SUMMARY REPORT

OTKA101661

ELUCIDATION OF CHARACTERISTICS OF INTRACELLULAR PROCESSES
DURING ACTIVATION OF
TH1, TH2, REGULATORY T AND TH17 CELL TYPES
WITH A NOVEL FLOW CYTOMETRY TECHNIQUE.

PRINCIPAL INVESTIGATOR

BARNA VASARHELYI

2015

Final scientific summary report OTKA No 101661

The OTKA101661 project entitled ’Elucidation of characteristics of intracellular processes during activation of Th1, Th2, regulatory T and Th17 cell types with a novel flow cytometry technique’ has the following aims

.

AIM 1: Calcium handling of regulatory T cells (Tregs) and Th17 cells.

AIM 2: Comparison of intracellular NO and O2 production, plasma membrane potencial, mitochondrial calcium and membrane potential in Th1, Th2, Treg and Th17 cells.

AIM 3: Characterization of activation properties of Th1, Th2, Treg and Th17 cells in immune mediated disorders

Using the 3-years OTKA grant support we reached significant results in each of these field as it is reflected by the 15 related articles published in international periodicals (cumulative impact factor: 32.28)

Based on these results 3 PhD students (Ambrus Kaposi, Gergő Mészáros & Balázs Szalay) completed their Theses.

Ambrus defended his Theses in 2013, Gergő & Balázs will keep their PhD lectures until Summer, 2015.

Final scientific summary report OTKA No 101661

AIM 1: Calcium handling of regulatory T cells (Tregs) and Th17 cells.

related paper:

Orbán C1, Bajnok A, Vásárhelyi B, Tulassay T, Toldi G. Different calcium influx characteristics upon Kv1.3 and IKCa1 potassium channel inhibition in T helper subsets. Cytometry A. 2014 Jul;85(7):636-41.

DIFFERENT CALCIUM INFLUX CHARACTERISTICS UPON KV1.3 AND IKCA1 POTASSIUM CHANNEL INHIBITION IN T HELPER SUBSETS

Functional imbalance between T helper subsets plays important role in the pathogenesis of autoimmune disorders. Transient increase of cytoplasmic calcium level, and sustention of negative membrane potential by voltage sensitive Kv1.3 and calcium-dependent IKCa1 potassium channels are essential for short-term lymphocyte activation, thus present possible target for selective immunomodulation. We aimed to investigate calcium influx sensitivity to the inhibition of potassium channels in the main T helper subsets. Peripheral blood from 11 healthy individuals was drawn and calcium influx kinetics following activation with phytohemagglutinin in Th1, Th2, Th17 and Treg cells were evaluated. Alteration of calcium influx induced by specific inhibitors of Kv1.3 and IKCa1 potassium channels, and the expression of Kv1.3 channels were also assessed. Highest cytoplasmic calcium concentration was observed in stimulated Th1 cells, while the lowest level was measured in Treg cells. In Th1 and Th17 cells, inhibition of both investigated potassium channels decreased calcium influx. In Th2 cells only the inhibitor of Kv1.3 channels, while in Treg cells none of the inhibitors had significant effect. Upon the inhibition of IKCa1 channels, short-term activation of pro-inflammatory cells was specifically decreased without affecting anti-inflammatory subsets, indicating that selective immunomodulation is possible in healthy individuals.
Keywords: potassium channels, lymphocyte activation, Th17, Treg

Introduction

Allergic and autoimmune diseases are characterized by a dysregulation of cytokine production and a dysbalance between T helper lymphocyte subsets. The local cytokine milieu promotes the differentiation of naive CD4+ T helper cells into specific lineages, such as Th1, Th2, Th17 and regulatory T (Treg) cells. According to their physiological role, Th1 cells promote the clearance of viruses and intracellular bacteria, Th2 cells are involved in the clearance of extracellular parasites and activation of eosinophils, Th17 cells protect against fungal infections and also mediate tissue inflammation and autoimmunity, while Treg cells are important in the maintenance of self-tolerance and in the regulation of inflammation (1).

In spite of their specific roles, important developmental and functional links exist between these cell lines. For instance, Treg and Th17 cells share a requirement for transforming growth factor (TGF)-b to develop from naive T cells. However, terminal differentiation of Th17 cells also requires IL-1b and IL-6 in humans (2). Previous studies also demonstrated a complex functional collaboration between Th1 and Th17 cells (3).

Earlier studies raised the pathophysiologic role of the dysbalance between T helper subsets in the development of asthma and allergic disease. Besides the well-described role of Th2 shift (4), increased prevalence of Th17 cells and increased level of serum IL-17 have been demonstrated in allergic asthma (5). Th17 associated cytokines were found to promote the proliferation and decrease the apoptosis rate of airway smooth muscle cells (6). In allergic asthmatic children, the percentage of Th17 cells was also found to be higher, and the level of asthma control was related to the frequency of this cell type (7). On other hand, the prevalence of Treg cells was decreased in asthmatic children (8). A similar imbalance was found in allergic rhinitis (9).

The activation and cytokine production patterns of T helper lymphocytes are tightly related to the increase of the cytoplasmic calcium concentration and the influx of calcium from the extracellular space through ORAI channels. Cytokine expression can be differentially induced depending on the pattern (amplitude, duration, oscillations) of calcium signaling. Since distinct subsets exert different actions and produce different sets of cytokines, they presumably also show different characteristics in their calcium influx and short-term activation. Studies comparing the calcium profile of subsets showed that Th17 cells have different calcium influx characteristics than Th1 and Th2 cells. Upon stimulation, the populations of all three T cell subtypes have identically high cytoplasmic calcium peaks. However, while in Th1 cells this is followed by high sustained calcium levels that oscillate numerous times, Th2 cells exert a more rapid decrease with few oscillations. The initial high spike of cytoplasmic calcium in Th17 cells is followed by sustained calcium levels that are intermediate between Th1 and Th2 levels and oscillations that are similar to Th1 cells and larger and more numerous than Th2 cells (10).

In order to maintain the driving force for further calcium entry, potassium ions need to leave the cell via potassium channels (11). Therefore, the voltage dependent Kv1.3 and the calcium dependent IKCa1 potassium channels are important regulators of calcium influx kinetics in lymphocytes. Since these channels are elemental to the functionality of T cells (12), and their specific inhibition might have important therapeutic consequences, our research group previously investigated their role on CD4+ and CD8+ cells in autoimmune diseases, such as rheumatoid arthritis (13). Other studies also found that pharmacologic modulation of these channels might be a possible therapeutic approach in T cell mediated disorders (14,15). Since the two different potassium channels are expressed at different ratios on different cell subsets, the possibility of selective immunomodulation has been raised via their specific inhibition (13,16).

In contrast with Th1 and Th2 cells, only limited information is available on the potassium channel characteristics of Th17 and Treg cells. Di et al. has shown that Th17 and Treg function is not altered in a mouse model of T cell induced colitis when IKCa1 channels are missing, while Th1 and Th2 cells showed decreased calcium influx and cytokine production functions (17). Due to their important role in the development of T cell mediated disease, further investigations of calcium influx kinetics and its alteration by Kv1.3 and IKCa1 channel inhibitors are required, especially in human samples.

In this study, we aimed to characterize calcium influx kinetics in human Th1, Th2, Th17 and Treg subsets isolated from peripheral blood samples, and to describe the alterations of calcium influx induced by the inhibition of Kv1.3 and IKCa1 potassium channels.

Materials and Methods:

Peripheral blood samples were taken from 11 healthy individuals free from immune-mediated or any other disease (6 men age, 5 women; age: 24.6 [22-28] years). Informed consent was obtained from all subjects, and our study was reviewed and approved by an independent ethical committee of the institution. The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

Peripheral blood mononuclear cells (PBMCs) were separated by a standard density gradient centrifugation (25 min, 400 g, 22 ^{o}C) from freshly drawn blood. This cell suspension was washed twice in PBS. From then on, cells were kept throughout staining with fluorescent markers, treatment with inhibitors and measurement on flow cytometer in a modified RPMI-1640 medium. The calcium concentration of this medium was set to 2 mM by addition of crystalline $CaCl_2$.

PBMCs were incubated with the following anti-human mAbs: anti-CD4 PE-Cy7 (Cat:344612, BioLegend, San Diego, CA, USA), anti-CD25 APC-Cy7 (Cat:557753, BD Biosciences, San Jose, CA, USA), anti-CXCR3 APC (Cat:550967,BD Biosciences), anti-CCR4 PE (Cat:551120,BD Biosciences) and anti-CCR6 PerCP (Cat:560467, BD Biosciences), as well as anti-Kv1.3 channel FITC (Cat:SAB4501619, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacters' instructions. Cytoplasmic free calcium level was detected by loading the cells with 2.6 μM Fluo-3-AM (Cat:50016, Biotium, Hayward, CA, USA) and

0.02 % Pluronic F-127 (Molecular Probes, Karlsbad, CA, USA) for 20 minutes at 30 ^{o}C. Cells were washed once before measurement.

PBMCs were divided into four vials with equal cell numbers. One vial was treated with margatoxin (MGTX, 4 nM; Sigma-Aldrich, St.Louis, MO, USA), a selective blocker of the Kv1.3 channel, for 15 min before measurement. Another vial was treated with a triarylmethane compound, TRAM-34 (240 nM; Sigma-Aldrich), a specific inhibitor of the IKCa1 channel, for 10 min before measurement. The third vial was used as control. The fourth vial was incubated with 1 µg anti-Kv1.3 (Sigma-Aldrich) besides the mAbs used for surface staining. In all cases, samples were kept at 30 ^{o}C in dry bath, until the measurements.

At the beginning of the control, MGTX, and TRAM-34 treated sample measurements, a 2 min baseline of calcium level was recorded. T cell activation was initiated by phytohemagglutinin (15 µg/ml final concentration) as described earlier (18). Fluorescence emission of sequentially measured cells was monitored for 15 minutes. Average cell acquisition rate was 1000 cells/s.

In the case of Kv1.3 expression measurements, 500.000 cells were recorded, and the mfi (mean fluorescence intensity) values were calculated. All measurements were performed on a BD FACSAria flow cytometer (BD Biosciences, San Jose, CA) at 30^{o} C. The population of lymphocytes was gated according to forward and side scatter characteristics. CD4+ CXCR3+ cells were regarded as Th1 cells, CD4+ CCR4+ cells were regarded as Th2 cells, CD4+ CD25hi cells were regarded as Treg cells and CD4+ CCR4+ CCR6+ cells were regarded as Th17 cells.

