**FINAL REPORT – OTKA Project 112011**

**Finishing the project, we accomplished our aims, which yielded 12 full-length publications in leading journals of the field, several talks and presentations at national and international conferences, and 3 PhD dissertations which were successfully defended during the period of the project. One more paper is under review and two additional manuscripts will be sent for publication soon.**

We started the investigations as described in our proposal, but as the project advanced, two of our aims had to be revised. We decided to replace these by better-developing topics, which emerged during our studies in the other sub-projects. These new topics fit well into our project entitled *Regulation of adaptive immune responses by complement and Toll-like receptors in health and disease*.

*Explanation why two sub-projects had to be replaced*

1. The sub-project described in Specific Aim 3 ("*To reveal and characterize the expression and function of C3aR and its cross-talk with TLRs in human B cells"*) had to be dropped, because Mariann Kremlitzka - a doctoral student at the time of the beginning of these experiments -, left the group shortly after obtaining her PhD, to start her post-doc studies in Sweden. While still in Hungary, she obtained promising results, which were presented as award winning abstract at the XXVth International Complement Workshop in Rio de Janeiro (Kremlitzka et al, 2014, XXV ICW, Rio de Janeiro, Abstr. n. 177, Mol.Immunol. page 278,). Furthermore, she obtained an EMBO-fellowship to work at the University of Lübeck on the same topic. A scientific report on the work, carried out under the supervision of Prof. Jörg Köhl and Dr. Christian Karsten, with constant consultation with me, is available. Thus, although we began our studies as planned in the proposal, and it progressed well, unfortunately we could not bring it to publishing the results in the form of an original paper, due to the lack of a researcher who could have finished the work.

*Here is a brief summary of our results obtained investigating Specific Aim 3:* We have shown that human B cells express C3aR as well as intracellular C3 - both at the mRNA and at the protein level. We also found that while C3 appeared both intracellularly and at the cell surface, C3aR was expressed only inside the cell. C3a was chemotactically active on B cells even at nanomolar concentrations. Activation of the cells via the B cell receptor (BCR) or the Toll-like receptor 9 (TLR9) resulted in a twofold increase in the amount of intracellular C3 as well as C3aR, however the receptor could not be detected at the cell surface even after activation. Incubation of BCR- and/or TLR9-activated B lymphocytes with C3a resulted in enhanced phosphorylation of the mitogen-activated protein kinases, like p38 and ERK, however IL-6 and IL-10 production was suppressed in a dose-dependent manner.

2. Although we have had promising preliminary data, we also had to drop studies aimed in the proposal, conceived as follows: "*To get a deeper insight into the mechanism of Rituximab treatment and to reveal the fate of non-lysed („surviving") human B cells"*. We started our studies on the effect of Rituximab- and serum-treatment using human B cells, and set up the optimal experimental conditions regarding utilization of the CD20 targeting antibody. We demonstrated C3-deposition by FACS-analysis and assessed the survival of the treated cells. The problem, however emerged later, as unexpectedly and unfortunately we could not recover enough cells for further functional studies. Importantly however, while working on this project, we stumbled upon an interesting finding, namely the expression of CR3 and CR4 on human B-cells, which we considered important to pursue. So we concentrated on a new line of research, related to the differential role of CR3 and CR4 on human B cells and phagocytes (see later).

**The driving hypothesis throughout our studies** has been that the complement system, particularly the split products of its major component C3, play several, so far unrevealed roles in the regulation of adaptive responses. *(Our review summarizes the versatile functions of complement C3-derived ligands. Erdei et al., Immunol Rev., 2016).* We also assumed the existence of an interplay between two key element of innate immunity, namely the complement system and Toll-like Receptors (TLRs). So we set out to decipher how this might influence adaptive immune responses.

In our present OTKA-project we answered several questions, focusing on how different elements of innate immunity collaborate with each other, and how these events influence adaptive immune responses. More precisely we revealed how various B-cell functions (such as cytokine release, antibody production) are influenced by complement and Toll Like Receptors (TLRs), and dissected various functions of dendritic cells and macrophages (such as phagocytosis, adherence and migration), initiated by their interaction with different C3-derived ligands.

It is important to emphasize that we performed all our experiments employing cells of human origin – namely B lymphocytes isolated from human blood and tonsil, and monocytes isolated from human buffy coats. Macrophages and dendritic cells (DC) were differentiated from freshly isolated monocytes *in vitro*.

