FINAL REPORT

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The **major goal** of the project was the systematic analysis of dendritic cell (DC) activation by ligands of various pattern recognition receptors (PRR) that involve Toll-like receptor (TLR), Nod-like receptor (NLR) and RIG-like receptor (RLR) family molecular sensors. These receptors exhibit overlapping ligand specificities and show typical localization patterns in various cell types. TLR are integrated to cell- or vesicular membranes, whereas RLR and NLR proteins reside in the cytosol. Recent studies revealed the subtype and subset specific expression of these PRR family members in human DC subtypes and subsets. In this project we focused to:

- studying the functional role of these PRR in the activation of the previously identified and characterized humun monocyte-derived $CD14^{low}$DC-SIGN+CD1a-PPARγ+ (**CD1a+)** and the CD14-DC-SIGN+CD1a+PPARγ- (**CD1a-**) monocyte-derived DC subsets;
- investigate the collaboration of these receptors in conventional and plasmacytoid DC subtypes.

1. Collaboration of vesicular TLR and intracellular RLR in conventional DC

1.1 Activation of moDC subsets by TLR ligands

Conventional DC express vesicular TLR3, TLR4 and the structurally related TLR7/8 receptors. To assess the response of CD1a- and CD1a+ monocyte-derived DC (moDC) to ligands targeting these receptors (polyI:C, LPS, CL075, respectively) we studied the phenotypic changes and cytokine secretion of activated DC derived from 5 – 7 healthy blood donors. We found statistically significant differences in the rate of activation monitored by the activation molecule CD83 and the chemokine receptor CCR7, as well as by the secretion of the inflammatory cytokines TNF-α, IL-6, IL-10 and IL-12. The T-cell polarizing cytokine IL-12 was predominantly produced by CD1a+, whereas IL-10 was produced by CD1a- cells indicating the inflammatory and regulatory functions of these subsets. To analyze the effect of differential TLR expression in these subsets we compared mRNA expression levels in these isolated DC subsets. We found that TLR3 and TLR7/8 are expressed at higher levels in CD1a+ cells than in the CD1a- ones, but TLR4 was expressed at higher levels in CD1a- cells. Furthermore, the response to LPS required DC-conditioned medium and showed high variations among the tested donors. These results suggested complex LPS-mediated signaling in DC subsets and prompted us to study additional factors involved in LPS-mediated stimulation of CD1a- and CD1a+ DCs. To this end we studied the dependence of DC activation on the presence of membrane bound and soluble CD14 (sCD14) protein that id preferentially expressed by monocytes, to a lower extent by CD1a- DC and not by CD1a+ DC. Both proteins bind LPS in a molecular complex with the MD2 protein, when transferred by the LPS binding protein (LBP). Soluble CD14 was detected in the supernatants of both moDC subsets but was shown to be released predominantly by CD1a- cells in line with the low CD14 positivity of this subset. As sCD14 redirects LPS from cells to the extracellular spane, the decreased activation of CD1a- cells by LPS may be caused by this phenotypic difference.

These results were summarized in the diploma work of Varga Rita Éva awarded by the prizes **TDK II.** 2008, **OTDK I.** 2009.

These results altogether revealed that CD1a- and CD1a+ cells responded differentially to various TLR signals. Furthermore, LPS-mediated activation of DC subsets depends on additional extra- and/or intracellular regulatory factors. Describing the underlying mechanisms involved in signal transduction of TLR4-mediated signals in these functionally distinct DC subsets is still in progress.

1.2. LPS signaling in monocyte-derived dendritic cell subsets

Our gene array studies focusing to TLR signaling pathways revealed that the expression pattern of the tested genes in resting DC were higher in CD1a- cells than in their CD1a+ counterpart. However, TLR3 receptor expression but not that of TLR4 and TLR7/8 was up-regulated by its ligand-induced activation suggesting that TLR-mediated signaling is not directly linked to the more inflammatory nature of the CD1a+ subset. To uncover the molecular background of differential activation and inflammatory potential of CD1a- and CD1a+ DC we sought to perform global gene expression profiling and identified the genes involved in the different TLR signaling pathways. Our results indicated marked differences between the signaling pathways involved in TLR4- and TLR3-mediated signal transduction and identified the cytosolic sensor melanoma differentiation associated gene 5 (MDA5), which signals through interferon regulatory factor 3 (IRF3) and leads to type I interferon secretion in CD1a+ DC. Our data also demonstrated the involvement of different sets of genes induced by LPS and polyI:C, targeting TLR4 and TLR3, respectively.