Data acquired from the measurements were evaluated with specific software developed at our laboratory. Gating strategy is shown in Supplementary Figure 1. The core of this software is an algorithm (19) based on the calculation of logistic functions for each recording. The software also calculates parameter values describing each function, such the area under the curve (AUC), the End value, describing the maximal fluorescence of the calcium binding dye, and the time to reach 50% of the maximal fluorescence value ($t_{50\%}$). AUC values correspond to the sum cytoplasmic calcium increase. Data are expressed as median [IQR]. Comparisons between two sample populations were made with Mann-Whitney tests, as a test of normality (performed according to Kolmogorov–Smirnoff) indicated non-normal distribution of data. Comparisons between the paired values (with or without treatment of specific channel blockers) in the same population were made with Wilcoxon tests. p-values less than 0.05 were considered as significant. Statistics were calculated using the GraphPad Prism 5 software (La Jolla, CA, USA).

Results

Our results are summarized in Table 1, demonstrating the AUC, End and t50% parameters from the kinetic measurements, as well as basal calcium levels and Kv1.3 channel expression.

AUC values were the highest in Th1 cells compared with other subpopulations, among which no significant difference could be demonstrated. Of note, however, the lowest AUC value was ibserved in Treg cells, followed by the Th2 subset. In case of the End parameter, we could not observe a significant difference between the investigated T cell subpopulations. t50% values were either not significantly different across the investigated subsets, however, the highest value was detected in Treg cells, indicating that these cells need more time to achieve the maximal calcium influx.

Inhibition of Kv1.3 channels with MGTX decreased the AUC value in Th1, Th2 and Th17 but not in Treg cells. The End value was also decreased by this inhibitor in Th2 and Th17, as wells as in Treg cells.

The effect of TRAM on the inhibition of IKCa1 channels is less characteristic. TRAM decreased the AUC value in Th1 and Th17 cells, as well as the End value in Th17 cells.

Our measurements indicated no significant difference in the basal calcium levels across the investigated T helper cell subsets, although the lowest initial concentration was detected in Th17 cells.

The surface expression of Kv1.3 channels was higher in Th2, Th17 and Treg cells than in the Th1 subset. The highest amount of Kv1.3 channels was detected on Th17 cells.

Discussion

In this study we examined calcium influx characteristics upon PHA stimulation in human Th1, Th2, Th17 and Treg cells, and its sensitivity to the inhibition of Kv1.3 or IKCa1 potassium channels.

Our results indicate no difference in the maximal cytoplasmic calcium concentration (End value) following the addition of PHA between the investigated subsets. This corresponds with the findings of Weber et al obtained in mouse T cells (10). In their study, calcium levels were the most sustained in Th1 cells, compared to Th17 and Th2 cells. In line with this, we detected the highest cytosolic calcium level (AUC value) in Th1 cells, while AUC values were decreased in the other three T helper subsets. Of note, when data from human and mouse T cells are compared, it has to be taken into account that the potassium channel repertoire in these two might be different. Although not statistically significant, it is worth to notice that the lowest AUC value and the highest t50% value were found in the Treg subset, indicating that this subset shows the slowest and least reaction compared to other subsets upon an identical activating stimulus (Figure 1). This is in line with the physiological regulatory role of this subset controlling lymphocyte activation. Of note, our method is not appropriate for the demonstration of delicate oscillations in calcium levels; therefore, this feature of the calcium signalling pattern could not be compared with the results of Weber et al (10). Interestingly, our results indicate that the basal cytoplasmic calcium concentration is comparable across the investigated T lymphocyte subsets.

While MGTX unequivocally decreased calcium influx in Th1, Th17 and Th2 cells, the inhibitory effect of TRAM was not so homogenous. Calcium influx was decreased by TRAM to a similar extent as by MGTX in Th1 cells, to a lower extent compared to MGTX in Th17 cells, and showed no statistically significant decrease in Th2 cells. This might be related to the low AUC value observed in this subset upon activation, since the function of IKCa1 channels is regulated by cytoplasmic calcium levels (12). While cytoplasmic calcium concentrations are sufficient for IKCa1 channels in Th1 and Th17 cells to react to inhibition (though they are lower in the latter, as already demonstrated by Weber et al.), cytoplasmic calcium levels might not be sustained enough in Th2 cells to cause a significant alteration upon blocking IKCa1 channels. The differences observed in the sensitivity of potassium channel inhibition between Th1 and Th2 cells are in line with our earlier results (13) and the findings of others (20). In case of Treg cells, the total amount of calcium influx (represented by AUC values) are not altered upon the inhibition of Kv1.3 and IKCa1 channels. This finding might also be related to the physiological regulatory role of this subset controlling lymphocyte activation.

In order to test whether the sensitivity to the inhibition of Kv1.3 channels is due to alterations in their expression, we tested the presence of these channels on the cell surface. Although the inhibitory effect of MGTX is similar in Th1, Th2 and Th17 cells, Kv1.3 channels are expressed at a considerably higher level in the Th2, and especially in the Th17 subset, raising the notion that their significance is smaller in balancing calcium influx in Th2 and Th17 compared to Th1 cells. This is further supported by the fact that a higher amount of calcium influx (represented by a higher AUC value) is counterbalanced by a smaller amount of Kv1.3 channels expressed on the cell surface in the Th1 subset compared to the other investigated cell types.

The limitation of this study is that our findings were obtained solely in healthy individuals; therefore it is still of question whether lymphocytes of patients with immune-mediated disorders present with similar characteristics. If so, these results indicate that Kv1.3 channels have a higher role in balancing calcium influx in T helper subpopulations compared to IKCa1 channels, however, their specific inhibition by MGTX is not selective for a particular subset in healthy adults. In contrast, blocking IKCa1 channels with TRAM yields a less drastic alteration in calcium influx kinetics, but it seems to be more selective, since the decrease of AUC values was only significant in Th1 and Th17 cells. This finding might be of particular interest in autoimmune disorders, because inhibition of IKCa1 channels alters the calcium profile of the pro- inflammatory subpopulations without affecting anti-inflammatory cells. These results are in partial agreement with the observations of Di et al, who also found that IKCa1 inhibition decreased calcium influx and cytokine production of Th1 cells (17). However, in their study, IKCa1 was also

important in Th2, but not in Th17 cells. This difference might be due to the fact that their experiments were performed on a mouse model, while we studied human T cells.

In conclusion, upon the inhibition of IKCa1 channels, the short-term activation of Th1 and Th17 cells is specifically decreased without affecting the Th2 and Treg subsets, indicating that selective immunomodulation can be reached in healthy individuals under experimental conditions. Further studies are needed to evaluate the potential therapeutic application of IKCa1 inhibitors in immune-mediated disorders.

Literature cited

1. Romagnani S, Maggi E, Liotta F, Cosmi L, Annunziato F. Properties and origin of human Th17 cells. Mol Immunol 2009;47:3-7.
2. Weaver CT, Hatton RD. Interplay between the TH17 and TReg cell lineages: a (co-)evolutionary perspective. Nat Rev Immunol 2009;9:883-9.
3. Damsker JM, Hansen AM, Caspi RR. Th1 and Th17 cells: adversaries and collaborators. Ann N Y Acad Sci 2010.
4. Robinson DS. The role of the T cell in asthma. J Allergy Clin Immunol 2010;126:1081-91; quiz 1092-3.
5. Bajoriuniene I, Malakauskas K, Lavinskiene S, Jeroch J, Gasiuniene E, Vitkauskiene A, Sakalauskas R. Response of peripheral blood Th17 cells to inhaled Dermatophagoides pteronyssinus in patients with allergic rhinitis and asthma. Lung 2012;190:487-95.
6. Chang Y, Al-Alwan L, Risse PA, Halayko AJ, Martin JG, Baglole CJ, Eidelman DH, Hamid Q. Th17-associated cytokines promote human airway smooth muscle cell proliferation. FASEB J 2012;26:5152-60.
7. Kerzel S, Dehne J, Rogosch T, Schaub B, Maier RF, Zemlin M. TH17 cell frequency in peripheral blood from children with allergic asthma correlates with the level of asthma control. J Pediatr 2012;161:1172-4.
8. Wei B, Zhang H, Li L, Li M, Shang Y. T helper 17 cells and regulatory T-cell imbalance in paediatric patients with asthma. J Int Med Res 2011;39:1293-305.
9. Zhang C, Hong S, Hu G. [The expression of Treg/Th17 cells related transcription factors and cytokines in PBMCs and plasma in patients with allergic rhinitis]. Lin Chung Er Bi Yan Hou Tou Jing Wai Ke Za Zhi 2012;26:209-11.
10. Weber KS, Miller MJ, Allen PM. Th17 cells exhibit a distinct calcium profile from Th1 and Th2 cells and have Th1-like motility and NF-AT nuclear localization. J Immunol 2008;180:1442-50.
11. Robert V, Triffaux E, Savignac M, Pelletier L. Singularities of calcium signaling in effector T-lymphocytes. Biochim Biophys Acta 2013;7:20.
12. Grissmer S, Nguyen AN, Cahalan MD. Calcium-activated potassium channels in resting and activated human T lymphocytes. Expression levels, calcium dependence, ion selectivity, and pharmacology. J Gen Physiol 1993;102:601-30.
13. Toldi G, Bajnok A, Dobi D, Kaposi A, Kovacs L, Vasarhelyi B, Balog A. The effects of Kv1.3 and IKCa1 potassium channel inhibition on calcium influx of human peripheral T lymphocytes in rheumatoid arthritis. Immunobiology 2013;218:311-6.
14. Grgic I, Wulff H, Eichler I, Flothmann C, Kohler R, Hoyer J. Blockade of T-lymphocyte KCa3.1 and Kv1.3 channels as novel immunosuppression strategy to prevent kidney allograft rejection. Transplant Proc 2009;41:2601-6.
15. Lam J, Wulff H. The Lymphocyte Potassium Channels Kv1.3 and KCa3.1 as Targets for Immunosuppression. Drug Dev Res 2011;72:573-584.
16. Beeton C, Pennington MW, Wulff H, Singh S, Nugent D, Crossley G, Khaytin I, Calabresi PA, Chen CY, Gutman GA and others. Targeting effector memory T cells with a selective peptide inhibitor of Kv1.3 channels for therapy of autoimmune diseases. Mol Pharmacol 2005;67:1369-81.
17. Di L, Srivastava S, Zhdanova O, Ding Y, Li Z, Wulff H, Lafaille M, Skolnik EY. Inhibition of the K+ channel KCa3.1 ameliorates T cell-mediated colitis. Proc Natl Acad Sci U S A 2010;107:1541-6.
18. Meszaros G, Szalay B, Toldi G, Kaposi A, Vasarhelyi B, Treszl A. Kinetic measurements using flow cytometry: new methods for monitoring intracellular processes. Assay Drug Dev Technol 2012;10:97-104.
19. Kaposi AS, Veress G, Vasarhelyi B, Macardle P, Bailey S, Tulassay T, Treszl A. Cytometry-acquired calcium-flux data analysis in activated lymphocytes. Cytometry A 2008;73:246-53.
20. Fanger CM, Neben AL, Cahalan MD. Differential Ca2+ influx, KCa channel activity, and Ca2+ clearance distinguish Th1 and Th2 lymphocytes. J Immunol 2000;164:1153-60.

