Task 1. Detection of anti-citrullinated peptide antibodies – monitoring antibody affinities by Surface Plasmon Resonance biosensor (SPR)

We received the ethical permission (ETT TUKEB a 49468-/2013/EKU (576/2013) for carrying out the experiments with serum and cell samples from human blood. Blood samples were collected from healthy persons and RA patients with established disease, sera and lymphocytes were isolated. Lymphocytes were used immediately; sera were aliquoted and frozen at -70^{o}C for further experiments. All together we collected serum samples from 338 RA patients, 46 CCPnegative, non-RA patients and 200 healthy controls.

1.1) Synthesis of the peptide epitopes and the N- or C-terminal biotinylated peptide conjugates.

The synthetic work was constantly ongoing throughout years, according to the needs of the experiments and was carried out by the consortial partner, the MTA-ELTE Research Group of Peptide Chemistry lead by Prof. Ferenc Hudecz. Peptides corresponding to known and modified epitopes of citrullinated proteins were synthesized, purified and analyzed by the consortial partner. Unlabeled and biotinylated, arginine or citrulline containing peptides were synthesized, corresponding to selected sequences in the beta chain of fibrin (Ac-RPAPPPISGGGYRAR-NH2 and Ac-XPAPPPISGGGYXAX-NH2), filaggrin (5mer: Ac-TRGRS-NH2 and Ac-TXGRS-NH2, 19mer: Ac-SHQESTRGRSRGRSGRSGS-NH2 and Ac-SHQESTXGXSXGRSGRSGS-NH2), vimentin 65-77 (Ac-SAVRARSSVPGVR-NH2 and Ac-SAVRAXSSVPGVR-NH2) and collagen 359–369 (Ac-ARGLTGRPGDA-NH2 and Ac-AXGLTGXPGDA-NH2). X stands for citrulline. The α-enolase 5-21 and the Epstein-Barr virus nuclear antigen (EBNA) peptides, VCP1 and VCP2 were synthesized in the second half of the project.

The arginine- or citrulline containing derivatives were built up using solid phase peptide synthesis. The N-terminus of the peptides was acetylated or biotinylated, the C-terminus was always amidated. The biotinylated forms were made using biotinyl-6-aminohexanoic acid. In case of the C-terminus biotinylated peptide a lysine was built into the C-terminal to conjugate the biotin derivatives to its side chain (epsilon-amino group). The derivatives were built up on Rink Amide-4-methylbenzhydrylamine (Rink Amide-MBHA) resin using Fmoc/tBu chemistry. Amino acids were coupled as Fmoc derivatives by the DIC/HOBt coupling method using a 3 molar excess over the resin capacity in DMF. The N-terminal Fmoc group was removed by treatment of the resin-bound peptide with piperidine/DMF (30:70, v/v) mixture. After removal of the last Fmoc group, the N-terminus was acetylated by acetic anhydride and DIEA (5:5 equiv) or biotinylated. After washing, the peptide-resin was dried in a desiccator. The peptides were cleaved from the resin with a mixture of TFA water-TIS (95:2.5:2.5, v/v/v) at room temperature for 2 h. The crude products were purified by semipreparative RP-HPLC and the purified compounds were characterized by analytical RP-HPLC and mass spectrometry. The yields were ~ 40-50 % to the resin capacity.

The Cit or Arg containing peptides were applied in the following studies:

- for the affinity purification of Cit-peptide-specific IgG: non-biotinylated, citrulline containing filaggrin306-324 (19-mer), fibrinβ60-74, collagen359–369, vimentin65-77, and α-enolase5-21 were used;
- for ELISA and ELISPot experiments and for the equilibrium dissociation constant (K_D) determination by surface plasmon resonance on neutravidin coated (NLC) sensor chip:

biotinylated citrulline containing filaggrin306-324 (19-mer), filaggrin311-315 (5-mer), fibrin β60-74, collagen359–369, vimentin65-7;

- for covalent-binding to GLH sensor chip: non-biotinylated citrulline containing filaggrin306-324 (19-mer), fibrinβ60-74, collagen359–369, vimentin65-77, α-enolase5-21, and EBNA2 341-361 peptides were used. Fibrin β, α-enolase5-21 and EBNA peptides were used in non-biotinylated form in ELISA.