## 1. BCR – TLR9 interaction on B cells

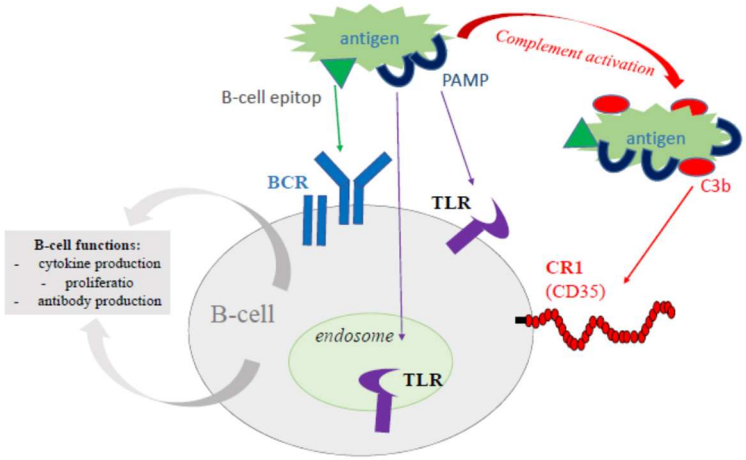
In the frame of the present project we studied the B cell receptor (BCR) induced, Toll-like receptor 9 (TLR9) mediated activation and differentiation of human B cells. It has been known that B cells are efficiently activated by CpG oligodeoxynucleotides (ODNs) to produce pro-inflammatory cytokines and antibody (Ab). As a result of our studies we described a so far unidentified, spleen tyrosine kinase (Syk)-dependent pathway, which is indispensable for CpG-induced human B cell activation. We showed that triggering of B cells by CpG results in Syk and src kinase phosphorylation, proliferation, as well as cytokine and Ab production independent of the BCR. Notably, all these functions are abrogated when Syk is inhibited. We demonstrate that CpG-induced Syk activation originates from the cell surface in a TLR9-dependent manner. While inhibition of Syk does not influence the uptake of CpG ODNs, activation of the kinase is a prerequisite for the delivery of CpG into TLR9-containing endolysosomes and for the CpG-induced upregulation of TLR9 expression. Our results revealed an alternative, Syk-dependent pathway of CpG-induced B cell stimulation, which is initiated at the plasma membrane and seems to be an upstream requirement for endosomal TLR9-driven B cell proliferation and differentiation.
*(Publication: Kremlitzka et al, CMLS, 2015)*

## 2. BCR – CR1 - TLR interaction on B cells

Earlier we have shown that complement receptor type 1 (CR1, CD35) is a potent inhibitor of the B cell receptor (BCR)- induced functions of human B lymphocytes (Józsi et al, JI, 2003). Our recent findings on the interaction between BCR and TLR9 prompted us to investigate the interaction between the BCR, TLRs and complement receptor type 1 (CR1, CD35). The complement system and TLRs are two major elements of innate immunity, which are critical not only for first line host defense, but also in shaping adaptive immunity. Several microbial components or damage products, such as zymosan, LPS, and nucleic acids trigger the complement cascade and initiate TLR-signaling simultaneously, immediately upon encounter

with such substances (Figure 1.). The developing crosstalk between the two key innate elements may profoundly modulate adaptive immune responses. Rearding the interaction between the complement system and TLRs only the outcome of separate activation of these systems had been investigated earlier. Now we demonstrated that occupancy of complement receptor type 1 (CR1, CD35) by its natural, complement component C3-derived ligand significantly and dose dependently reduces the TLR9-induced expression of activation markers, cytokine production, proliferation, and antibody production by human B cells, but has no effect on the TLR7-induced functions. The synergistic response to the simultaneous engagement of either TLR9 or TLR7 along with the BCR however, is significantly inhibited by CR1 occupancy. These findings imply that both under physiological and pathological conditions, when complement- and TLR-activating microbial and damage products are present in the B cell environment, the cooperation between CR1 and TLR7 or TLR9 provides additional levels of the regulation of human B cell functions.
*(Publication: Mácsik-Valent et al, Frontiers in Immunology, 2019)*