		No inhibitor		*MGTX (4 nM)*		*TRAM (240 nM)*	
Th1	**AUC (U)**	2795 [2477-3005]		2380[b] [2014-2457]	p = 0.01	2431[b] [2081-2570]	p = 0.032
	End (rpv)	1.459 [1.334-1.799]		1.281 [1.071-1.379]		1.255 [1.121-1.444]	
	$t_{50\%}$ (s)	500 [221-526]		362 [146-506]		465 [294-885]	
	Kv1.3 (mfi)	323 [167-497]			**Basal Ca^{2+} (mfi)**	1.60 [0.59-2.69] X 10^4	
		No inhibitor		*MGTX (4 nM)*		*TRAM (240 nM)*	
Th2	**AUC (U)**	2309[a] [1921-2772]	p = 0.044	2078[b] [1600-2195]	p = 0.005	2053 [1829-2506]	
	End (rpv)	1.379 [1.068-1.624]		1.050[b] [0.844-1.118]	p = 0.003	1.191 [1.029-1.611]	
	$t_{50\%}$ (s)	324 [208-964]		652 [192-904]		299 [201-696]	
	Kv1.3 (mfi)	577[a] [296-974]	p = 0.001		**Basal Ca^{2+} (mfi)**	1.59 [1.07-1.93] x 10^4	
		No inhibitor		*MGTX (4 nM)*		*TRAM (240 nM)*	
Th17	**AUC (U)**	2429[a] [2236-2556]	p = 0.014	2099[b] [2002-2410]	p = 0.003	2211[b] [2169-2491]	p = 0.024
	End (rpv)	1.290 [1.148-1.828]		1.107[b] [1.036-1.225]	p = 0.008	1.263[b] [1.037-1.299]	p = 0.047
	$t_{50\%}$ (s)	448 [347-532]		774 [320-817]		508 [157-977]	
	Kv1.3 (mfi)	1608[a] [543-3321]	p = 0.001		**Basal Ca^{2+} (mfi)**	1.16 [0.47-1.90] X 10^4	
		No inhibitor		*MGTX (4 nM)*		*TRAM (240 nM)*	
Treg	**AUC (U)**	2264[a] [2147-2822]	p = 0.042	2177 [2048-2279]		2435 [2248-2486]	
	End (rpv)	1.248 [1.094-2.106]		1.117[b] [1.044-1.174]	p = 0.01	1.302 [1.149-1.425]	
	$t_{50\%}$ (s)	657 [281-806]		392 [219-638]		616 [349-819]	
	Kv1.3 (mfi)	845[a] [211-1523]	p = 0.001		**Basal Ca^{2+} (mfi)**	1.61 [0.85-2.49] x 10^4	

Table 1. The effects of margatoxin (MGTX) and triarylmethane (TRAM) application on parameter values (AUC – Area Under the Curve in units (U), End – Ending value in relative parameter value (rpv), $t_{50\%}$ – Time to reach 50% value in seconds (s)) of calcium influx kinetics, as well as mean fluorescence intensity (mfi) of the anti-Kv1.3 channel antibody and calcium binding Fluo-3 dye in T helper lymphocytes obtained from 11 healthy individuals. Data are expressed as median [IQR]. [a] vs. Th1, [b] MGTX and TRAM treated samples were compared with samples with no inhibitor application within the investigated T helper subsets

AIM 2: Comparison of intracellular NO and O2 production, plasma membrane potencial, mitochondrial calcium and membrane potential in Th1, Th2, Treg and Th17 cells.

related paper: Mészáros G, Orbán C, Kaposi A, Toldi G, Gyarmati B, Tulassay T, Vásárhelyi B: Altered mitochondrial response to activation of T-cells in neonate, Acta Physiologica Hungarica, accepted for publication

ALTERED MITOCHONDRIAL RESPONSE TO ACTIVATION OF T-CELLS IN NEONATE

Abstract

Mitochondrial functions have a major impact on T-cell functionality. In this study we characterized whether mitochondrial function in the neonatal T-cells differs from that in the adult T-cells during short T-cell activation. We used flow cytometry methods to test mitochondrial mass and to monitor mitochondrial Ca^{2+} levels, mitochondrial potential and superoxide generation in parallel with cytoplasmic Ca^{2+} levels during phythohaemagglutinine-induced activation of CD4+ and CD8+ T cells of 12 term neonates and 11 healthy adults.

Baseline mitochondrial mass of CD4+ and CD8+ cells was lower in the neonate than in the adult. In comparison with the adult, neonatal resting CD4+ T-cells had lower cytoplasmic Ca^{2+}- levels and this was associated with normal activation induced Ca^{2+}-response. During short-term activation cytoplasmic Ca^{2+} response was lower in neonatal than in adult CD8+ T-cells. Mitochondrial Ca^{2+}-uptake was increased in CD4+ neonatal T cells while decreased in CD8+ T-cells. Mitochondrial depolarization was increased in CD4+ and decreased in CD8+ neonatal T-cells compared to adults. Superoxide generation was higher and equal in neonatal CD4+ and CD8+ cells, respectively, compared to the adult.

Our data suggest that neonatal T-cells exhibit marked differences in mitochondrial function and superoxide generation compared to adult T-cells.

Background

Clinical experience and experimental data support that neonatal T-cells are immunologically less competent than those in the adult (1). Upon activation the production of inflammatory cytokines in CD3+ T-cells is lower in the neonate than in the adult (7, 13). The major factors explaining the reduced response of neonatal T-cells is probably the absence of pre-existing immunological memory (2) and increased number of CD71+ erythroid cells (5). However, we also observed that the intracellular Ca^{2+} response of the neonatal T-cells to activation also presents specific characteristics (23).

Proper T-cell activation is a result of coordinated work of intracellular signaling machinery (6). As a result of T-cell receptor (TCR) stimulation, cytoplasmic Ca^{2+} level ($[Ca^{2+}]_c$) is transiently increased and activates Ca^{2+}-dependent signaling pathways (8). We found that Ca^{2+} response during short-term activation of T-cells is lower in the neonate than in the adult. The immaturity of potassium channels on neonatal cell membrane may be an explanation for this finding (23).

Now we focused on mitochondrion, another central player of T-cell activation. Changes in $[Ca^{2+}]_c$ are associated with alterations in mitochondrial Ca^{2+} levels ($[Ca^{2+}]_m$) (4). Mitochondria have an important role in T-cell activation as they modulate the Ca^{2+}-signaling during TCR induced activation. Additionally mitochondrial Ca^{2+}-signal regulates energy metabolism and has also a major impact on other specific T-cell functions (19).

Appropriate generation of reactive oxygen species (ROS) is a fundamental element of T-cell activation (10, 22). ROS modulates signaling pathways and transcription of several genes (25). TCR-signaling induces mitochondrial O_2^- generation in a Ca^{2+} dependent manner (3). Phagocyte-type NADPH-oxidase and mitochondria are the major sources of ROS generation during the first 15 minutes of T-cell activation (10).In this study we compared mitochondrial mass and function including mitochondrial Ca^{2+} kinetics

and mitochondrial membrane potential and ROS generation in CD4+ and CD8+ T cells of the neonate with those of the adult.

Materials and Methods

Patients

We used cord blood samples of twelve healthy term neonates [10 girls and 2 boys, gestational age: 39 (38–40) weeks, median (range), birth weight: 3375 (2510–4040) grams, median (range)] and peripheral blood samples of 11 healthy adults [3 women and 8 men, age: 25 (22–44) years, median (range)] (Table1).

Ethical Statement

The study adhered to the tenets of Declaration of Helsinki. Written informed consent was obtained from the adults and the mothers of the neonates. An independent institutional ethical committee, Semmelweis University Regional and Institutional Committee of Science and Research Ethics, approved this study.

Solutions and reagents

Ficoll Paque; Amersham Biosciences AB, Uppsala, Sweden; phosphate buffered saline (PBS) (2 mM KH_2PO_4, 9.5 mM $Na_2HPO_4.2H_2O$, 136.7 mM NaCl); anti-human mAbs: anti-CD4 PE-Cy7 (cat. no. 557852), anti-CD8 APC-Cy7 (cat. no. 557834) (both from PharMingen, San Diego, CA, USA); Fluo-3-AM, Rhod2/AM, tetramethylrhodamine methylester (TMRM), MitoTracker Green and Dihydroethidium (DHE) dyes as well as Pluronic F-127 were purchased from Molecular Probes (Karlsbad, CA) and they were freshly dissolved in dimethyl sulfoxide (DMSO) or ethanol according to the manufacturer's protocol before the measurements. Phytohemagglutinin (PHA), ionomycin, carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP), RPMI-1640 medium were purchased from Sigma–Aldrich (St.Louis, MO, USA).

Cell isolation

Peripheral blood mononuclear cells (PBMC) and cord blood mononuclear cells (CBMC) were separated by a standard density gradient centrifugation with Ficoll Paque (27 min, 400 g, 22 ^oC) from freshly drawn blood collected in lithium heparin-treated tubes (BD Vacutainer and BD Biosciences, San Jose, CA, USA). This cell suspension was washed twice in PBS. From then on, cells were kept throughout staining with fluorescent markers and measurement on flow cytometer in a modified RPMI medium. The Ca^{2+} concentration of the modified RPMI medium was set to 2 mM by addition of crystalline $CaCl_2$.

Surface staining

For surface marker staining, PBMCs and CBMCs were incubated with anti-CD4 PE-Cy7 and anti-CD8 APC-Cy7 anti-human conjugated mAbs according to the manufacturers' instructions.

Cell activation

At the beginning of each measurement 2 min baseline was recorded. T-cell activation was initiated by phytohemagglutinin (15 □g/ml in final concentration). In each experiment fluorescence emission of sequentially measured cells was monitored for 12 minutes, the time which is needed for the decline of the PHA induced calcium signal in adult and cord blood human T-cells (23). In average, about 1 x 10^6 cells were measured.