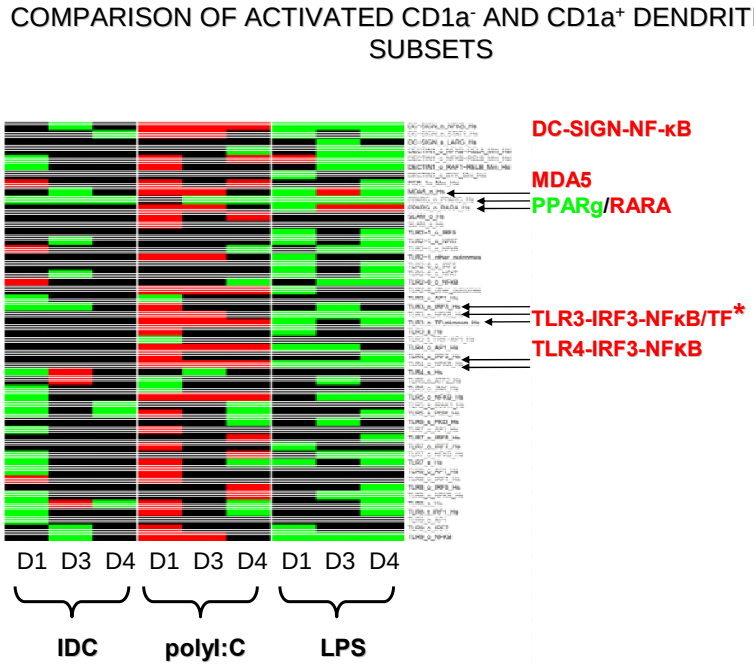

Figure 1 Dendritic cells isolated from donors D1, D3, D4 were sorted for CD1a- and CD1a+ fractions and gene expression profiles were generated by using the Illumina platform[1]. The results are shown as relative ratios of expression levels in CD1a+ vs CD1a- cells. Red color identifies up-regulation, green color indicates down-regulation as compared to resting DC.

1.3. Role of RLR family helicases in type I interferon production by a subset of monocyte-derived dendritic cells

To search for mechanisms that support inflammatory DC activation we compared the expression levels of the RLR family retinoic acid-induced gene I (RIG-I) and MDA5 helicases as a result of activation by various signals and monitored the expression and activation of the signaling pathways, connected to these sensors, in CD1a- and CD1a+ cells. Cytosolic RLR are PRR involved in interferon production and anti-viral immunity. Functional comparison of the previously identified $CD14^{-}$DC-$SIGN^{+}PPAR\gamma^{low}CD1a^{+}$ and $CD14^{low}$DC-$SIGN^{+}PPAR\gamma^{high}CD1a^{-}$ human moDC revealed that i) RIG-I and MDA5 gene and protein expressions are higher in $CD1a^{+}$ moDC than in their $CD1a^{-}$ counterpart and components of the RLR-related signalling pathway are expressed preferentially in the $CD1a^{+}$ subset. ii) The

RLR-coupled signalling pathway is more active in the $CD1a^+$ subset as compared to $CD1a^-$ cells and results in the production of IFNβ in an IRF3-dependent manner. iii) We also provided the first evidence that the type I interferon response in human moDC is primarily mediated by the RLR sensor system, whereas the regulation of pro-inflammatory cytokine production is under the control of the TLR3-NF-κB pathway that challenges the current paradigm concerning the collaborative or dichotomical regulation of type I interferon and inflammatory cytokine responses in human DC. iv) The increased activity of the RLR – IRF3 – IFNβ signalling pathway was shown to result in efficient priming of naïve $CD8^+$ T lymphocytes by the $CD1a^+$ subset and silencing of RIG-I/MDA5 abrogated this effect. Furthermore, the presence of RIG-I/MDA5 positive cells with DC morphology in tonsil, reactive lymph nodes (LN) and in adult respiratory distress syndrome caused by A(H1N1)-2009 influenza virus infection verified the *in vivo* importance of $CD1a^+$ RIG-I and MDA5 expressing DC. These findings not only describe the underlying mechanism of IFNβ production in two moDC subsets with complementary functions but also identify them as possible targets of modulating immune pathology or improving the efficacy of prophylactic and/or therapeutic vaccines against intracellular pathogens or tumors. As a summary our results identify the CD1a- and CD1a+ DC subsets with complementary functions against viruses as potential targets for modulating immune responses[2].