*Figure 1. Crosstalk between the BCR, TLRs and CR1*



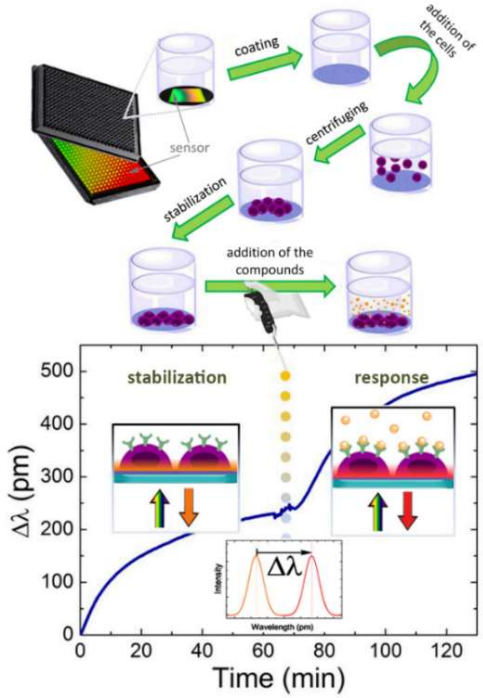

We found that CR1 regulates the TLR9-initiated B cell responses in both healthy individuals and SLE patients. We found that treatment of B cells with the TLR9-ligand CpG oligonucleotide, results in activation, proliferation and Ig- production, which are all inhibited upon ligation of CR1. Our results show that despite the fact that B lymphocytes from SLE patients express half as many surface CR1 as normal B cells, clustering of the complement receptor results in efficient inhibition of the main B cell functions similar to the healthy controls. Our results provide a new possibility for the inhibition of autoreactive B cells in SLE patients and reveal a novel link between complement and TLRs in the regulation of humoral immunity.
*(Publication: Kremlitzka et al., Journal of Immunology Research, 2016)*

### 3. B cell activation studied by a novel, label-free method

In another set of experiments, we examined B cell activation by a novel, label-free method using the evanescent field based resonance waveguide grating technology (Figure 2.). We successfully immobilized non-adherent B cells on the surface of the biosensors, without the ligation of any specific receptors or adhesion molecules. This way we were able to demonstrate that engagement of the antigen specific B cell receptors (BCR) induced reproducible dynamic mass redistribution (DMR) inside the cells as a measure of receptor activation. The initiated DMR response proved to be specific, since only antibodies recognizing the BCR could gener-

ate the response; neither the assay-buffer, nor high concentration of indifferent proteins or non-specific antibodies had any effect. The measure of cell activation was sensitive, concentration dependent, and specifically and dose-dependently inhibited by the Syk inhibitor BAY 61-3606. The BCR-triggered DMR response was evoked from three human Epstein-Barr virus (EBV) negative B cell lines, but could not be elicited in two EBV-positive BL cell lines, where the presence of the EBV-derived LMP2A protein desensitizes the cells' response to the BCR-induced signaling. Parallel engagement of the inhibitory FcRIIB together with the BCR resulted in the inhibition of the activation of B cells demonstrating that the DMR response mirrors the expected interaction between the downstream signaling events of the two receptors. As a multi-target profiling procedure, this label-free RWG technology is applicable for the study of complex cellular signaling via the analysis of integrated real time signature of DMR. Therefore, our work opens new avenues to study complex signaling events and to decipher interactions within the signaling network during B cell activation.
*(Publication: Kurucz et al, Sensors and Actuators, 2017)*

*Figure 2. The protocol and experimental design of our studies*




Lymphocytes were seeded on special 384 well microplates, where each well is embedded with and optical biosensor sensitive to the change of the refractive index above the sensor surface;

