

**In this issue: FLOW CYTOMETRY IN THE CLINICAL LABORATORY**

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**CALCIUM INFLUX CHARACTERISTICS DURING T LYMPHOCYTE ACTIVATION MEASURED WITH FLOW CYTOMETRY**

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**ABSTRACT**

T lymphocytes are of paramount importance in many intercellular reactions, such as the regulation of the inflammatory response and immune reactivity. Until the recent past, single-cell techniques were used for the investigation of calcium influx during T lymphocyte activation. Therefore, over the recent years we have created a novel approach that allows simultaneous recording of calcium influx in several lymphocyte subsets using flow cytometry. Our research group developed a robust algorithm (FacsKin) for the evaluation of the acquired data that fits functions to median values of the fluorescent marker of interest and calculates relevant parameters describing each function.

Over the recent years, we have investigated calcium influx characteristics applying this method in a number of autoimmune disorders and under different physiological conditions (such as the neonatal period and pregnancy). In this review, we aim to give a brief summary of our findings and of the common characteristics of calcium influx in the investigated disorders, namely: multiple sclerosis (MS), rheumatoid arthritis (RA), type 1 diabetes mellitus (T1DM), ankylosing spondylitis (AS), and preeclampsia (PE). Based on our results, a number of dominant features were identified that were present in most of the investigated autoimmune diseases.

**INTRODUCTION**

T lymphocytes are of paramount importance in many intercellular reactions, such as the regulation of the inflammatory response and immune reactivity. Upon the engagement of the T cell receptor (TCR), a number of signal transduction pathways culminate in the transient elevation of the cytoplasmic calcium concentration ([Ca<sup>2+</sup>]<sub>cyt</sub>). First, Ca<sup>2+</sup> is released from intracellular stores that is followed by further Ca<sup>2+</sup> entry from the extracellular space through the store-operated calcium release activated calcium (CRAC) channels. In the course of lymphocyte activation, K<sup>+</sup> channels maintain the driving force for sustained Ca<sup>2+</sup> influx as they grant the efflux of K<sup>+</sup> from the cytoplasm, thus conserving an electrochemical potential gradient between the intra- and extracellular spaces. There are two major types of K<sup>+</sup> channels in T cells: the voltage-gated Kv1.3 and the Ca<sup>2+</sup>-activated IKCa1 channels. The relation between the Ca<sup>2+</sup> currents through CRAC channels and the efflux of K<sup>+</sup> makes the proliferation and activation of lymphocytes sensitive to pharmacological modulation of Kv1.3 and IKCa1 channels, and provides an opportunity for targeted intervention. Specific inhibition of these channels results in a diminished Ca<sup>2+</sup> influx in lymphocytes and a lower level of lymphocyte activation.