Based on the cross-reactivity of affinity purified ACPA with citrullinated peptides derived from filaggrin, fibrin β chain, collagen, vimentin, and α-enolase, a novel, multi-Cit-epitope peptide: AXAXGSGSGXGXG (X stands for citrulline) was designed and synthesized in different forms:

- for covalent coupling to affinity matrix and to ProteOn XPR36 GLH sensor chip the non-biotinylated citrulline or arginine containing "multi-epitope" peptides coupled to Ttds linker (4,7,10-trioxa-1,13-tridecane-diamino-succinic acid), and

- for ELISA the biotinylated citrulline or arginine containing "multi-epitope" peptide coupled with Ttds linker was used.

1.2) Measurement of the ACPA specificity and affinity to citrulline containing peptides by surface plasmon resonance (SPR) analysis

1.2.1. Recognition of citrulline-containing peptide epitopes by autoantibodies (ACPA) produced in vivo and in vitro by B cells of rheumatoid arthritis patients

Anti-citrullinated peptide/protein antibodies (ACPAs) are highly sensitive and specific markers of rheumatoid arthritis (RA). Identification of peptide epitopes that may detect different subgroups of RA patients might have diagnostic and prognostic significance. We have investigated citrulline- and arginine-containing peptide pairs derived from filaggrin, collagen or vimentin, and compared this citrulline-peptide panel with the serological assays conventionally used to detect ACPAs. Furthermore, we studied if the same citrulline-peptides identify antibody-secreting cells in the in vitro cultures of RA B cells. Recognition of citrulline- and arginine-containing filaggrin, vimentin and collagen peptide epitopes were tested by Multipin ELISA system, indirect ELISA and by a peptide-specific microarray.

The combined citrulline-peptide panel including the new short epitope of filaggrin, fil311-315, identified nearly one-third of RA cases that were negative for ACPA, mutated citrullinated vimentin or for rheumatoid factor detected by the conventional ELISA.

The results with the peptide-specific microarray have shown that although most ACPA recognizing the four citrulline peptides are IgG, some of them specifically recognizing citrulline-containing filaggrin peptides (fil311–315 and fil306–326) are IgM, and so may be produced either by newly formed activated B cells or by unswitched B memory cells. Furthermore, the **Cit-peptides of filaggrin and vimentin detected ACPA-producing cells (in ELISpot assay), and thus could also be applied to study the B cells of RA patients**. (Published in: Szarka et al. Immunology, 141, 181–191, 2014. doi:10.1111/imm.12175), Babos et al. Bioconjugate Chem. 24, 817−827, 2013. dx.doi.org/10.1021/bc400073z)

1.2.2. Purification of citrulline-peptide (Cit-peptide) specific antibodies by affinity chromatography

Cit-peptid specific autoantibodies were separated from RA sera via affinity chromatography on HiTrap NHS-activated HP columns (GE healthcare, NHS-activated agarose bead medium). Cit-peptides were coupled covalently to the affinity matrix. IgG fractions were purified from RA sera on Protein G column,

and then the eluted fractions were loaded to the Cit-peptide coated matrices. The affinity purified IgGs were re-tested by ELISA and have shown an extensive cross-reaction, some of them recognized 3-4 other Cit-peptides beside the one used for the purification. Based on this, we designed a multi-epitope peptide that we tested in ELISA and in a functional assay, see later.

