated whether the alterations in activated cT_{FH} cells and T_{FH} cell subsets associated with disease etiology and aberrant B cell distribution in SLE. Correlations analysis between certain B cell subsets and the percentages of T_{FH} subsets and serological markers was demonstrated on Fig.5A, while the correlation between the ratio of cT_{FH} cells and serum levels of anti-dsDNA, immune complexes, complement C3 and C4 were displayed on Fig.5B. Positive correlation is indicated by green colour and negative correlation showed in red colour.

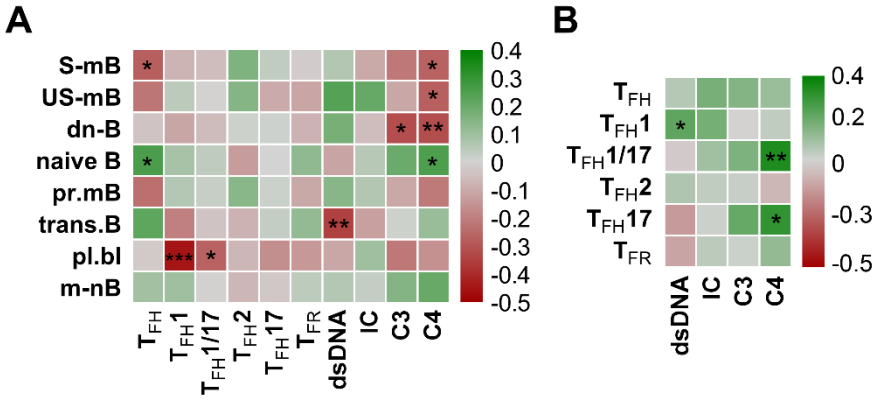

Figure 5.

In the functional analysis of T_{FH} dependent B-cell differentiation in SLE, at first we followed the method that we used in pSS and isolated $CD4^+CXCR5^+$ T_{FH} cells and $CD19^+$ B cells then co-cultured them as detailed above. However, for the neutralization we used a recombinant Hu IL-21R His tag protein instead of a combination of anti-IL-21 and anti-CD40. Blockage of IL-21R decreased both IgM and IgG antibody production in cT_{FH}-B cell co-culture system but no difference was found between patients and controls, however IgG production was more affected indicating that IL-21 is critical for T_{FH}-cell dependent B-cell responses (Fig.6).

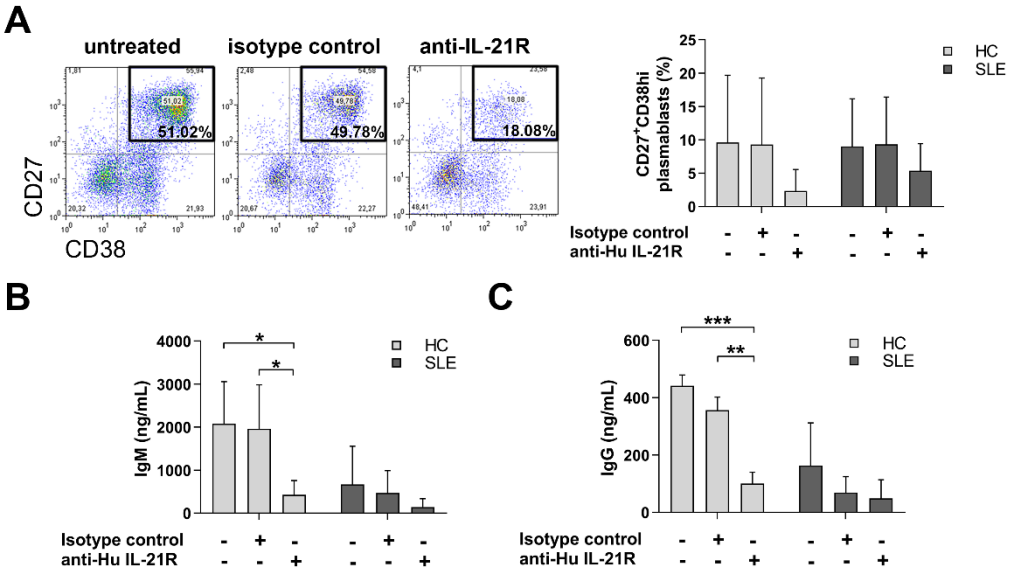

Figure 6.

To gain a more specified result, we altered our protocol, and isolated $CD19^+CD27^-$ naïve B cell and $CD19^+CD27^+$ memory B cell subpopulations and cultured them with $CD4^+CXCR5^+$ T_{FH} cells. At the present, we are expanding the sample size in case of both controls and patients, however, we are struggling to recruit suitable lupus patients from the Outpatient Clinic of the Division of Clinical Immunology due to the SARS-CoV2 pandemic.

However, a significant part of the manuscript has already been written and after the *in vitro* functional analysis is completed, the manuscript will be prepared for publication in a short duration of time.