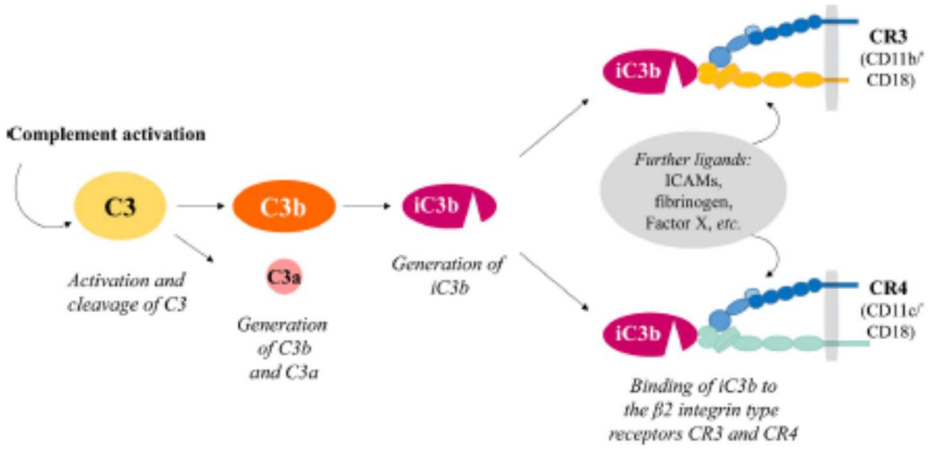
Specific wavelength of the broadband light source couples into the resonant waveguide grating (RWG) and gets detected. Activation induced intracellular macromolecular redistribution results in the change of the refractive index above the sensor surface, ultimately causing the shift of the detected wavelength. These cellular changes can be recorded real-time.

Recently, in cooperation with the EK-MFA Nanobiosensorics Group we successfully applied a label-free optical biosensor for real-time measurement of the integrated cellular response of primary human B cells induced by simultaneous receptor engagement. The ligand binding of certain inhibitory receptors (FcγRIIb, CR1) results in specific, dose-dependent modulation of BCR-induced B cell activation. This non-invasive whole-cell assay enables the detection of complex cellular changes simultaneously induced by multiple ligands, imitating the *in vivo* environment of the lymphocytes. Results have been presented in conferences held in Hungary, and publication is presently in preparation.
*(Manuscript containing these results is in preparation. The tentative title is: Label-free real-time monitoring of the BCR-triggered activation of primary human B cells modulated by the simultaneous engagement of inhibitory receptors)*

## 4. CR3 and CR4 on B cells

While performing the studies planned in our research proposal, we found that in addition to CR1 (CD35) and CR2 (CD21) further complement receptors - such as CR3 (CD11b/CD18) and (CR4 CD11c/CD18) - also play important roles in various B cell functions. Ligands to these receptors are generated when the complement system is activated and split-products of C3 bind to the activating surface (Figure 3.).

Figure 3. Generation of complement C3-derived ligands of CR3 and CR4




Thus, we decided to extend our studies to these beta2-integrins, too, aiming to reveal the expression and role of these complement receptors on human B lymphocytes and their possible interaction with TLRs. First we studied isolated B cells of two patients with chronic lymphocytic leukemia (CLL), characterized by a peculiar immune-phenotype containing both CD5-positive and CD5-negative B cell populations. We found that CD11b and CD11c were expressed on both CD5-positive and CD5-negative B cells, albeit to different extents. Our data suggest that these receptors are involved in spreading, since this activity of TLR9 (CpG)-activated B cells on fibrinogen could be partially blocked by monoclonal antibodies specific for CD11b or CD11c. CpG-stimulation lead to proliferation of both CD5-positive and CD5-negative B cells of the patients with a less pronounced effect on the CD5-positive cells. In contrast to normal B cells, CLL B cells of both patients reacted to CpG-stimulation with robust IL-10 production. The concomitant, suboptimal stimulus via the BCR and TLR9 exerted either a synergistic enhancing effect or resulted in inhibition of proliferation and IL-10 production of patients' B cells. Our data point to the possible role of CR3 in the interaction of tumor cells with the microenvironment and suggest the involvement of IL-10 producing B cells in the pathologic process.
*(Publication: Uzonyi et al, Immunology Letters 189, 73-81, 2017)*