In 2007 – 2011 these results were presented at international meetings in **7 lectures** and **8 posters** by **Attila Szabó,** a pre-doctoral fellow. His poster presented at the Semmelweis PhD Scientific Days was awarded as the **Best Poster Presentation** of the Section, 2009). Two lectures were presented by **Krisztián Bene**, the MSc student of Attila Szabó at Student Conferences in 2009 and 2010.

1.4. Human inhibitory complement receptors are part of the TLR4 receptor complex

Complement inIn a scientific collaboration with István Andó (Biological Research Centre, Szeged) we showed that the composition of the LPS complex is reorganized upon monocyte to DC transition, and two complement inhibitory membrane proteins, CD55 and CD59 are also acting ascomponents of the LPS receptor complex in human moDC. Down regulation of these proteins by small interfering RNA (siRNA), or inactivating these receptors by neutralizing monoclonal antibodies (mAb) inhibited DC activation as monitored by CD83 and CCR7 expression, inflammatory cytokine secretion tested by ELISA and chemokine-induced DC migration.

These results were summarized in the diploma work of Edit Posta, a medical student and awarded by the prizes **TDK I.** 2009, **OTDK V.** 2009, **OTDK VI.** 2011.

1.5. Early activation of monocyte-derived dendritic cells triggers inhibitory mechanisms

We have shown that early activation of developing moDC triggers both immediate and persisting inhibitory mechanisms leading to aborted cytokine production and a non-migratory phenotype. Dendritic cells migrate towards lymphoid tissues in response to activation signals, whereas monocytes are able to enter inflamed tissues and give rise to newly generated inflammatory DC. These DC can participate in local immune responses or may differentiate towards migratory DC that maintain homeostatic naïve T cell activation. Long-term activation of DC however, has been associated with impaired functionality of these cells. How moDC developing in inflamed tissues avoid functional exhaustion due to persistent stimulatory signals is a question yet to be solved. We have shown that early activation of developing moDC leads to transient cytokine production accompanied by the up-regulation of the

inhibitory factors SOCS1, IL-10, STAT-3, miR146a and CD150 (SLAM), which conferred short term inhibitory effects on cytokine production but did not lead to persistent DC inactivation. The transient cytokine production was accompanied by IRAK-1 downregulation, inactivation of MyD88-dependent pathways and the inability of the cells to respond to further TLR ligation with IL-12 or TNF-α production. Furthermore, early activation during moDC differentiation was not accompanied by migratory DC differentiation, as reflected by the lack of CCR7 induction and the maintenance of CCR5 expression. Stimulation of monocytic DC precursors may thus lead to the rapid down-regulation of effector functions and the preservation of a tissue resident non-migratory phenotype.[3]

These results were presented at **3 international meetings** and at Student Conference (2008, 2009) by **Tünde Fekete**, PhD student.

2.Collaboration of vesicular and cytosolic PRR and other factors in activation of plasmacytoid and conventional dendritic cells

2.1. TLR and RLR cross-talk in pDC

Plasmacytoid DC express vesicular TLR7 and TLR9 in combination with the cytosolic sensors RIG-I and MDA5. Specific ligands of all these receptors trigger signaling pathways resulting in type I interferon (IFN) production. We addressed the question, which receptor(s) is/are responsible for the high amounts of IFNα secretion by pDC, also referred to as natural interferon producing cells (NIPC). Studies on pDC are hampered by their scarcity, short survival time in culture and difficulties to collect them. Using primary pDC available at limited amounts, and leukemic pDC cells (pDC-L) we provided ample evidence that the malignant cells isolated from bone marrow samples of a patient suffering from pDC leukemia are suitable for pDC research. Phenotypic maturation and cytokine secretion of pDC-L cells following TLR9 ligand (type A and type B CpG) or TLR7 ligand (imiquimod) stimulation were identical with those described for primary pDCs. Although pDC-L cells lack the ability to release IFNα due to the side effect of cryopreservation, the IFN signaling pathway was shown to be functional in them and they are able to activate T-cells. The expression of cytosolic RLR RNA receptors is established in conventional DC, but their expression and function in pDCs has not been characterized sofar. We showed that both pDC-L cells and primary pDCs express RIG-I and MDA5 under steady-state conditions at similar low levels, whereas the expression of these RLR is dramatically up-regulated upon stimulation with imiquimod or type A CpG. We also demonstrated for the firs time that TLR7-mediated signals efficiently block the expression of type I IFN genes induced by RIG-I/MDA5 ligands and thus regulate RLR functions through concomitantly switching on the ubiquitin-like IFN stimulated gene 15 (ISG15)-mediated inhibitory pathway to keep the inflammatory responses under control. The role of this regulation in the pathology of pDC leukemia, in virus induced pDCs stimulation and in autoimmune diseases remains to be explored[4].