Cytoplasmic Ca^{2+} kinetics measurement

Cytoplasmic free Ca^{2+} level was detected by loading the cells with Fluo-3-AM (emission maximum, 526 nm, recorded with a 530/30 nm band pass filter (BP filter)). Cells were loaded with 2.6 μM Fluo-3-AM and 0.02% Pluronic F-127 for 30 minutes at 30 ^oC. Cells were washed once before measurements. At the end of the measurements ionomycin was added to the samples to verify the specificity of the signal (17).

Measurement of superoxide generation

Dihydroethidium (DHE), a redox-sensitive probe reacts with superoxide and results in the formation of a fluorescent dye (emission maximum, 605 nm, recorded with 610/20 nm BP filter). Cells were loaded with 1 μM DHE for 18 minutes at 37 ^{o}C (17).

Measurement of baseline mitochondrial mass and investigation of the kinetics of mitochondrial Ca^{2+} level, mitochondrial membrane potential upon activation

Mitochondrial mass was estimated by staining with potential insensitive mitochondrial dye, MitoTracker Green (emission maximum: 516 nm recorded in with a 530/30 nm BP filter). Cells were loaded with 150 nM MitoTracker Green for 30 minutes at 37 ^{o}C in the dark. Cells were washed once before measurements. Mitochondrial Ca^{2+} level was monitored using Rhod2/AM (emission maximum, 581 nm, recorded with a 575/26 nm BP filter). Cells were loaded with 2.5 μM Rhod2/AM and 0.02% Pluronic F-127 for 20 minutes at 30 ^{o}C and washed once before measurements. At the end of each measurement FCCP was added to the sample to verify the specificity of the signal (17).

Mitochondrial membrane potential was estimated by staining with TMRM (emission maximum, 567 nm recorded with 575/26 nm BP filter) in quench/dequench mode. Cells were loaded with 1 μM TMRM for 20 min at 37 ^{o}C in the dark and washed once before measurement. At the end of the measurements FCCP was added to the sample to verify the specificity of the signal.

Equipment and gating methods

All flow cytometric measurements were performed on a BD FACSAria flow cytometer (BD Biosciences, San Jose, CA) equipped with 488- and 633-nm excitation lasers and data were processed using the FACSDiVa software.

The population of lymphocytes was gated according to forward scatter characteristics and side scatter characteristics. PE-Cy7 conjugated anti-CD4 and APC-Cy7 conjugated anti CD8 antibodies wwere used for the gating of CD4+ and CD8+ T-cell subpopulations. The gating strategy of flow cytometry data is shown in Fig. 1.

Data analysis

Data acquired from the measurements were evaluated with specific software developed at our laboratory. The core of this software is an improved version of an algorithm (15) based on fitting of logistic or double logistic functions to each recording (14). The logistic function was used to characterize continuously increasing fluorescence values, while the double logistic function was used to describe measurements that have an increasing phase, a peak and a decreasing phase as time passes. The software also calculates parameter values describing each function, such as the maximum value (Max), the time to reach maximum value (t_{max}), slope at 50% of maximum (Slope), ending value (End) and the area under the curve (AUC). One unit (U) of the AUC value is defined as one relative intensity value in 1 s, where relative intensity values are the rate of actual intensity values divided by intensity values at 0 s.

Comparison of parameter distributions of the two groups was made by probability binning method (21), Mann–Whitney tests was performed as a test of normality (performed according to Kolmogorov–Smirnoff) indicated non-normal distribution of data. Mann–Whitney tests were used to test the difference between baseline parameters in the different cell populations. Two tailed p-values obtained with less than 0.05, were considered as significant.

Results

Mitochondrial mass

To examine the mitochondrial mass in resting CD4+ and CD8+ T-cell populations isolated from newborn cord blood and adult peripheral-blood, we measured the whole cell fluorescent intensity of MitoTracker Green, a mitochondrial specific fluorescent dye. Mitochondrial mass was significantly decreased in the neonatal CD4+ T-cell populations compared to the adult CD4+ T-cells, while the difference in the CD8+ T-lymphocytes was not significant (Figure 2 A).

Baseline cytoplasmic Ca^{2+} levels, mitochondrial Ca^{2+} levels, mitochondrial membrane potential

Lower cytoplasmic Ca^{2+}-levels in neonatal CD4+ T-cells

In order to compare the cytoplasmic Ca^{2+}-levels of unstimulated adult peripheral and neonatal cord blood CD4+ and CD8+ T-cells, T-cells were loaded with Fluo-3-AM, a calcium sensitive fluorescent dye. Baseline cytoplasmic Ca^{2+}-levels of neonatal CD4+ T –cells were lower compared to adult CD4+ lymphocytes whereas no difference was observed between the 2 groups in CD8+ T-cells (Figure 2 B). The addition of the calcium ionophore ionomycin indicating that the capacity for pharmacologically induced calcium flux in neonatal CD4+ T cell was reduced compared to adult CD4+ T-cells, while it was comparable in neonatal and adult CD8+ T-cells (Figure 2 C).

Comparable basal mitochondrial Ca^{2+}-levels and membrane potential in neonatal and adult CD4+ and CD8+ T-cells

When investigating the role of mitochondria in Ca^{2+}-metabolism of neonatal and adult T-cells, first we measured basal mitochondrial Ca^{2+}-levels. Mitochondrial Ca^{2+}-levels of unstimulated neonatal and adult CD4+ or CD8+ T-cells were comparable (Figure 2 D).

Mitochondrial membrane potentials of resting CD4+ or CD8+ T-cells were equivalent in the two analyzed groups (Figure 2 E).

PHA induced changes in cytoplasmic Ca^{2+} levels, mitochondrial Ca^{2+} levels, mitochondrial membrane potential and superoxide generation

Decreased cytoplasmic Ca^{2+}-levels in neonatal CD8+ cells following PHA induced activation

We stimulated T-cells with PHA and monitored the initial fluctuation of cytoplasmic Ca^{2+} levels. AUC, Max or End and Slope parameters values were calculated of the fitted functions and compared between the two groups. During short-term activation cytoplasmic Ca^{2+}-response (in term of parameters AUC and Slope) was lower in neonatal than in adult CD8+ T-cells (Figure 3 A and C) while no difference was observed in neonatal and adult CD4+ T-cells (Figure 3 A and B).

Elevated mitochondrial Ca^{2+}-levels in neonatal CD4+ following PHA induced activation

In order to examine the effect of cytoplasmic Ca^{2+} signaling on mitochondrial Ca^{2+} uptake and mitochondrial function we measured mitochondrial Ca^{2+} levels and mitochondrial membrane potential after stimulation with PHA. The extent and kinetics of mitochondrial Ca^{2+}-uptake induced by PHA addition (characterized by AUC, Slope and End) was increased in CD4+ T cells obtained from neonates compared to adults (Figure 4 A and B), while neonatal CD8+ cells contained less Ca^{2+} than adult CD8+ T-cells at the end of the measurement (End value) (Figure 4 A and C).

Increased mitochondrial depolarization in neonatal CD4+ and decreased in CD8+ T-cell following PHA stimulation

In parallel to mitochondrial calcium uptake, mitochondrial membrane potential showed similar alterations following PHA stimulation: increased depolarization (AUC and Max) in neonatal CD4+ T-cells compared to adult (Figure 5 A and C) and reduced depolarization in CD8+ neonatal T-lymphocytes compared to that one of adult (AUC, Slope and Max) (Figure 5 A and C).

Superoxide generation

To see whether these changes in mitochondrial calcium metabolism and function affect the mitochondrial superoxide generation, we measured the fluorescent intensity changes of DHE after PHA stimulation. Surprisingly superoxide generation was also higher (based on AUC parameter) in neonatal than in adult CD4+ T-cells (Figure 6 A and B) whereas no differences were observed between neonatal and adult CD8+ T-cells (Figure 6 A and C).

Table 2 summarizes our results acquired by PHA stimulation of CBMC and PBMC T-cells.

Discussion

In this study, we observed that in addition to earlier observations done on cytoplasmic Ca^{2+} response, neonatal T-cells present other functional characteristics distinct from T-cells of the adult (11, 23). Now we measured reduced baseline cytoplasmic Ca^{2+}-levels and reduced Ca^{2+}-influx capacity in CD4+ neonatal T-cells.

After these observations in Ca^{2+}-handling we focused on mitochondrial function during T-cell activation. The rationale is the previous data suggesting that mitochondria are central players of T-cell activation and Ca^{2+}-signaling (9).

Indeed we measured lower mitochondrial mass in neonatal CD4-cells than in those from the adult. Previous study showed that CD8+ memory T cells contain higher amount of mitochondria compared to naïve T-cells (24). Neonates, compared to adults, have higher ratio of CD45RA+ naive T-cells (~90% and ~55% respectively). The mitochondrial mass of CD4+ naïve and memory T cells was not yet investigated. Although we did not distinguish naïve and memory T-cells in our experiments, the higher ratio of naïve T-cells might contribute to the lower mitochondrial mass of neonatal CD4+ and CD8+ T-cells.

The mass of mitochondria has a major effect on intracellular Ca^{2+} handling. Mitochondria act not only as Ca^{2+} buffers but they have a major impact on Ca^{2+}-channel regulation (20). Mitochondrial Ca^{2+} uptake reduces cytoplasmic Ca^{2+} peaks, but delays the return of $[Ca^{2+}]_c$ to the baseline. This feature was strikingly different in CD4+ and CD8+ T-cell populations. In the presence of lower mass of mitochondria, neonatal CD4+ T-cells took up more, while CD8+ cells took up less Ca^{2+} than those in the adult. These altered mitochondrial Ca^{2+}-handling characteristics might shape the cytosolic Ca^{2+}-signal leading to an earlier decline of cytosolic Ca^{2+}-signal. As there are no available data concerning the mitochondrial Ca^{2+} handling mechanism in neonatal T-cells further studies are required to explain these differences. This issue however is of major importance for immune modulation, as the diminished cytoplasmic Ca^{2+} levels were reported to be responsible for the reduced NFAT-associated gene expression (9, 13).

Of note, mitochondrial depolarization in T-cells upon activation is associated with the altered mitochondrial Ca^{2+} uptake in the neonatal T cells. Increased mitochondrial depolarization upon PHA induced T-cell activation was detected in neonatal CD4+ cells, as a consequence of elevated mitochondrial Ca^{2+} sequestration. On the other hand, mitochondrial depolarization was lower in neonatal CD8+ T-cells characterized by smaller Ca^{2+} uptake. These findings suggest that the difference in mitochondrial depolarization of T-cells between the neonate and the adult is attributable, at least partly to different Ca^{2+}-handling.