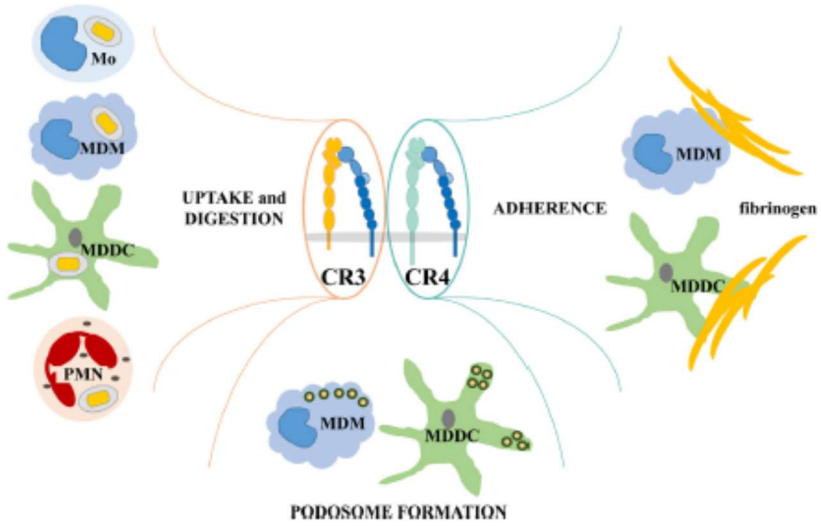
Our further goal is to decipher the function of CR3 and CR4 on B cells under physiological conditions, as well, since the role of these β2-integrins in human B lymphocytes have not been extensively studied so far. We investigated their expression and function on B cells isolated from healthy donors in addition to cells of CLL patients. Measuring the expression of these receptors by flow cytometry on normal human B cells we found, that resting B cells do not express CR3 and CR4, after activation however, they become positive for CR4. Analysing the function of this $\beta_2$-integrin by classical adhesion- and transwell-assay we found, that it contributes to the adhesion and migration of these cells. Although it might be assumed that these integrins contribute to the elevated adhesive and migratory capacity of the malignant cells, this has not been proven so far. We found that the B cells of all patients expressed CR4, while

CR3 could be detected rarely. While the role of CR3 on CLL-B cells is still undefined, we found that CR4 clearly contributes to the adhesion and migration towards the chemokine SDF-1. Thus our finding suggests that CR4 participates in the pathomechanism of the disease, since it might help the malignant cells to reach the protective niches of the bone marrow more efficiently. *(Z. Nagy-Baló et al, Abstracts of the 5th European Congress of Immunology - ECI 2018 - Amsterdam, The Netherlands, p. 248, 2018, manuscript in preparation)*

## 5. CR3 and CR4 on macrophages and dendritic cells

CR3 and CR4, the leukocyte specific $\beta_2$-integrins are involved in cellular adherence, migration and phagocytosis. These receptors are generally expected to mediate similar functions, due to structural homology, overlapping ligand specificity and parallel expression on human phagocytes. We have however shown recently that there is a division of labor between these two β2-integrins expressed by different phagocytes (Figure 4.) *(Our recent review (Erdei et al, Sem. in Cell and Developmental Biol., 2019) summarizes available data on these receptors, emphasizing the division of labour between these two β2 integrins.).*

Figure 4. Division of labour between CR3 and CR4




In the frame of this OTKA-project we studied the participation of CR3 and CR4 in binding, internalization and ingestion of Staphylococcus aureus by normal human phagocytes, namely monocyte derived macrophages (MDMs,) monocyte derived dendritic cells (MDDCs), monocytes and neutrophils. We found that CR3 plays a dominant role in the phagocytosis of iC3b opsonized S. aureus by all of these cell types. We also investigated the participation of CR3 and CR4 in the podosome formation of human phagocytes. Confocal microscopy analysis shows that both CD11b and CD11c are located in the podosome adhesion ring of the studied cell types. These data highlight a difference in the uptake and ingestion of complement opsonized antigen between the function of human CR3 and CR4 while in the process of podosome formation both receptors take part. *(Publications: Sandor et al, PLOS ONE 11: (9), 2016, Lukácsi et al., Immunology Letters 189, 64-72, 2017)*

After these studies carried out under physiological conditions we provided further evidence that the expression and function of CR3 and CR4 are not identical in these cell types. We found that LPS treatment changes their expression differently on MDMs and MDDCs, suggesting a cell type specific regulation. Using mAb24, specific for the high affinity conformation of CD18, we proved that the activation and recycling of $\beta_2$-integrins is

significantly enhanced upon LPS treatment. Adherence to fibrinogen was assessed by two fundamentally different approaches: a classical adhesion assay and a computer-controlled micropipette, capable of measuring adhesion strength. While both receptors participated in adhesion, we demonstrated that CR4 exerts a dominant role in the strong attachment of MDDCs. Studying the formation of podosomes we found that MDMs retain podosome formation after LPS activation, whereas MDDCs lose this ability, resulting in a significantly reduced adhesion force and an altered cellular distribution of CR3 and CR4. Our results suggest that inflammatory conditions reshape differentially the expression and role of CR3 and CR4 in macrophages and dendritic cells.
*(S. Lukácsi et al, Abstracts of the 5th European Congress of Immunology - ECI 2018 - Amsterdam, The Netherlands, p182, 2018, Manuscript is under review)*