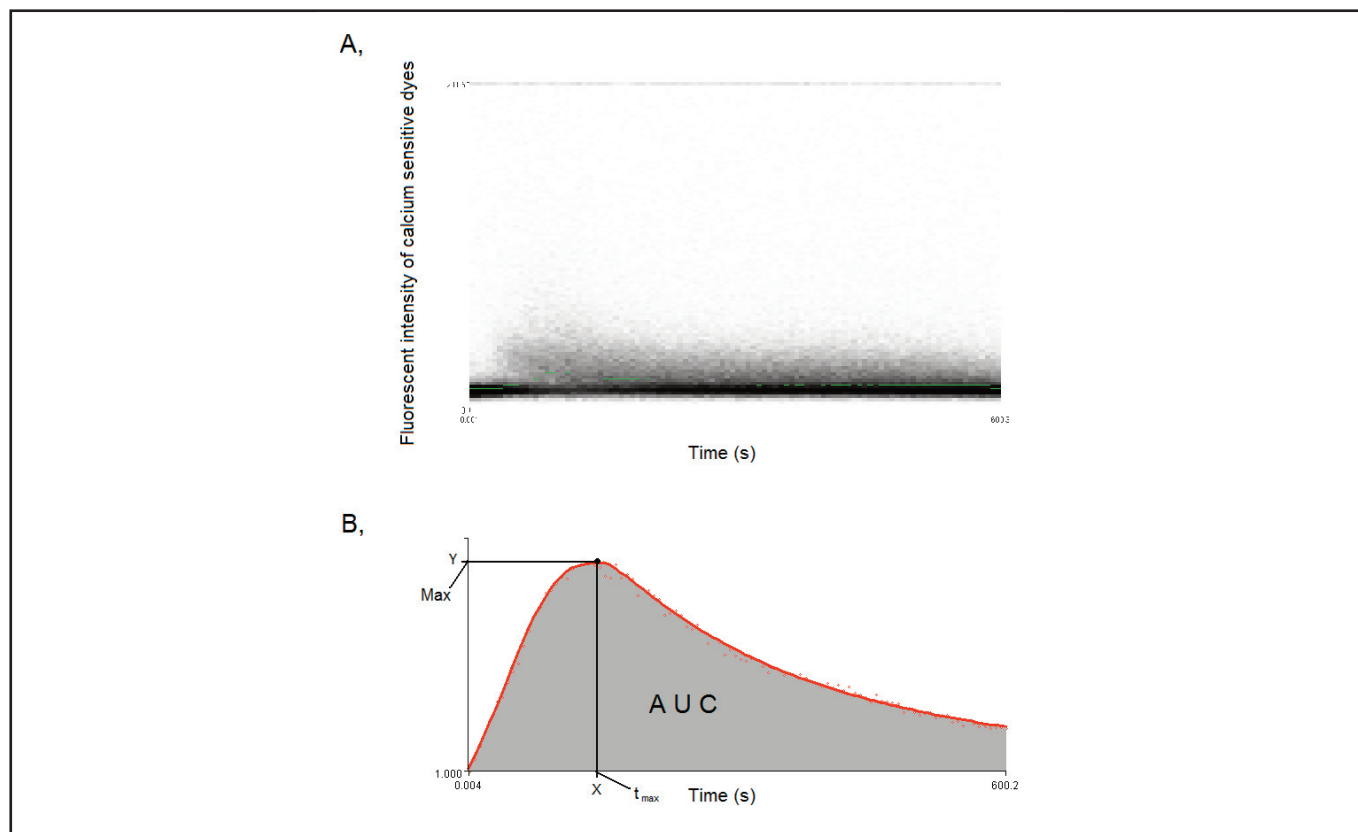
Previous data suggest that selective modulation of lymphocyte activation through specific inhibition of K<sup>+</sup> channels may be a possible therapeutic approach for the treatment of autoimmune disease [1-6]. Beeton et al. showed that terminally differentiated effector memory T (TEM) cells play a pivotal role in the pathogenesis of autoimmunity [1]. For instance, Wulff et al. suggest that disease causing TEM cells are able to home to inflamed tissues in the CNS and exhibit immediate effector function in autoimmune disease. They described that the characteristic K<sup>+</sup> channel phenotype of TEM cells in MS is Kv1.3<sup>high</sup> IKCa1<sup>low</sup>, contrasting naïve and central memory T (TCM) cells, which exhibit a Kv1.3<sup>low</sup> IKCa1<sup>high</sup> channel phenotype [3]. Therefore the therapeutic relevance of specific Kv1.3 channel inhibitors is of outstanding interest, as they may offer the possibility for selective modulation of pathogenic TEM cells, while naïve and TCM cells (needed for physiological immune responses) would escape the inhibition through upregulation of IKCa1 channel expression. Beeton et al. demonstrated that the symptoms of experimental autoimmune encephalitis, a murine model of MS, significantly improved after treatment with selective Kv1.3 inhibitors [1, 4]. Besides naïve and memory cells, helper (CD4) and effector (CD8) T lymphocytes modulate the autoimmune response in different ways. CD4 cells influence the immune system by producing cytokines, while CD8 cells are capable of causing immediate cell destruction. The two main arms of CD4 lymphocytes are Th1 and Th2 cells. Th1 cells mainly produce pro-inflammatory cytokines, thus sustaining autoimmune reactions. However, Th2 cells reduce the inflammatory response by producing anti-inflammatory cytokines. Until the recent past, single-cell techniques were used for the investigation of Ca<sup>2+</sup> influx during lymphocyte activation. There has been no high-throughput method available to study the kinetics of lymphocyte activation in more subsets simultaneously. Single-cell techniques are restricted by not being capable of characterizing this process in complex cellular systems, therefore ignoring the interaction between the different lymphocyte subsets that may modulate the course of their activation. Therefore, over the recent years we have created a novel approach that allows simultaneous recording of Ca<sup>2+</sup> influx in several lymphocyte subsets. Our research group developed a robust algorithm (FacsKin) that fits functions to median values of the data of interest and calculates relevant parameters describing each function. By selecting the best fitting function, this approach provides an opportunity for the mathematical analysis and statistical comparison of kinetic flow cytometry measurements of distinct samples. For example, in case of Ca<sup>2+</sup> influx measurements, the software fits a double-logistic function on each recording. This function is used to describe measurements that have an increasing and a decreasing intensity as time passes. The software also calculates parameter values describing each function, such as the Maximum value, the Time to reach maximum value or the Area Under the Curve (AUC). These parameters represent different characteristics of lymphocyte Ca<sup>2+</sup> influx kinetics (Figure 1). Details of the method of measurements were described earlier [7, 8]. Briefly, peripheral blood mononuclear cells (PBMCs) were separated from freshly drawn peripheral venous blood of the investigated subjects. Cell surface markers were applied to separate the lymphocyte subsets of interest. Cells were loaded with Ca<sup>2+</sup> sensitive dyes and activated with aspecific stimuli. Cell fluorescence data were measured and recorded for 10 minutes in a kinetic manner on flow cytometer. Our studies were adhered to the tenets of the most recent revision of the Declaration of Helsinki. Over the recent years, we have investigated Ca<sup>2+</sup> influx characteristics in a number of autoimmune disorders and under different physiological conditions. In this review, we aim to give a brief summary of our findings and of the common characteristics of Ca<sup>2+</sup> influx in autoimmune disease.

## MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is an autoimmune disease affecting the central nervous system. The autoimmune reaction primarily destroys the myelin sheath covering the nerve cells. T lymphocytes play a key role in the pathogenesis of MS. They regulate the ongoing inflammatory process of the central nervous system (CNS) which leads to the damage of the myelin sheath and axons. However, only a small part of T lymphocytes are myelin-specific autoreactive cells. Besides the demyelinating action of these cells of the CNS, the activation of peripheral lymphocytes also contributes to the pathogenesis and disease progression [9]. In our investigations we measured samples of healthy individuals and MS patients with no immunomodulatory therapy, as well as MS patients treated with interferon beta (IFN- $\beta$ ), currently regarded as the most effective therapy of MS.

In our study we discovered increased sensitivity of CD8 cells to Kv1.3 channel inhibition in MS. Therefore, from the CD4-CD8 point of view, we demonstrated specific immunomodulation that may be beneficial in the therapy of MS via the selective suppression of CD8 effector lymphocytes over CD4 helper cells. However, this specificity is not present within the CD4 subset, since our results suggest that Th1 and Th2 cells are similarly suppressed upon the inhibition of Kv1.3 channels. Since the cytokine balance is of utmost importance in the regulation of the autoimmune reaction, the inhibition of the Th2 subset would probably result in a setback of therapeutic efforts in MS. Our findings are relevant in the light of observations regarding the contribution of TEM cells to the development of MS, as described above. Although the Kv1.3<sup>high</sup> IKCa1<sup>low</sup> pattern is found in both CD4<sup>+</sup> and CD8<sup>+</sup> TEM cells, we can assume that the majority of TEM cells are CD8<sup>+</sup>, since TEM cells express immediate effector function. This provides an explanation for the increased sensitivity of CD8 cells to Kv1.3 channel inhibition in MS in our study.

We have also demonstrated important differences in Ca<sup>2+</sup> influx kinetics and lymphocyte K<sup>+</sup> channel function in MS patients without IFN- $\beta$  compared with healthy individuals. The selective blocker of the lymphocytes Kv1.3 channel might be a promising drug in combination therapy, supplementing the presently used IFN- $\beta$  treatment. Our results indicate that IFN- $\beta$  therapy causes



**Figure 1**

A: Dot-plot of a kinetic flow cytometry recording, where each dot represents one cell of the measured sample. Horizontal axis: Time (in seconds), Vertical axis: Fluorescent intensity of Ca<sup>2+</sup> sensitive dyes (relative parameter value). Green line: median value of the fluorescent parameter of interest.

B: FacsKin software fits a double-logistic function (red line) on each recording. Horizontal axis: Time (in seconds), Vertical axis: Fluorescent intensity of Ca<sup>2+</sup> sensitive dyes (relative parameter value). The calculated parameters: Max: the peak value of Ca<sup>2+</sup> influx, t<sub>max</sub>: time to reach Max value, AUC: Area Under the Curve.

compensatory changes only in the Th1 subset in MS regarding Ca<sup>2+</sup> influx kinetics and the function of K<sup>+</sup> channels. However, the increased function of the Th2 subset, and therefore the production of anti-inflammatory cytokines is less affected. This might contribute to a more effective treatment of the autoimmune process in this disorder [10].

## RHEUMATOID ARTHRITIS

The short-term activation of peripheral blood and synovial fluid T lymphocytes, especially that of autoreactive T cells plays a crucial role in initiating and maintaining the chronic inflammation in the joints of patients suffering from rheumatoid arthritis (RA). These cells regulate the inflammatory process resulting in the destruction of the articular cartilage and also play a role in extra-articular damage. We investigated T lymphocyte Ca<sup>2+</sup> influx kinetics following activation in peripheral blood of recently diagnosed RA patients compared to healthy individuals.