Mitochondrial calcium uptake has a major impact on ROS generation. Activation of T-cells is accompanied by increased reactive oxygen species generation (in a Ca^{2+} dependent manner (3)). ROS facilitate the expression of the oxidation-dependent nuclear transcription factors NF-κB and AP-1 in T-cells (18). In spite of the diminished CD4+ T-cell mitochondrial mass in the neonatal T-cell, we observed increased ROS production in these cells. This finding suggests altered regulation of mitochondrial ROS production in the very early phase of cord blood T-cell activation and/or the dysfunctionality of intracellular antioxidant defense mechanisms. Additionally, a previous work demonstrated that between

15 and 75 minutes of T cell activation in the neonate, these cells produce increased amount of ROS after chemical stimulation (ionomycin + phorbol-dibutyrate) (16) and decreased amount after anti-CD28 mAb stimulation. We propose that the elevated initial and then decreased ROS generation contribute to the different activation characteristics of neonatal T-cells However we have to note some limitations regarding our superoxide measurements done in a period when flow cytometry technique was a widely accepted approach for the detection of superoxide generation. However, today HPLC based methods are recommended to detect 2-hydroxyethidium (2-OH-E+), the fluorescence product formed from the DHE/O2•− reaction (12). Further studies applying these techniques are required to confirm our results about superoxide generation in T-cells; however, our observations provide a sound basis for these experiments.

Upon evaluation of our findings, we are to consider the limitations of our experiments. Number of samples measured and blood amount (25 ml) of adult and newborn groups were limited, and the study was performed in an in vitro setting.

Conclusion

Our data suggest that neonatal T-cells present different characteristics during short-term activation. In addition to reduced cytoplasmic Ca^{2+} signaling the mitochondrial mass is decreased in neonatal CD4+ T-cells. We measured elevated mitochondrial Ca^{2+} sequestration and mitochondrial depolarization in CBMC T-cells. All these alterations might contribute to the elevated O_2^- production and ROS signaling (Figure 5). These findings indicate that intracellular machinery in its complexity presents different features between neonatal and adult T-cells that may contribute to the immaturity of adaptive immunity of the neonate.

Acknowledgements

This work was supported by OTKA Grants 84087 and 101661.

References

1. Adkins B: Neonatal immunology: responses to pathogenic microorganisms and epigenetics reveal an "immunodiverse" developmental state. Immunol. Res. 57, 246-257 (2013)
2. Adkins B, Leclerc C, and Marshall-Clarke S: Neonatal adaptive immunity comes of age. Nat. Rev. Immunol. 4, 553-564 (2004)
3. Devadas S, Zaritskaya L, Rhee SG, Oberley L, Williams MS: Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: selective regulation of mitogen-activated protein kinase activation and fas ligand expression. J. Exp. Med. 195, 59-70 (2002)
4. Duchen MR: Mitochondria and calcium: from cell signalling to cell death. J. Physiol. 529 Pt 1, 57-68 (2000)
5. Elahi S, Ertelt JM, Kinder JM, Jiang TT, Zhang X, Xin L, Chaturvedi V, Strong BS, Qualls JE, Steinbrecher KA, Kalfa TA, Shaaban AF, Way SS: Immunosuppressive CD71+ erythroid cells compromise neonatal host defence against infection. Nature 504, 158-162 (2013)
6. Feske S: Calcium signalling in lymphocyte activation and disease. Nat. Rev. Immunol. 7, 690-702 (2007)
7. García Vela JA, Delgado I, Bornstein R, Alvarez B, Auray MC, Martin I, Oña F, Gilsanz F: Comparative intracellular cytokine production by in vitro stimulated T lymphocytes from human umbilical cord blood (HUCB) and adult peripheral blood (APB). Anal. Cell. Pathol. 20, 93-98 (2000)
8. Hogan PG, Lewis RS, Rao A: Molecular basis of calcium signaling in lymphocytes: STIM and ORAI. Annu. Rev. Immunol. 28, 491-533 (2010)

9. Hoth M, Button DC, Lewis RS: Mitochondrial control of calcium-channel gating: a mechanism for sustained signaling and transcriptional activation in T lymphocytes. Proc. Natl. Acad. Sci. U.S.A. 97, 10607-10612 (2000)
10. Jackson SH, Devadas S, Kwon J, Pinto LA, Williams MS: T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation. Nat. Immunol. 5, 818-827 (2004)
11. Jullien P, Cron RQ, Dabbagh K, Cleary A, Chen L, Tran P, Stepick-Biek P, Lewis DB: Decreased CD154 expression by neonatal CD4+ T cells is due to limitations in both proximal and distal events of T cell activation. Int. Immunol. 15, 1461-1472 (2003)
12. Kalyanaraman B, Darley-Usmar V, Davies KJ, Dennery PA, Forman HJ, Grisham MB, Mann GE, Moore K, Roberts LJ, Ischiropoulos H: Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. Free Radic. Biol. Med. 52, 1-6 (2012)
13. Kaminski BA, Kadereit S, Miller RE, Leahy P, Stein KR, Topa DA, Radivoyevitch T, Veigl ML, Laughlin MJ: Reduced expression of NFAT-associated genes in UCB versus adult CD4+ T lymphocytes during primary stimulation. Blood 102, 4608-4617 (2003)
14. Kaposi AS, Toldi G, Mészáros G, Szalay B, Veress G, Vásárhelyi B (2012): Experimental Conditions and Mathematical Analysis of Kinetic Measurements Using Flow Cytometry – The FacsKin Method. In: Flow Cytometry - Recent Perspectives, ed Schmid I, InTech.
15. Kaposi AS, Veress G, Vásárhelyi B, Macardle P, Bailey S, Tulassay T, Treszl A: Cytometry-acquired calcium-flux data analysis in activated lymphocytes. Cytometry A 73, 246-253 (2008)
16. Kilpinen S, Hurme M: Low CD3+CD28-induced interleukin-2 production correlates with decreased reactive oxygen intermediate formation in neonatal T cells. Immunology 94, 167-172 (1998)
17. Mészáros G, Szalay B, Toldi G, Kaposi AS, Vásárhelyi B, Treszl A: Kinetic measurements using flow cytometry: new methods for monitoring intracellular processes. Assay Drug Dev. Technol. 10, 97-104 (2012)
18. Quintana A, Griesemer D, Schwarz EC, Hoth M: Calcium-dependent activation of T-lymphocytes. Pflugers Arch. 450, 1-12 (2005)
19. Quintana A, Schwindling C, Wenning AS, Becherer U, Rettig J, Schwarz EC, Hoth M: T cell activation requires mitochondrial translocation to the immunological synapse. Proc. Natl. Acad. Sci. U.S.A. 104, 14418-14423 (2007)
20. Rizzuto R, De Stefani D, Raffaello A, Mammucari C: Mitochondria as sensors and regulators of calcium signalling. Nat. Rev. Mol. Cell Biol. 13, 566-578 (2012)
21. Roederer M, Treister A, Moore W, Herzenberg LA: Probability binning comparison: a metric for quantitating univariate distribution differences. Cytometry 45, 37-46 (2001)
22. Sena LA, Li S, Jairaman A, Prakriya M, Ezponda T, Hildeman DA, Wang CR, Schumacker PT, Licht JD, Perlman H, Bryce PJ, Chandel NS: Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling. Immunity 38, 225-236 (2013)
23. Toldi G, Treszl A, Pongor V, Gyarmati B, Tulassay T, Vásárhelyi B: T-lymphocyte calcium influx characteristics and their modulation by Kv1.3 and IKCa1 channel inhibitors in the neonate. Int. Immunol. 22, 769-774 (2010)
24. van der Windt GJ, Everts B, Chang CH, Curtis JD, Freitas TC, Amiel E, Pearce EJ, Pearce EL: Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development. Immunity 36, 68-78 (2012)
25. Williams MS, Kwon J: T cell receptor stimulation, reactive oxygen species, and cell signaling. Free Radic. Biol. Med. 37, 1144-1151 (2004)

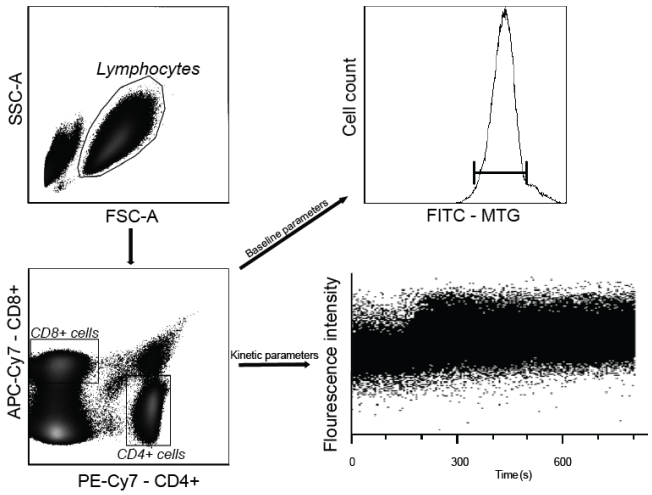

Figure 1. Staining and gating strategy. The population of lymphocytes was gated from PBMCs according to Forward Scatter Characteristics and Side Scatter Characteristics. The CD4+ or CD8+ subpopulations were differentiated according to the surface staining and used either for the measurement of baseline parameters (for example mitochondrial mass by MitoTracker Green (MTG)) or for kinetic measurements.