1.2.3. Affinity measurements of purified anti-peptide IgG and serum antibodies from RA patients

Binding affinities of IgG and serum samples from RA patients were tested on citrullinated peptides covalently coupled to the surface of ProteOn XPR36 GLH sensor chip or on biotinylated peptides bound to neutravidin coated (NLC) chip. Biosensor analysis has shown that ACPA in sera of RA patients are very heterogeneous regarding both specificities and affinities. **KD values of affinity-purified ACPA IgGs varied between 10^{-6} and 10^{-8} M and inversely correlated with disease activity**. Based on their cross-reaction with citrulline-peptides, **we designed a novel multi-epitope peptide, containing Cit-Gly and Ala-Cit motifs in two–two copies, separated with a short, neutral spacer.** This peptide detected antibodies in RA sera with 66% sensitivity and 98% specificity in ELISA and was recognized by 90% of RA sera, while none of the healthy samples in SPR. When coupled to nanoparticles, the multi-epitope peptide specifically targeted and, in the presence of complement activation, depleted ACPA-producing B cells ex vivo. Conclusions: **The unique multi-epitope peptide that we designed based on ACPA cross-reactivity might be suitable to develop better diagnostics and novel therapies for RA.** (Published in: Szarka et al. International Journal of Molecular Sciences, 19, 326, 2018; doi:10.3390).

1.3) Parallel measurements of the specificities and the complement activation potential of ACPA.

We set up a bead array-based assay for a multiplexed determination of antigen-specific antibody levels in parallel with their properties for complement activation. Deposition of C3 fragments from serum samples was measured to reflect the degree of complement activation via all three complement activation pathways. We utilized the assay on a bead array containing native and citrullinated peptide antigens to investigate the levels of IgG, IgM and IgA autoantibodies along with their complement activating properties in serum samples of 42 rheumatoid arthritis patients and 40 controls. **Significantly higher IgG reactivity against the citrulline containing fibrin β and filaggrin peptides was detected and C3 deposition from RA patients' sera was observed. This mechanism may contribute to autoantibody-associated immune pathologies.** (Published in: Ayouglu, Szarka et al. PLosOne, 9 (5), e96403. 2014. doi:10.1371/journal.pone.0096403)

Task 2. Analysis of B cell subpopulations in health and RA.

2.1) Activation of signaling pathways in naïve and memory B cells from healthy individuals and RA patients

In this part of the project we investigated the cross-talk between B cell receptor (BCR), the receptors for the B-cell activating factor of the tumor necrosis factor family (BAFF-R) and the toll-like receptor-9 (TLR-9) stimulated pathways and upon the Fcγ receptor type IIb (FcγRIIb) – BCR – TLR-9 mediated signaling, finally, we studied the selective depletion of auto-antigen specific RA B cells in ex-vivo cultures.

B cell development and activation are regulated by combined signals mediated by BCR, BAFF-R and the innate receptor, TLR9. However, the underlying mechanisms by which these signals cooperate in human B cells remain unclear. Our aim was **to elucidate the key signaling molecules at the crossroads of BCR, BAFF-R and TLR9 mediated pathways and to follow the functional consequences of**

costimulation. Therefore we stimulated purified human B cells by combinations of anti-Ig, B-cell activating factor of the tumor necrosis factor family (BAFF) and the TLR9 agonist, CpG oligodeoxynucleotide. Phosphorylation status of various signaling molecules, B cell proliferation, cytokine secretion, plasma blast generation and the frequency of IgG producing cells were investigated. We have found that **BCR induced signals cooperate with BAFFR- and TLR9-mediated signals at different levels of cell activation.** BCR and BAFF- as well as TLR9 and BAFF-mediated signals cooperate at NFkB activation, while BCR and TLR9 synergistically costimulate mitogen activated protein kinases (MAPKs), ERK, JNK and p38. We show here for the first time that the **MAP3K7 (TGF beta activated kinase, TAK1) is responsible for the synergistic costimulation of B cells by BCR and TLR9**, resulting in an enhanced cell proliferation, plasma blast generation, cytokine and antibody production. Specific inhibitor of TAK1 as well as knocking down TAK1 by siRNA abrogates the synergistic signals. We conclude that **TAK1 is a key regulator of receptor crosstalk between BCR and TLR9, thus plays a critical role in B cell development and activation.** We set up and applied the phospho-flow analysis to compare the level of p38 MAPK phosphorylation in CD19+ CD27- naïve B cells from healthy blood donors and RA patients. BCR and TLR9 double signals induced a significantly higher p38 MAPK phosphorylation as compared to single stimuli. However, since the basal level of p38 MAPK phosphorylation was higher in RA patients as compared to healthy control, **the relative phosphorylation of p38 MAPK in the single or double stimulated samples was significantly lower in RA B cells.** (Published in: Szili et al. PLoS ONE 9(5): e96381. 2014. doi:10.1371/journal.pone.0096381)