These results show that the expression of various combinations of TLR and RLR family members by itself does not correlate with their functional activity that seems to be under tight control to prevent exacerbated inflammation.

2.2. Activation of dendritic cells by the coordinate action of NOD2 and inferferon-γ

Interferon-gamma (IFNγ) is produced by NK- and inflammatory Th1 cells and acts as an autocrine master cytokine driving inflammatory Th1 polarization. IFNγ triggers cells expressing type II interferon receptors (IFNRII) and signals through the Jak1 – Stat1 pathway.

To assess the interplay of MDP-induced NLR- and IFNγ cytokine-mediated signaling in moDC we exposed the cells to MDP in the presence or absence of IFNγ and monitored the expression of NLR family genes and proteins (Nalp3, NOD2 and caspase-1) and the functional characteristics of the activated cells. Our experiments showed that MDP alone has no effect on the expression levels of NOD2, Nalp3 or caspase-1, but results in IκB phosphorylation and activation of the NFκB pathway one hour after stimulation. IFNγ up-regulates NOD2 and caspase-1 mRNA, but the expression of Nalp3 mRNA and NOD2 protein requires both IFNγ- and MDP-mediated signaling suggesting the synergistic effects of the two pathways. Expression of the CD83 activation marker in the plasma membrane and the production of high levels of inflammatory citokines (TNF-α, IL-6, IL-1β, IL-8, IL-12) also was dependent on the presence of both ligands. The highest levels of biologically active cytokines were secreted when the cells were pre-treated by IFNγ followed by MDP stimulation as compared to simultaneous activation by IFNγ and MDP. These results demonstrate that the secretion of biologically active IL-1β requires the collaboration of the NLR sensor system with exogenous IFNγ that sensitizes DC to MDP, a known ligand of Nod2.
It has previously been described in resting murine bone marrow macrophages that Nod2 co-localizes with actin in the perinuclear region, but IFNγ is able to induce its dissociation from the actin cytoskeleton. These data suggest that IFNγ is able to prime cells for Nod2 redistribution to the cytosol and prompted us to test the role of IFNγ on the expression of components of the Nod2-signalosome. Our results indicated that in moDC IFNγ not only triggers NOD2 but also induces caspase-1 expression and thus allows the production of biologically active IL-1β. Furthermore, MDP was shown to trigger rapid modification of IKKγ/NEMO and degradation of phospho-IκB-α, however these events were not sufficient to induce enhanced cell surface expression of CD83 and the secretion of cytokine secretion. These functional changes required the interplay of MDP and IFNγ. To demonstrate the contribution of the Nod2-signalosome to inflammatory cytokine production we used the siRNA approach. Silencing of the Nod2 gene, the receptor component of the signallosome was performed by a mixture of specific and control siRNA. When differentiating DC were treated by Nod2-specific siRNA and were stimulated by MDP + IFNγ, a 60±10 % decrease in Nod2 gene expression was shown as compared to control siRNA, while had marginal effect on electroporated cells indicating that down-regulation of NOD2 mRNA affected RIP2 and IκB expression. As Nod2 binds to ubiquitinylated RIP2, an obligate component of the Nod2 signalosome to induce NF-κB activation, we suggest that NOD2 expression regulates the amount of NOD2:RIP2 complexes, lysine-63 (K63) polyubiquitination of lysine-285 on IKKγ/NEMO and consequently IκB degradation. As a consequence, the levels of NF-κB induced cytokines are decreased as a compared to the control samples where NOD2 expression is high. Thus NOD2 gene and protein expression has a critical role in regulating RIP2 – IKKγ/NEMO-mediated activation of the NF-κB pathway, while caspase-1 activity is ensured by IFNγ.