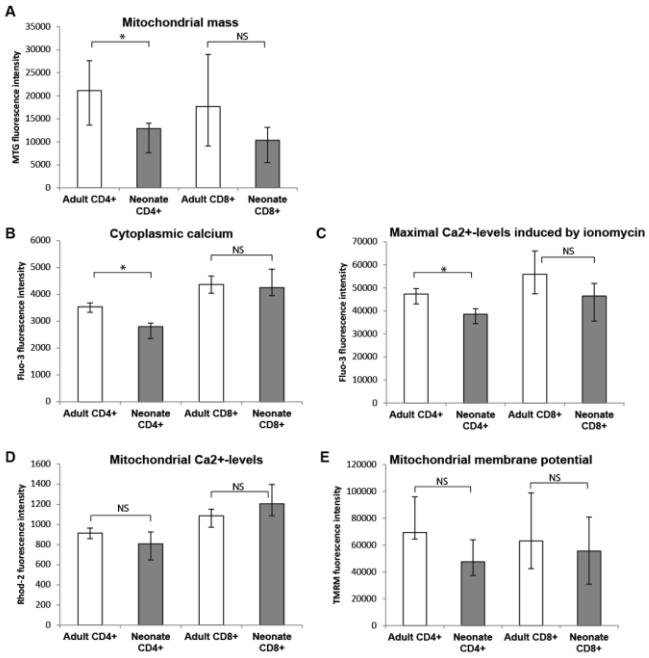

Figure 2. Comparison of resting T-cell parameters. Median fluorescence values of the mitochondrial specific fluorescence dye MitoTracker Green (MTG) **(**A); baseline and ionomycin induced cytoplasmic Ca^{2+}-levels measured by Fluo-3 (B and C respectively); mitochondrial Ca^{2+}-levels estimated by Rhod-2 (D); mitochondrial membrane potential measured by TMRM (E) in adult and neonate CD4+ or CD8+ T-cell subpopulations. Abbreviations: A-CD4+ - adult CD4+ T-cells; A-CD8+ - adult CD8+ T-cells; N-CD4+ - neonatal CD4+ T-cells, N-CD8+ - neonatal CD8+ T-cells. Data are expressed as median and interquartile range. * $p < 0.05$

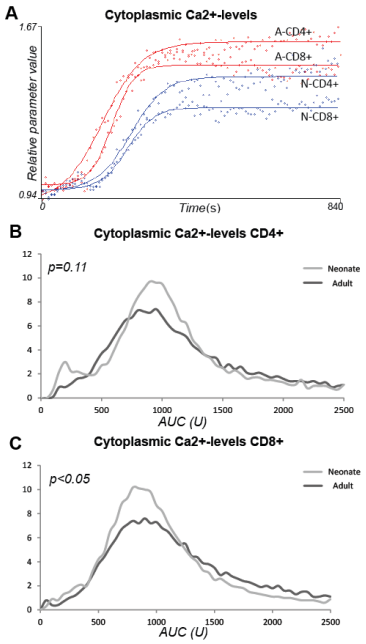

Figure 3. PHA induced kinetic changes in cytoplasmic Ca^{2+} levels. Figure shows the calculated median functions (A) (records of representative samples). Histograms showing the distributions of Area Under the Curve (AUC) parameter of fitted functions in CD4+ (B) and CD8+ (C) T-cells. Abbreviations: A-CD4+ - adult CD4+ T-cells; A-CD8+ - adult CD8+ T-cells; N-CD4+ - neonatal CD4+ T-cells, N-CD8+ - neonatal CD8+ T-cells.

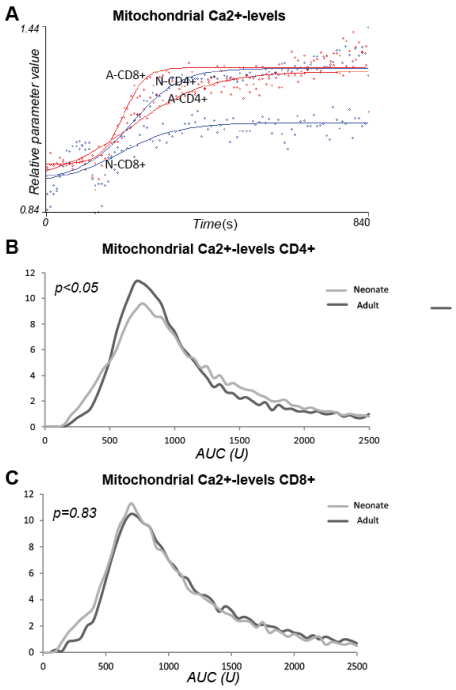

Figure 4. PHA induced kinetic changes in mitochondrial Ca^{2+} levels. Figure shows the calculated median functions (A) (records of representative samples). Histograms showing the distributions of Area Under the Curve (AUC) parameter of fitted functions in CD4+ (B) and CD8+ (C) T-cells. Abbreviations: A-CD4+ - adult CD4+ T-cells; A-CD8+ - adult CD8+ T-cells; N-CD4+ - neonatal CD4+ T-cells, N-CD8+ - neonatal CD8+ T-cells.

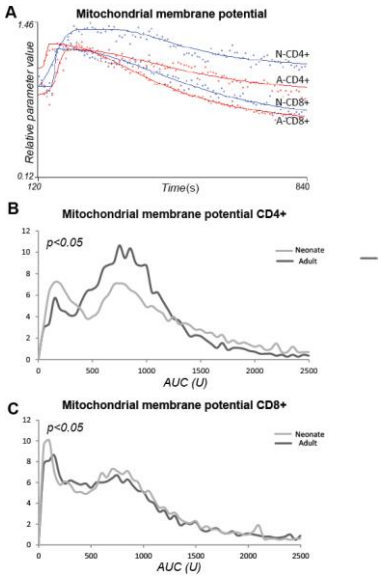

Figure 5. PHA induced kinetic changes in mitochondrial membrane potential. Figure shows the calculated median functions (A) (records of representative samples). Histograms showing the distributions of Area Under the Curve (AUC) parameter of fitted functions in CD4+ (B) and CD8+ (C) T-cells. Abbreviations: A-CD4+ - adult CD4+ T-cells; A-CD8+ - adult CD8+ T-cells; N-CD4+ - neonatal CD4+ T-cells, N-CD8+ - neonatal CD8+ T-cells.

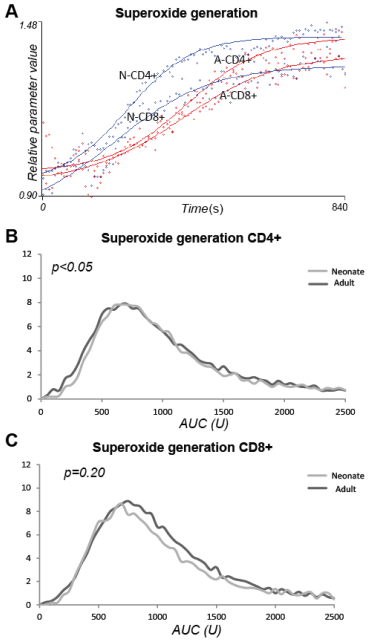

Figure 6. PHA induced kinetic changes in superoxide generation. Figure shows the calculated median functions (A) (records of representative samples). Histograms showing the distributions of Area Under the Curve (AUC) parameter of fitted functions in CD4+ (B) and CD8+ (C) T-cells. Abbreviations: A-CD4+ - adult CD4+ T-cells; A-CD8+ - adult CD8+ T-cells; N-CD4+ - neonatal CD4+ T-cells, N-CD8+ - neonatal CD8+ T-cells.

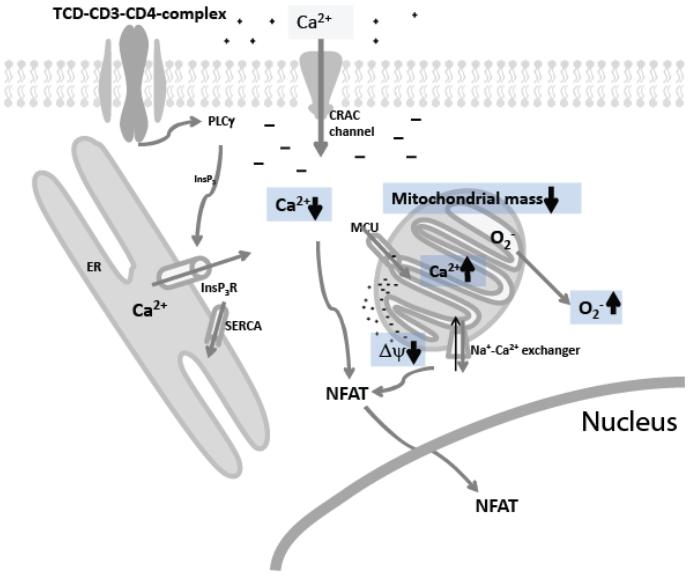

Figure 7 Schematic representation of neonatal CD4+ T-cell activation. Schematic representation of CD4+ T-cell activation showing the parameters altered in neonate compared to adult. In addition to reduced cytoplasmic Ca^{2+} signaling the mitochondrial mass is decreased in neonatal CD4+ T-cells. Decreased mitochondrial mass is responsible for the elevated mitochondrial Ca^{2+} sequestration and mitochondrial depolarization which contribute to the elevated O_2^- production. All these changes might contribute to reduced NFAT-associated gene expression in neonatal CD4+ T-cells.

	Number	**Gender (F/M)**	**Birth weight**	**Gestational age (neonate) or age**	**Ethnicity**
Neonates	12	10/2	3375 (2510–4040) grams	39 (38–40) weeks	Caucasian (Hungarian)
Adults	11	3/8	N.A.	25 (22–44) years	Caucasian (Hungarian)

Table 1. Clinical data and patient characteristics. Data are expressed as median (range).

Parameter	T-cell subpopulation			
	Adult CD4+ (n=10)	**Neonate CD4+** (n=11)	**Adult CD8+** (n=10)	**Neonate CD8+** (n=11)
Cytoplasmic Ca^{2+}-levels (CD4+-double logistic+ function; CD8+ logistic+ function)				
AUC	1193 [799-2263]	1059 [789-1760]	1155 [791-1881]	982 [b] [726-1445]
Slope	0.0046 [0.0016-0.0177]	0.0030 [0.0014-0.0136]	0.0022 [0.0010-0.0080]	0.0015 [b] [0.0006-0.0075]
Max or End	1.62 [1.06-3.26]	1.39 [1.02-2.53]	1.62 [1.16-2.58]	1.32 [0.96-2.01]
Mitochondrial Ca^{2+}-levels (logistic+ function)				
AUC	939 [691-1541]	989 [a] [693-1582]	960 [687-1497]	899 [645-1577]
Slope	0.0009 [0.0004-0.0041]	0.0018 [a] [0.0009-0.0043]	0.0008 [0.0004-0.0060]	0.0024 [b] [0.0007-0.0260]
End	1.37 [1.02-2.19]	1.46 [a] [0.94-2.43]	1.46 [1.04-2.11]	1.19 [b] [0.85-1.79]
Mitochondrial membrane potential (double logistic+ function)				
AUC	797 [518-1101]	880 [a] [465-1507]	773 [558-1415]	767 [b] [353-1270]
Slope	0.0245 [0.0029-0.2203]	0.0061 [0.0010-0.0255]	0.0326 [0.0037-0.1707]	0.0289 [b] [0.0036-0.1460]
Max	1.15 [0.78-1.53]	1.27 [a] [0.69-2.13]	1.13 [0.52-2.01]	1.10 [b] [0.60-1.77]
Superoxide generation (O_2^-) (logistic+ function)				
AUC	1001 [634-1909]	1058 [a] [676-2412]	955 [652-1474]	1008 [656-1960]
Slope	0.0010 [0.0006-0.0023]	0.0022 [0.0007-0.0082]	0.0008 [0.0005-0.0013]	0.0029 [0.0008-0.0175]
End	1.51 [0.99-2.89]	1.49 [0.96-3.68]	1.36 [0.94-2.06]	1.39 [0.89-2.94]

Table 2. Effects of PHA stimulation on cord blood and adult CD4+ and CD8+ T cell populations. The measured parameter values of logistic+ and double logistic+ functions were AUC (area under the curve in units [U]), Max (double logistic+ function - maximum value in relative parameter value [rpv]), Slope (slope at the first 50% value of Max), and End (logistic+ function - ending value). Data are expressed as median and interquartile range.