The Fcγ receptor type IIb (FcγRIIb) inhibits B cell responses when co-engaged with B cell receptor (BCR), and has become a target for new autoimmune disease therapeutics. For example, BCR and FcγRIIb co-engagement via the Fc-engineered anti-CD19 monoclonal antibody, XmAb5871 that binds to human FcγRIIb with >400 higher affinity than native IgG1, suppresses humoral immune responses. We assessed the effects of XmAb5871 on other activation pathways, including the pathogen-associated molecular pattern receptor, TLR9. Since TLR9 signaling is implicated in autoimmune diseases, we asked if XmAb5871 could inhibit TLR9 costimulation. We have found that **XmAb5871 decreases ERK and AKT activation, cell proliferation, cytokine, and IgG production induced by BCR and/or TLR9 signals. XmAb5871 also inhibited differentiation of citrullinated peptide-specific plasma cells** from rheumatoid arthritis patients. **The Fc-engineered anti-CD19 XmAb5871 may therefore have potential to suppress pathogenic B cells in autoimmune diseases.** (Published in: Szili et al. mAbs 6:4, 991–999, 2014; doi: 10.4161/mabs.28841)

The **fibrin β60-74Cit peptide** is a major epitope recognized by ACPA in RA patients. This peptide was selectively recognized by a small subset of B cells from RA patients having high level of peptide specific serum antibody, suggesting that the peptide **can target diseased B cells**. The modified gp120 peptide of HIV envelope protein covalently coupled to NPs can activate the complement mainly on the classical pathway and induce the formation of the complement membrane attack complex, C5b-9 in human serum. We showed for the first time that bifunctional NPs coupled to multiple copies of both the targeting peptide and the complement activating effector peptide significantly reduce fibrin β60-74Cit peptide-specific ex vivo ACPA production by inducing complement dependent lysis of the cit-peptide specific B cells of seropositive RA patients. Conclusions: **Bifunctional NPs covalently coupled to autoantigen epitope peptide and to a lytic peptide may specifically target and deplete the peptide specific autoreactive B-cells.** (Published in: Pozsgay et al. Arthritis Research & Therapy 18:15, 2016. DOI

10.1186/s13075-016-0918-0, and Pozsgay et al. Nature Reviews Rheumatology, 13(9): 525-537, 2017. doi:10.1038/nrrheum.2017.107)

2.2) Investigation of cytokine secretion by B cell subsets and the regulatory capacity of Breg cells

The most important feature of B cells is the production of Abs upon activation; additionally, B cells produce pro- and anti-inflammatory cytokines in response to certain stimuli. IL-10–producing B cells represent a major subset of regulatory B cells (Bregs) that suppress autoimmune and inflammatory responses. B cells play a crucial role in the development and maintenance of the chronic inflammatory autoimmune disease, rheumatoid arthritis (RA); however, controversial data are available on IL-10 producing Bregs in RA. Our aim was to identify the optimal conditions that induce IL-10+ Bregs and, furthermore, to shed light on the signaling pathways that are responsible for their expansion. The results have shown that dual stimulation by CpG and CD40L for 48 h is optimal for IL-10 induction, and this can be synergistically boosted by IL-21. We identified **the CD19+CD27+ memory B cell population as the major source of IL-10+ Bregs**.We detected **significantly fewer CD19+CD27+IL-10+ cells in RA patients compared with healthy controls, and these were functionally defective in suppressing IFN-γ production by CD4+ T cells in co-culture.** IL-21 drastically increased the number of IL-10+ Bregs within the CD19+CD27+ and CD19+CD27- populations; furthermore, it induced the appearance of IL-10+Blimp-1+ plasmablasts. Monitoring the phosphorylation of key signaling molecules revealed that **activation of ERK, p38, and CREB is indispensable for the induction of IL-10 production, whereas phosphorylation of STAT3 further enhances IL-10 expression in human Bregs. We conclude that CREB and STAT3 are the key transcription factors responsible for the expansion and differentiation of human IL-10–producing Bregs.** The lower frequency of activation-induced IL-10 producing Bregs and the increased ratio of IL-6 and TNFα secreting B cells in RA patients may contribute to the exacerbation of the disease. (Bankó et al, J. Immunol. 18, 2017, doi:10.4049/jimmunol.1600218)