Based on these results we suggest that Nod2-mediated NFκB signaling and the Jak-Stat pathway used by IFNγ is linked in moDC and may have an impact on inducing inflammatory T cell responses.

3. The effect of apoptotic cell internalization on dendritic cell functions

The means how phagocytes handle apoptotic cells has a great impact on the outcome of immune responses. Here we show that phagocytosis of apoptotic neutrophils by human moDC is slow and less efficient than that of macrophages, and $CD1a^-$ DC are more active in the

engulfment of apoptotic neutrophils than $CD1a^+$ DC. Blocking DC-SIGN function partially interferes with the uptake of apoptotic cells and long term interaction of apoptotic neutrophils with DC make them prone to pro-inflammatory cytokine responses. Engulfment of apoptotic cells sensitizes $CD1a^-$ DC for high IL-8, TNF-α, IL-6, and $CD1a^+$ cells for IL-12 and IL-10 cytokine secretion elicited by additional inflammatory stimuli, which also result in polarization of autologous T lymphocytes to Th1 effector cells. However, as a result of PPARγ activation by rosiglitasone, the inflammatory response of $CD1a^-$ DC with high PPARγ expression and their capacity to trigger Th1 cell activation is down modulated. These results demonstrate that DC are able to respond to apoptotic neutrophils with inflammatory cytokine and T-cell responses in a subtype specific manner that can be modulated by the anti-inflammatory effects of PPARγ.[5]
Glucocorticoids are powerful anti-inflammatory compounds inhibiting inflammatory cell recruitment and production of pro-inflammatory cytokines. As we found that DC, the key-players of T cell priming and polarization respond to allogeneic apoptotic neutrophils with proinflammatory cytokine release and Th1 cell activation, we also tested the the capacity of moDC to engulf apoptotic cells and up-regulate genes of the apopto-phagocytic system. This gene expression pattern was reprogrammed when moDC differentiation took place in the presence of the synthetic glucocorticoid dexamethasone (Dex), which increased the expression of phagocytosis receptors MERTK and CD14, the bridging molecule C1QA, DNASE2 and the adenosine A3 receptor (ADORA3). The increased phagocytosis was attenuated by the addition of ADORA3 antagonist and could not be observed when bone marrow-derived DC of ADORA3 knockout mice were treated with Dex. The glucocorticoid treated human moDC loaded with apoptotic neutrophils secreted, in response to LPS and IFNγ, the inflammatory cytokine TNF-α. Furthermore, Dex-treated moDC could activate autologous T lymphocytes toward Th1 effector cells and this was enhanced by their exposure to allogeneic apoptotic neutrophils.[6]

4. Role of ion channels in dendritic cell functions

It has been reported that various voltage gated sodium channel (VGSCs) antagonists are able to influence the activation of lymphocytes, modify volume regulation, migration and apoptosis and impair phagocytosis and inflammatory responses of macrophages. However, the function of VGSCs in immune cells is still elusive. Previously we characterized a novel VGSC Nav1.7 channel expressed by monocyte-derived DCs and demonstrated a developmental switch of the Nav1.7 and VGPC Kv1.3 expression and function in the course of *in vitro* monocyte-derived DC maturation.[7] Expression of CD1a protein defines a human moDC subset with unique functional activities. We aimed to study the expression of the Nav1.7 sodium channel and the functional consequences of its activity in $CD1a^-$ and $CD1a^+$ DC. Single-cell electrophysiology (patch-clamp) and Q-PCR experiments performed on sorted $CD1a^-$ and $CD1a^+$ resting DC showed that the frequency of cells expressing Na^+ current, current density and the relative expression of the *SCN9A* gene encoding Nav1.7 were significantly higher in $CD1a^+$ cells than in their $CD1a^-$ counterparts. We also showed that the presence and activity of active Nav1.7 channels results in a depolarized resting membrane potential (–8.7±1.5 mV) in $CD1a^+$ IDC as compared to $CD1a^-$ cells lacking Nav1.7 (–47±6.2 mV). Stimulation of DC by inflammatory signals or by increased intracellular Ca^{2+} levels resulted in reduced Nav1.7 expression. Silencing of the *SCN9A* gene shifted the membrane potential to a hyperpolarizing direction in $CD1a^+$ IDC resulting in decreased cell migration, whereas pharmacological inhibition of Nav1.7 by tetrodotoxin sensitized the cells for activation signals. Fine tuning of IDC functions by a voltage-gated sodium channel emerges

as a new regulatory mechanism modulating the migration and cytokine responses of these DC subsets.[8]
In line with these studies, the importance and regulatory function of other membrane channels in DC and other immune cells has also been described.[9] [10]