[a] Neonatal cord blood samples were compared with adult samples (CD4+ T-cell population), $p < 0.05$.

[b] Neonatal cord blood samples were compared with adult samples (CD8+ T-cell population), $p < 0.05$.

AIM 3: Characterization of activation properties of Th1, Th2, Treg and Th17 cells in immune mediated disorders

Within this aim we analyzed lymphocyte activation characteristics in specific Th1 and Th2 mediated disorders and conditions: rheumatoid arthritis, stroke and pregnancy

RHEUMATOID ARTHRITIS:

Bajnok A, Kaposi A, Kovács L, Vásárhelyi B, Balog A, Toldi G.: Analysis by flow cytometry of calcium influx kinetics in peripheral lymphocytes of patients with rheumatoid arthritis., Cytometry A, 2013 Mar;83(3):287-93.

The transient increase of the cytoplasmic free calcium level in T lymphocytes plays a key role in initiating and maintaining the autoimmune reaction in rheumatoid arthritis (RA). Kv1.3 and IKCa1 potassium channels are important regulators of the maintenance of calcium influx during lymphocyte activation and present a possible target for selective immunomodulation. We aimed to compare peripheral T lymphocyte calcium influx kinetics upon activation in patients with recently diagnosed and established RA, and to demonstrate the differences in analysis of kinetic flow cytometry data when using two different algorithms. We took peripheral blood samples from nine patients with recently diagnosed and six patients with established RA. We evaluated calcium influx kinetics following activation in CD4, Th1, Th2, and CD8 cells applying an approach based on smoothing of median fluorescence values (FlowJo) and an algorithm based on function fitting (FacsKin). We assessed the sensitivity of the above subsets to specific inhibition of the Kv1.3 and IKCa1 potassium channels. Th2 cells of patients with established RA react slower to activating stimuli, whereas CD8 cells show a faster reaction than in patients with recently diagnosed RA. While initially Th1 cells are less sensitive to the inhibition of Kv1.3 and IKCa1 channels in RA, their sensitivity increases along with the duration of the disease. With the algorithm of function fitting instead of smoothing, more statistically significant differences of potassium channel inhibition between the two RA groups could be demonstrated. The function fitting algorithm applied by FacsKin is suitable to provide a common basis for evaluating and comparing flow cytometry kinetic data.

Szalay B; Vásárhelyi B; Cseh A; Tulassay T; Deák M; Kovács L; Balog A: The impact of conventional DMARD and biological therapies on CD4+ cell subsets in rheumatoid arthritis: a follow-up study., Clin Rheumatol. 2014 Feb;33(2):175-85., 2014

Rheumatoid arthritis (RA) is an autoimmune disease characterized by abnormal prevalence of Th1, Th2, Th17, and regulatory (Treg) subsets. Some data suggest that these subsets are influenced by anti-RA agents. Follow-up studies monitoring T cell phenotype in response to therapy are limited. We investigated the alteration of CD4+ T cell subset distribution after the initiation of disease-modifying antirheumatic drug (DMARD) (with glucocorticosteroid (GCS) and methotrexate (MTX)) and anti-TNFα therapy. We enrolled 19 treatment naive (early) RA patients and initiated GCS (in a dose of 16 mg/day for 4 weeks; then 8 mg/day). MTX, 10 mg/week, was started at week 4. We also enrolled 32 RA patients unresponsive toDMARD and initiated anti-TNFα therapy: adalimumab (ADA), 40 mg/2 weeks, n = 12; etanercept (ETA), 50 mg/weeks, n = 12; or infliximab (IFX) on week 0, 2, and 6, 3 mg/kg bw, n = 8. Blood was taken before and 4 and 8 weeks after the initiation of therapy. Ten volunteers served as controls. The T cell phenotype was assessed with flow cytometry. In early RA, Th1, Th2, and Th17 prevalence was higher, while Treg prevalence was lower than normal. GCS alone decreased Th2 prevalence. GCS + MTX decreased Th17 prevalence. Immune phenotype in unresponsive RA before anti-TNF therapy was as in early RA. Four and 8 weeks after initiating anti-TNF therapy, Th1 prevalence was higher than baseline in ETA or IFX, while it was stable in ADA groups. Th2 prevalence was higher than normal in ADA or IFX, while normalized in ETA group. In each group, Treg prevalence increased, while Th17 prevalence was at the baseline. The

proinflammatory immune phenotype is normalized only under GCS + MTX combination in early RA. Anti-TNFα therapy exhibit marked effects on all the cell populations investigated (except Th17); some slight differences in this action exist between ADA, ETA, and IFX therapy.

Szalay B; Cseh A; Mészáros G; Kovács L; Balog A; Vásárhelyi B.: The impact of DMARD and anti-TNF therapy on functional characterization of short-term T-cell activation in patients with rheumatoid arthritis--a follow-up study Plos One 2014 Aug 6;9(8):e104298.

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by a systemic dysfunction of T-cells. In this study we tested the impact ofDMARD and anti-TNF agents on short-term activation characteristics of T-cells. We enrolled 12 patients with newly diagnosed RA (naïve RA) who were treated with methothrexate (MTX) and glucocorticsteroid (GCS) and 22 patients with established RA non responding to conventional DMARDtherapy who were treated with different anti-TNF agents. Nine healthy volunteers served as controls. Blood samples were taken at baseline, then at 4th and 8th week of therapy. The characteristics of several intracellular activation processes during short-term activation of T-cells including cytoplasmic Ca(2+) level, mitochondrial Ca(2+) level, reactive oxygen species (ROS) and nitric oxide (NO) generation were determined by a novel flow-cytometry technique. At baseline, the tested processes were comparable to controls in naïve RA. During GCS therapy, cytoplasmic Ca(2+) level and ROS generation decreased. After the addition of MTX to GCS cytoplasmic Ca(2+) level became comparable to controls, while ROS generation decreased further. In DMARD non responders, cytoplasmic Ca(2+) level was higher than controls at baseline. The cytoplasmic Ca(2+) level became comparable to controls and ROS generation decreased during each of the three anti-TNF-α agent therapies. Mitochondrial Ca(2+) level and NO generation were unaltered in all of the patient groups. These results indicate that intracellular machinery is affected in T-cells of RA patients. This may alter the behavior of T-cells during activation. Different therapeutic approaches may modulate the abnormal T-cell functions.

PREGNANCY:

Toldi G, Saito S, Shima T, Halmos A, Veresh Z, Vásárhelyi B, Rigó J Jr, Molvarec A.: The frequency of peripheral blood CD4+ CD25high FoxP3+ and CD4+ CD25- FoxP3+ regulatory T cells in normal pregnancy and pre-eclampsia., Am J Reprod Immunol, 2012 Aug;68(2):175-80.

Regulatory T cells (Tregs) play an important role in the development of pregnancy-specific immune tolerance. We aimed to determine the peripheral frequency of a recently described Treg subpopulation, the CD4+ CD25- FoxP3+ Treg subset, and its correlation with the conventional CD4+ CD25high FoxP3+ Tregs in normal pregnancy (NP) and pre-eclampsia (PE) compared to non-pregnant (non-P) women. We also examined the proportion of the activated CD4+ CD25high FoxP3high Treg subset within conventional Treg cells. We took peripheral blood samples from 20 PE, 20 NP, and 12 non-P women and determined the frequency of the above Treg subsets using flow cytometry. The frequency of conventional CD4+ CD25high FoxP3+ Tregs and activated CD4+ CD25high FoxP3high Tregs, but also that of non-conventional CD4+ CD25- FoxP3+ Tregs was higher in NP compared to non-P women, but lower again in PE, reaching comparable levels to the non-P group. The ratios of CD4+ CD25high FoxP3+ and CD4+ CD25- FoxP3+ Treg subsets were constant in all three investigated groups. Our results indicate that the frequency of conventional and non-conventional Tregs alters simultaneously, and the presence in circulation of both of these Treg subsets is similarly important in the adequate development of pregnancy-specific immune tolerance.

STROKE

Folyovich A, Biro E, Orban C, Bajnok A, Vasarhelyi B, Toldi G: Kv1.3 lymphocyte potassium channel inhibition as a potential novel therapeutic target in acute ischemic stroke., CNS Neurol Disord Drug Targets., 2014;13(5):801-6.

Stroke-induced immunosuppression (SIIS) leads to severe complications in stroke patients, including an increased risk of infections. However, functional alterations of T lymphocytes during SIIS are poorly described in acute ischemic stroke (AIS). We aimed to characterize Ca(2+) influx kinetics in major lymphocyte subsets (CD4, Th1, Th2, CD8) in AIS patients without infection 6 hours and one week after the CNS insult. We also assessed the sensitivity of the above subsets to specific inhibition of the Kv1.3 and IKCa1 lymphocyte K(+) channels. We took peripheral blood samples from 12 non-stroke individuals and 12 AIS patients. We used an innovative flow cytometry approach to determine Ca(2+) influx kinetics and the surface expression of Kv1.3 channels. Our results indicate that Ca(2+) influx kinetics is altered in the Th2 and CD8 subsets in AIS which may play a role in the development of SIIS. Specific inhibition of Kv1.3 channels selectively decreased Ca(2+) influx in the CD8 and Th2 subsets of AIS patients. The surface expression of Kv1.3 channels is also altered compared to non-stroke individuals. Kv1.3 channel inhibition might have beneficialtherapeutic consequences in AIS, selectively targeting two distinct T cell subsets at two different time points following the CNS insult. Within hours after the insult, it might prevent excessive tissue injury through the inhibition of CD8 cells, while at one week after the insult, it may improve the inflammatory response through the inhibition of Th2 cells, thus reducing the unwanted clinical consequences of SIIS.

ADDITIONAL ACHIEVEMENTS

Using the technical developments and/or biobanks established during our research on AIMS1-3 we performed several additional substudies.

These include the

- analysis of clinical utility of suPAR, a novel inflammatory marker
- investigation of immune modulatory cell surface receptors in different conditions.

I. Analysis of clinical utility of suPAR, a novel inflammatory marker in Th1 & Th2 dominant disorders

RHEUMATOID ARTHRITIS

Toldi G, Bekó G, Kádár G, Mácsai E, Kovács L, Vásárhelyi B, Balog A.: Soluble urokinase plasminogen activator receptor (suPAR) in the assessment of inflammatory activity of rheumatoid arthritis patients in remission., Clin Chem Lab Med., 2013 Feb;51(2):327-32.