Task 3. Monitoring re-emerging B cell subsets in RA patients after receiving biological therapies

Cytokines, including tumor necrosis factor alpha (TNFα) are involved in Rheumatoid arthritis (RA) pathogenesis by augmenting autoimmunity, sustaining long term inflammation in the synovium, and promoting joint damage. Anti-TNF therapy is one of the most efficient and widely used therapies for RA, although its mechanism is not clarified yet. Earlier we demonstrated that RA patients have a reduced number of IL-10 producing regulatory B cells (B10 cells) as compared to healthy individuals and they are functionally impaired. Our aim was to study the influence of anti-TNF therapy on B10 cells in RA, to follow the alteration of B cell activation markers (CD25, CD69) and to monitor the level of cit-peptide-specific antibodies and the secreted IL-10 in patients'sera during the therapy. We have observed that **at six month after starting the therapy the frequency of B10 cells remarkably increased, while the expression of the activation marker, CD69 decreased on B cells.** In contrast, serum levels of IL-10 and anti-cit-peptide antibodies did not change post-treatment. The major weakness of our pilot study is the small number of patients. If confirmed in larger studies, **these data suggest that the reduced activation state of B cells and the increasing number of regulatory B10 cells might contribute to the therapeutic efficacy of anti-TNF agents in RA.** (Bankó, et al., Clin. Immunol. 184:63-69. 2017. http://dx.doi.org/10.1016/j.clim.2017.05.012). We could only follow patients receiving TNFα-blockers (Humira and Enbrel) in combination with methotrexate (MTX), since patients with B cell depletion therapy or other biological therapies were not available for us. The low amount of blood obtained from the patients did not allow further tests monitoring Th17 and Treg activity.

Task 4. Collagen induced arthritis (CIA): the role of co-signaling between BCR and TLR9 in the pathogenesis of the disease.

The CIA model. CIA is the most often studied animal model of RA, induced by immunization of DBA/1 mice with bovine collagen type II (CII) mixed with complete Freund`s adjuvant, and followed by re-immunization with CII emulsified in incomplete Freund's adjuvant. Mice were harvested from the acute and remission phases of CIA and B cells from the spleens and lymph nodes were analyzed.

In vitro experiments: how T-bet is regulated in naïve mature B cells? The importance of the BCR and TLR9 in autoimmunity and in the production of auto-antibodies is well established, but the underlying molecular mechanism still needs to be determined. We aimed to characterize the BCR-TLR9 cross-talk by its effect on T-bet, as T-bet is activated and regulated by both receptors and has an important role in class switching to pathological IgG2a in mice. Lymph node B cells were stimulated for different times by anti-IgM or TLR9 agonist, CpG-ODN or the combination of both and the expression of T-bet mRNA was analyzed for gene and protein expression. The simultaneous stimulation with anti-IgM and CpG-ODN caused a more intense T-bet up-regulation resulting in a 3-5 fold increase with the highest synergy at 24 hours. Gene expression experiments were **verified by Western blot data and showed the synergistic effect of receptor crosstalk on T-bet protein expression at as early as 3 hours**. **However, the activation of T-bet provoked by the synergistic BCR-TLR9 crosstalk is transient** and without additional signals cannot contribute to the induction of late response genes. (Published in: Kövesdi et al. Eur. J. Immunol. 44: 887–893. 2014. DOI: 10.1002/eji.201343841)

The role of co-signaling between BCR and Toll-like Receptors 9 (TLR9) in the pathogenesis of CIA. The receptor cross-talk between BCR and TLR9 could induce the activation of different proteins and the production of regulatory cytokines such as the suppressive IL-10. We found that **IL-10 expression was inducible by TLR9 agonist CpG-ODN, moreover simultaneous BCR cross-linking resulted in a synergistic and time-dependent IL-10 mRNA expression peaking at around 1-3 hours, which show a resemblance to T-bet kinetics.**