**Publications** (14 publications: 8 originals, 4 reviews, 2 abstracts - all acknowledging OTKA)

**Original, full-length papers:**


1. Kremlitzka M, Mácsik-Valent B, Erdei A.: *Syk is indispensable for CpG-induced activation and differentiation of human B cells*, Cell Mol Life Sci., 2015

2. Norbert Orgovan, Rita Ungai-Salánki, Szilvia Lukácsi, Noémi Sándor, Zsuzsa Bajtay, Anna Erdei, Bálint Szabó, Robert Horvath: *Adhesion kinetics of human primary monocytes, dendritic cells, and macrophages*, BIOINTERPHASES 11: (3), 2016

3. Kremlitzka M., Mácsik-Valent B., Polgár A., Kiss E., Poór G., Erdei A.: *Complement Receptor Type 1 Suppresses Human B Cell Functions in SLE Patients*, Journal of Immunology Research, 2016

4. Sandor N, Lukacsi S, Ungai-Salanki R, Orgovan N, Szabo B, Horvath R, Erdei A, Bajtay Z: *CD11c/CD18 Dominates Adhesion of Human Monocytes, Macrophages and Dendritic Cells over CD11b/CD18*, PLOS ONE 11: (9), 2016

5. Erdei A, Lukacsi S, Macsik-Valent B, Nagy-Balo Z, Kurucz I, Bajtay Z: *Non-identical twins: Different faces of CR3 and CR4 in myeloid and lymphoid cells of mice and men.*, SEMIN CELL DEV BIOL in press: 2017

6. Kurucz, I., Peter, B., Prosz, A., Szekacs, I., Horvath, R., & Erdei, A. (2017)..: *Label-free optical biosensor for on-line monitoring the integrated response of human B cells upon the engagement of stimulatory and inhibitory immune receptors*, Sensors and Actuators, B: Chemical, 240, 2017

7. Lukácsi Szilvia, Nagy-Balo Z, Erdei Anna, Sándor Noémi, Bajtay Z: *The role of CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in complement-mediated phagocytosis and podosome formation by human phagocytes*, IMMUNOL LETT 189: 64-72, 2017

8. Uzonyi B, Macsik-Valent B, Lukacsi S, Kiss R, Torok K, Kremlitzka M, Bajtay Z, Demeter J, Bodor C, Erdei A: *Functional studies of chronic lymphocytic leukemia*

*B cells expressing beta2-integrin type complement receptors CR3 and CR4*, IMMUNOL LETT 189: 73-81, 2017

**Reviews:**

1. Erdei A, Sándor N, Mácsik-Valent B, Lukácsi S, Kremlitzka M, Bajtay Z.: The versatile functions of complement C3-derived ligands, Immunol Rev., 2016 DOI: 10.1111/imr.12498

2. Kremlitzka M, Mácsik-Valent B, Erdei A.: Regulation of B cell functions by Toll-like receptors and complement, Immunol Lett., 178, 37-44, 2016 http://dx.doi.org/10.1016/j.imlet.2016.07.015

3. Erdei A, Lukácsi S, Mácsik-Valent B, Nagy-Baló Z, Kurucz I, Bajtay Z.: Non-identical twins: Different faces of CR3 and CR4 in myeloid and lymphoid cells of mice and men Sem. in Cell and Developmental Biol., 85. 110-121., 2019 doi.org/10.1016/j.semcdb.2017.11.025

4. Lukacsi S, Mácsik-Valent B. Nagy-Baló Z., Kovács G.K. Kliment K. Bajtay Z, Erdei A.: Utilization of complement receptors in immune cell - microbe interaction the role of various complement receptors, FEBS Letters 2020 Jan 27. doi: 10.1002/1873-3468.13743.

**Abstracts:**

1. Kremlitzka et al, 2014, XXV ICW, Rio de Janeiro, Abstr. n. 177, Mol.Immunol. page 278.
2. Z. Nagy-Baló1, S. Lukácsi1,2, B. Mácsik-Valent1,2, Z. Bajtay1,2, A. Erdei1,2: *Diverse functions of CR3 (CD11b/CD18) and CR4 (CD11c/CD18) β2-integrins expressed by human B lymphocytes*, ECI 2018 Amsterdam, Abstract book, p. 248, 2018