Soluble urokinase plasminogen activator receptor (suPAR) is a biomarker increasingly used for the assessment of systemic inflammation. We aimed to evaluate suPAR for the assessment of inflammatory activity in rheumatoid arthritis (RA) patients in remission.
In our cross-sectional study we measured plasma suPAR and C-reactive protein (CRP) levels as well as erythrocyte sedimentation rate (ESR) in 120 RA patients at various stages of disease activity and 29 healthy age-matched controls.
suPAR, CRP and ESR values were higher in RA patients compared to healthy individuals. When suPAR levels were analyzed according to DAS28 scores of RA patients, suPAR level in the subgroup with DAS28≤2.6 was lower than in the subgroup with DAS28>2.6, but still higher than in controls [4.45 (3.33-5.56) ng/mL vs. 3.66 (3.10-4.67) ng/mL vs. 2.80 (2.06-3.42) ng/mL, $p<0.0001$, median (interquartile range)]. In contrast, CRP and ESR values were comparable in the subgroup with DAS28≤2.6 and in healthy individuals. We further analyzed the correlation between the number of tender and/or swollen joints and suPAR levels in RA patients in remission. suPAR values were significantly higher in patients with four tender and/or swollen joints than in patients with 2-3 or 0-1 tender and/or swollen joints.
While CRP and ESR values indicate remission of the chronic inflammatory process in RA, suPAR values are still elevated compared to healthy individuals. suPAR might be particularly valuable in the recognition of inflammatory activity in patients who are in remission according to DAS28 scores but have symptoms of tender and/or swollen joints.

BECHTEREW'S DISEASE

Toldi G, Szalay B, Bekő G, Kovács L, Vásárhelyi B, Balog A.: Plasma soluble urokinase plasminogen activator receptor (suPAR) levels in ankylosing spondylitis., Joint Bone Spine, 2013 Jan;80(1):96-8.

Recent studies demonstrated that soluble urokinase plasminogen activator receptor (suPAR) is a valuable marker in the recognition of an inflammatory response. Ongoing inflammation leads to elevated plasma suPAR levels. We aimed to characterize plasma suPAR levels in ankylosing spondylitis (AS) patients compared to healthy individuals in order to reveal if suPAR could be used as a clinical marker of inflammation in AS. We measured plasma suPAR and C-reactive protein (CRP) levels as well as erythrocyte sedimentation rate (ESR) in 33 AS patients at various stages of disease duration and activity and 29 healthy controls. CRP and ESR values were higher in AS patients than in

healthy individuals, while suPAR values were comparable (median [interquartile range]: 2.97 [2.57-3.80] ng/mL vs. 2.80 [2.06-3.42] ng/mL, P>0.05). In AS patients, a correlation was detected between BASDAI scores and CRP as well as ESR values but not suPAR levels (P=0.0005, r=0.57 and P=0.01, r=0.43, respectively). Unlike in many other inflammatory conditions, plasma suPAR levels do not reflect inflammation in AS. To assess the inflammatory status in AS, ESR and particularly CRP values are still more appropriate clinical markers. In line with earlier findings, our results indicate that, unlike suPAR, both of these markers are positively correlated with disease activity in AS.

ASTHMA IN PREGNANCY

Ivancsó I; Toldi G; Bohács A; Eszes N; Müller V; Rigó J Jr; Vásárhelyi B; Losonczy G; Tamási L: Relationship of circulating soluble urokinase plasminogen activator receptor (suPAR) levels to disease control in asthma and asthmatic pregnancy., PLoS One. 2013;8(4):e60697.

Asthma has a high burden of morbidity if not controlled and may frequently complicate pregnancy, posing a risk for pregnancy outcomes. Elevated plasma level of the inflammatory biomarker soluble urokinase plasminogen activator receptor (suPAR) is related to a worse prognosis in many conditions such as infectious, autoimmune, or pregnancy-related diseases; however the value of suPAR in asthma and asthmatic pregnancy is unknown. The present study aimed to investigate the suPAR, CRP and IL-6 levels in asthma (asthmatic non-pregnant, ANP; N=38; female N=27) and asthmatic pregnancy (AP; N=15), compared to healthy non-pregnant controls (HNP; N=29; female N=19) and to healthy pregnant women (HP; N=58). The relationship between suPAR levels and asthma control was also evaluated. The diagnostic efficacy of suPAR in asthma control was analyzed using ROC analysis. IL-6 and CRP levels were comparable in all study groups. Circulating suPAR levels were lower in HP and AP than in HNP and ANP subjects, respectively (2.01 [1.81-2.38] and 2.39 [2.07-2.69] vs. 2.60 [1.82-3.49] and 2.84 [2.33-3.72] ng/mL, respectively, p=0.0001). suPAR and airway resistance correlated in ANP (r=0.47, p=0.004). ROC analysis of suPAR values in ANP patients with PEF above and below 80% yielded an AUC of 0.75 (95% CI: 0.57-0.92, p=0.023) and with ACT total score above and below 20 an AUC of 0.80 (95% CI: 0.64-0.95, p=0.006). The cut-off value of suPAR to discriminate between controlled and not controlled AP and ANP was 4.04 ng/mL. In conclusion, suPAR may help the objective assessment of asthma control, since it correlates with airway resistance and has good sensitivity in the detection of impaired asthma control. Decrease in circulating suPAR levels detected both in healthy and asthmatic pregnant women presumably represents pregnancy induced immune tolerance.

II. Investigation of immune modulatory cell surface receptors in different conditions.

NEONATE

Kollár S, Sándor N, Molvarec A, Stenczer B, Rigó J Jr, Tulassay T, Vásárhelyi B, Toldi G.: Prevalence of intracellular galectin-1-expressing lymphocytes in umbilical cord blood in comparison with adult peripheral blood., Biol Blood Marrow Transplant., 2012 Oct;18(10):1608-13

Umbilical cord blood (UCB) is a promising alternative for the treatment of hematological malignancies. The lower immune reactivity of UCB lymphocytes is a well-known phenomenon; however, immune tolerance mechanisms are not fully elucidated. Galectin-1 has strong immunosuppressive properties and plays a key role in the regulation of immune reactivity. We aimed to determine the properties of intracellular galectin-1 (Gal-1)-producing cells within CD3, CD4, CD8, regulatory T (Treg), and natural killer (NK) cells in UCB compared to adult peripheral blood (APB). We took peripheral blood samples from 22 healthy adults and cord blood samples from 19 healthy, term neonates. Intracellular Gal-1 expression was determined by flow cytometry in the above subsets. Furthermore, we assessed the prevalence of naive and memory T cells that play a role in the regulation of immune reactivity. We also performed functional analyses to assess the effect of exogenous Gal-1 on the rate of proliferation of T lymphocytes isolated from APB and UCB. The prevalence of intracellular Gal-1-expressing CD3, CD4, CD8, Treg and NK lymphocytes was lower in UCB than in APB. However, their capability to produce Gal-1 reaches the level seen in adults. The prevalence of naive cells was higher, whereas that of central and effector memory T cells was lower in UCB compared with APB. Lower Gal-1-producing cell proportion might be due to the naivety of neonatal lymphocytes, as indicated by the positive correlation detected between the number of CD3 lymphocytes expressing intracellular Gal-1 and the prevalence of memory T cells. The intracellular expression of Gal-1 may be down-regulated in neonatal lymphocytes due to the already reduced immune reactivity of UCB. In contrast with previous findings, our results indicate that the administration of exogenous Gal-1 failed to decrease the rate of proliferation in T lymphocytes isolated from either APB or UCB. This suggests that Gal-1-expressing lymphocytes are unlikely to play a major role in mitigating the immune reactivity of UCB.

PREGNANCY

Toldi G; Vásárhelyi B; Biró E; Fügedi G; Rigó J Jr; Molvarec A.: B7 costimulation and intracellular indoleamine-2,3-dioxygenase expression in peripheral blood of healthy pregnant and pre-eclamptic women, Am J Reprod Immunol. 2013 Mar;69(3):264-71, 2013

We determined the frequency of activated (CD11b+) monocytes expressing B7-1, B7-2, B7-H1, and B-7H2, and that of T cells and T helper cells expressing CD28, CTLA-4, PD-1, and ICOS in peripheral blood samples from normal pregnant (NP) and pre-eclamptic (PE) women. We also examined the intracellular expression of indoleamine-2,3-dioxygenase (IDO). We measured the expression of the above markers using flow-cytometry in peripheral blood samples from 20 NP and 20 PE women in the third trimester. The frequency of B7-1 and B7-2 expressing activated monocytes and that of IDO expressing T-lymphocytes was lower in PE than in NP.Lower expression of B7-1 and B7-2 proteins on peripheral monocytes in PE might indicate a secondary regulatory mechanism in response to the ongoing systemic maternal inflammation. IDO plays an important role in the pregnancy-specific immune tolerance, and might be a contributing factor in the pathogenesis of PE.

STROKE

Folyovich A; Biró E; Orbán C; Bajnok A; Varga V; Béres-Molnár AK; Vásárhelyi B; Toldi G: Relevance of novel inflammatory markers in stroke-induced immunosuppression., BMC Neurol. 2014 Mar 6;14:41.

Acute ischemic stroke (AIS) has a biphasic effect on the peripheral immune system. The initial inflammatory response is followed by systemic immunosuppression, referred to as stroke-induced immunosuppression (SIIS), leading to severe complications in stroke patients. We aimed to identify an inflammatory marker that best represents this biphasic immunological response after AIS. We investigated the alteration of CRP, WBC, neutrophil count, suPAR levels, CD4+ CD25high Tregs, CD64+ and CD177+ neutrophils and monocytes in 12 acute ischemic stroke patients free of infection within 6 hours and one week after the insult. As controls, 14 age-matched healthy individuals were included. CRP, WBC and neutrophil count values were comparable in stroke patients within 6 hours and controls, however, they were elevated in stroke one week after the insult. suPAR levels were higher in both stroke groups compared to controls. The prevalence of CD64+ neutrophils was higher in stroke patients within 6 hours than in controls and it decreased in stroke one week after the insult below the level in controls (5.95 [5.41-8.75] % vs. 32.38 [9.21-43.93] % vs. 4.06 [1.73-6.77] %, $p < 0.05$). Our pilot study identified that the prevalence of CD64+ neutrophils may reflect a biphasic alteration of the immune response following AIS. Since its level decreases below baseline after one week of the CNS insult in stroke patients without infection, it might serve as a reliable candidate to identify the developing inflammatory response due to infection after stroke in the future.