Regulatory B cells (Bregs) are specialized to suppress immune responses and control various immunological disorders, such as autoimmune diseases. Bregs have no specific cell surface markers and therefore they are characterized by the inducible IL-10 production. We studied three different subpopulations of B cells found in murine spleen (Transitional-2-Marginal Zone Precursors (T2-MZP), Marginal Zone (MZ) and Follicular (FO) B cells), which can be all natural sources of regulatory B cells. We found **that B cells from the marginal zone compartment were transformed most efficiently into Bregs and showed the highest capacity to produce IL-10.**

What genes are activated in B cells upon the onset of CIA? As both the BCR and TLR9-mediated signaling events are important for the initiation and maintenance of CIA we wanted to see whether T-bet or IL-10 is involved in CIA progression. We analyzed the expression pattern of T-bet and IL-10 by real-time RT-PCR and found that **both T-bet and IL-10 expression increased during the remission phase of arthritis in cells isolated as marginal zone (MZ) B cells.** These results clearly show that IL-10 producing regulatory B cells can be formed during the course of CIA, moreover suggest that **IL-10 production might be associated with T-bet expression.**

Does the expression of T-bet depend on any of the cytokines? We know that in mature B cells, T-bet is induced by IFNγ, and also by the BCR and TLR9 mediated double signals. Moreover, priming

autoimmune mice with IFNγ or treat simultaneously DBA/1 B cells with CpG and IFNγ results in an increased expression of IL-10, although B cells fail to produce IL-10 when stimulated only through the BCR. Therefore we used these signals alone or combined to induce T-bet and IL-10 expression in MZ B cells, isolated as CD23-, CD21high B cells from the spleen of healthy mice and subjected to real-time RT-PCR analysis. The kinetics of T-bet and IL-10 mRNA expressions were the same when anti-IgM, CpG and IFNγ were used simultaneously, showing a peak at 3 hours. These results were further verified by measuring the amount of secreted IL-10 in cultures supernatants, and by quantifying the number of IL-10 positive MZ B cells in cultures treated with anti-IgM, CpG or IFNγ. To check whether signals mediated by IFNγ and through the BCR or TLR9 could converge and generate IL-10/T-bet double positive cells we cultured sorted MZ B cells in the presence and combination of anti-IgM, CpG and IFNγ and performed double staining for IL-10 and T-bet. Flow cytometry analysis of intracellular IL-10 and T-bet showed the existence of the **IL-10/T-bet double positive MZ B cells that are generated by the simultaneous signals**. These results suggest that the **expression of T-bet could be a selective advantage for IL-10 positive MZ B cells in response to signals generated in vivo during CIA progression.**

Exploring the role of Tbet in B cells: identification of activation pathways, cytokine dependence
It is known that T-bet regulates the expression of the chemokine receptor CXCR3 on IL-10-producing regulatory T cells and promotes their accumulation at sites of Th1-mediated inflammation. Antibody-forming cells expressing CXCR3 are also recruited to inflammatory sites and their migration depends on B cells' intrinsic T-bet expression. To test whether T-bet upregulation induce CXCR3 expression on MZ B cells, sorted MZ B cells were cultured in the presence of anti-IgM, CpG, and IFN-γ for 48 h. Staining T-bet and CXCR3 clearly shows that almost **70% of cultured MZ B cells became T-bet/CXCR3 double-positive, reflecting the strong correlation between the two molecules**. We found that **CXCR3 expression is upregulated on a T-bet-dependent manner and showed that T-bet-expressing MZ B cells migrate toward CXCR3 ligand and secrete IL-10 by inflammatory stimuli.** Our data suggest that T-bet might contribute to the remission of CIA by facilitating the regulatory potential of IL-10-positive MZ B cells. (Huber et al. Eur J Immunol. 46(9):2239-46, 2016; DOI: 10.1002/eji.201546248)

The results of this project summarized here were published in 12 scientific papers in high level international journals, were presented in several local and international conferences; and besides, provided the basis of six PhD dissertations, Krisztina Babos (2014), Daniel Szili (2014), Eszter Szarka (2014), Judit Pozsgay (2018), Krisztina Huber (2018, submitted), Zsuzsanna Bankó (in preparation).