3. The role of altered expression of miRNAs in cT_{FH} and B cells in systemic autoimmune diseases

In case of miRNA measurements, formerly we have collected the precalculated sample size (13 individuals for each groups: pSS, SLE and healthy controls) of magnetically separated $CD19^+$ B cells and $CD4^+CXCR5^+$ cT_{FH} cells, however during the RNA isolation which was performed by our collaborating partner, unfortunately all of the RNAs degraded. Regrettably, for reasons beyond our control, we had to start collecting and preparing new samples. Unfortunately, it was considerably slower for the second time because the outpatient clinic of the Division of Clinical Immunology was suspended from March 2020 due to the SARS-CoV2 pandemic, and although it started to operate during the summer period, the vast majority of autoimmune patients refused to appear personally and asked the prescription of their medicaments through telemedicine. We completed the sample collection as well as RNA isolation for SLE (n = 13) and controls (n =

13) in our laboratory. The quality of the RNA was determined by Agilent 2100 Bioanalyzer System and RNA Integrity Number (RIN) was calculated by our collaborating partner.

	RIN cT_{FH} cells	**RIN B cells**
Patients with SLE	8.79 ± 0.87	9.03 ± 0.69
Controls	9.05 ± 0.35	9.18 ± 0.33

Data analysis are in progress with the help of our collaborating partner and the manuscript will be prepared for publication in a short duration of time.

Because some of the results of the project are not published yet, we kindly ask that the final scientific report not be made publicly available until the aforementioned results are in press.

Conference abstracts:

1. Jámbor I., **Szabó K.**, Papp G., Szántó A., Tarr T., Zeher M.: A B-sejtek és a follikuláris T-helper sejtek csoportjainak megoszlása autoimmun betegségekben: fenotípusos és funkcionális vizsgálatok. (oral presentation at 46th MAKIT Congress, Kecskemét, 10-12.05.2018)
2. **Szabó K.**, Jámbor I., Papp G., Zeher M.: A cirkuláló follikuláris T helper sejtek patológiás jelentősége autoimmun folyamatok kialakulásában. (oral presentation at 46th MAKIT Congress 10-12.05.2018)
3. **K. Szabó**, I. Jámbor, G. Papp, A. Szántó, M. Zeher: Investigating the subsets of circulating follicular T helper cells in patients with primary Sjögren's syndrome. (poster presentation at Autoimmunity 2018 Congress, Lisbon, 16-20.05.2018 (P33/110)
4. **Szabó, K.**, Jámbor, I., Tarr, T., Papp, G.: Alteration in the proportions of circulating follicular T helper cell subsets is related to aberrant B cell distribution and antibody production in patients with systemic lupus erythematosus. Allergy. 74 (S106), 243, 2019. (Abstract of poster discussion PD0434)
5. Jámbor, I., Papp, G., Zeher, M., Szántó, A., **Szabó, K.**: The imbalance of peripheral follicular T helper cell subsets is associated with abnormal B- cell distribution and disease severity in primary Sjögren's syndrome. Allergy. 74 (S106), 243, 2019. (Abstract of poster discussion PD0435)
6. **Szabó, K.**, Jámbor, I., Tarr, T., Papp, G.: Alteration in follicular T helper cell subsets and cytokine production contributes to dysregulated humoral immune response in systemic lupus erythematosus. Eur. J. Immunol. 49 (S4), 1-75, 2019. (Abstract of oral presentation)
7. Jámbor, I., Papp, G., Szántó, A., Horváth, I., **Szabó, K.**: Role of T_{FH} cells in abnormal B cell distribution and disease severity in primary Sjögren's syndrome. Eur. J. Immunol. 49 (S4), 27, 2019. (Abstract of oral presentation)

Supervision of diploma theses:

1. Anikó Bodnár: A cirkuláló follikuláris T helper sejtek fenotípusos vizsgálata primer Sjögren-szindrómában. (DE ÁOK KLK MSc, 2016)
2. Zsófia Szabó: Perifériás vérben keringő follikuláris T-helper sejt altípusok fenotípusos és funkcionális vizsgálata szisztémás lupus erythematosusban. (DE ÁOK KLK MSc, 2019)

Publications and manuscripts:

1. Jámbor, I., **Szabó, K.**, Zeher, M., Papp, G.: A mikro-RNS-ek jelentősége szisztémás autoimmun betegségek kialakulásában .Orv. hetil. 160 (15), 563-572, 2019.
2. **Szabó, K.**, Jámbor, I., Szántó, A., Horváth, IF., Tarr, T., Nakken, B., Szodoray, P., Papp, G.: The imbalance of circulating follicular T-helper cell subsets in primary Sjögren's syndrome associates with serological alterations and abnormal B-cell distribution. ICB-20-OA-0194.R1 (*under review*)

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1. Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu LC, Butcher E. Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. J Exp Med. 2001;193(12):1373-81.
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7. Szabo K, Papp G, Barath S, Gyimesi E, Szanto A, Zeher M. Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome. Clin Immunol. 2013;147(2):95-104.
8. Szabó K, Papp G, Szántó A, Tarr T, Zeher M. A comprehensive investigation on the distribution of circulating follicular T helper cells and B cell subsets in primary Sjögren's syndrome and systemic lupus erythematosus. Clin Exp Immunol 2016;183(1):76-89.