[1] Cavalieri D, Rivero D, Beltrame L, Buschow SI, Calura E, Rizzetto L, Gessani S, Gauzzi MC, Reith W, Baur A, Bonaiuti R, Brandizi M, De Filippo C, D'Oro U, Draghici S, Dunand-Sauthier I, Gatti E, Granucci F, Gündel M, Kramer M, Kuka M, Lanyi A, Melief CJ, van Montfoort N, Ostuni R, Pierre P, Popovici R, Rajnavolgyi E, Schierer S, Schuler G, Soumelis V, Splendiani A, Stefanini I, Torcia MG, Zanoni I, Zollinger R, Figdor CG, Austyn JM: *DC-ATLAS: a systems biology resource to dissect receptor specific signal transduction in dendritic cells.*, Immunome Res. 2010 Nov 19;6:10, 2010

[2] Szabo A, Krisztian Bene, Varga R E, Lanyi A, Bence R, Gogolak P, Dezso B, Rajnavolgyi E: *Szabo A, Krisztian Bene, Varga R E, Lanyi A, Bence R, Gogolak P, Dezso B, Rajnavolgyi E*, Mucosal Immunology (submitted for publication), 2011

[3] Fekete T, Szabo A, Beltrame L, Vivar N, Pivarcsi A, Lanyi A, Cavalieri D, Rajnavölgyi E, Rethi B: *Early activation of developing monocyte derived dendritic cells triggers both immediate and persisting inhibitory mechanisms leading to aborted cytokine production and non-migratory phenotype*, J Immunol (submitted for publication), 2011

[4] Magyarics Z, Szabo A, Pazmandi K, Gopcsa L, Rajnavolgyi E, Bacsi A: *Collaboration of TLR and RLH mediated signaling pathways in human primary and leukemic plasmacytoid dendritic cells*, J Immunol (submitted for publication), 2011

[5] Majai G., Gogolák P., Ambrus C., Vereb G., Hodrea J., Fésüs L., Rajnavölgyi É.: *PPARg modulated inflammatory response of human dendritic cell subsets to engulfed apoptotic neutrophils*, J Leukocyte Biol 88:981-991, 2010

[6] Hodrea J, Májai G, Doró Z, Zahuczky G, Pap A, Rajnavolgyi É, Fesüs L: *The glucocorticoid dexamethasone programs human dendritic cells for enhanced phagocytosis of apoptotic neutrophils with inflammatory response*, J Leukoc Biol (submitted for publication), 2011

[7] Zsiros, E., Kis-Toth, K., Hajdu, P., Gaspar, R., Bielanska, J., Felipe, A., Rajnavolgyi, E. and Panyi, G.: *Developmental switch of the expression of ion channels in human dendritic cells*, J Immunol 2009. 183: 4483-4492., 2009

[8] Kis-Toth K, Hajdu P, Bacskai I, Papp F, Szanto A, Posta E, Gogolak P, Szilagyi O, Panyi G, Rajnavolgyi E: *Voltage-gated sodium channel Nav1.7 maintains the membrane potential and regulates the activation and chemokine-induced migration of a monocyte-derived dendritic cell subset*, The Journal of Immunology (under revision), 2011

[9] Toth, B. I., Benko, S., Szollosi, A. G., Kovacs, L., Rajnavolgyi, E. and Biro, T.: *Transient receptor potential vanilloid-1 signaling inhibits differentiation and activation of human dendritic cells*, FEBS Lett 2009. 583: 1619-1624., 2009

[10] Petheő GL., Orient A., Baráth M., Kovács I., Réthi B., Lányi Á., Rajki A., Rajnavölgyi É., Geiszt M.: *Molecular and Functional Characterization of Hv1 Proton Channel in Human Granulocytes*, PLoS ONE, Volume 5, Issue 11, e14081, 2010