Our results indicate that margatoxin (MGTX), a specific blocker of the Kv1.3 channel acts differentially on Ca<sup>2+</sup> influx kinetics in major peripheral blood lymphocyte subsets of RA patients:

Th2 and CD8 cells are inhibited more dominantly than Th1 and CD4 cells. Kv1.3 channel expression in RA patients and healthy subjects Based on our results, the immunomodulatory effect of Kv1.3 channel inhibition is predominantly seen in cytotoxic (CD8) T cells in RA. However, this effect does not seem to be as specific as reported before by Beeton and colleagues in case of TEM cells [13], since anti-inflammatory Th2 cells are also affected to a noteworthy extent. This subset has an important role in counterbalancing the peripheral lymphocytes might be the differential distribution of disease-associated autoreactive T cells in RA patients on local and systemic levels. In the synovial fluid (locally), autoreactive TEM cells, expressing high numbers of Kv1.3 channels are abundantly present. However, this Kv1.3 pattern was not detected in peripheral blood T cells, because autoreactive TEM cells are infrequent in the circulation. Peripheral blood T cells were predominantly found to be naive and TCM cells [13]. A

reason for limited specificity of Kv1.3 inhibition in peripheral lymphocytes might be the differential distribution of disease-associated autoreactive T cells in RA patients in the synovial fluid and the circulation [11].

### **TYPE 1 DIABETES**

Type 1 diabetes mellitus (T1DM) is an autoimmune disease affecting the pancreas by destroying the insulin-producing beta cells. Due to the lack of insulin glucose levels increase in blood and in urine. Without insulin treatment T1DM is a fatal disease. In lymphocytes of healthy subjects both Kv1.3 and IKCa1 channels contribute to the maintenance of Ca<sup>2+</sup> influx upon activation. On the contrary, the sensitivity of T1DM lymphocytes to the inhibition of Kv1.3 channels is increased. Our data indicate that by specific inhibition of Kv1.3 channels, lymphocyte activation can be modulated in T1DM. It has been hypothesized that beneficial effects of Kv1.3 channel inhibition by MGTX are due to the dominance of Kv1.3 channels on activated TEM cells [12, 13]. It was also presumed that MGTX would inhibit the activation of CD8 lymphocytes responsible for cytotoxic destruction of pancreatic beta cells. Nevertheless, our findings obtained with a functional method (i.e. kinetic flow cytometry measurements) provide clear evidence for Kv1.3 channels to have an important role in each lymphocyte subset in T1DM, including Th2 lymphocytes acting as counterbalancing factors in the development of T1DM through the production of antiinflammatory cytokines [14]. Therefore, administration of Kv1.3 channel inhibitors would not have an exclusive effect on cells responsible for the autoimmune response in T1DM, but may have an impact on the activation characteristics of immune cells in general, leading to unpredictable alterations. For this reason, further studies are needed to describe the effects of the application of specific Kv1.3 inhibition and the extent of beneficial consequences in T1DM [8].

### **ANKYLOSING SPONDYLITIS**

Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disease, the best characterized of the diseases belonging to the concept of spondylarthritides. The pathogenesis of AS is still unclear, but it is considered to be a systemic autoimmune disease [15]. This is supported by the number of alterations found in lymphocyte subgroups in peripheral blood. Specifically, increased numbers of circulating Th2 helper lymphocytes [16] were reported in AS. Along with the alterations observed in cell prevalence one can assume that T cell activation properties may also be altered in AS. We investigated the short-term T cell activation characteristics in AS before and during infliximab (IFX) therapy [17].

CD4 and CD8 cells presented with a delayed increase in cytoplasmic Ca<sup>2+</sup> levels after activation in AS compared to controls. With IFX, therapy the delay in CD4+ cytoplasmic Ca<sup>2+</sup> levels did not normalize in AS. For CD8+ cells, cytoplasmic and mitochondrial Ca<sup>2+</sup> kinetics during activation normalized by week 6 on IFX (but not on week 2).

Of note, the increase of cytoplasmic Ca<sup>2+</sup> levels in CD4 and CD8 cells from AS is delayed compared to controls. The delayed Ca<sup>2+</sup> response of AS fits well into the observations done in *in vitro* tests with T cells exposed to TNF- $\alpha$ . Church et al. reported that TNF- $\alpha$  suppressed the Ca<sup>2+</sup> peak after PHA stimulation; they have suggested that either signalling pathways upstream of Ca<sup>2+</sup> mobilisation or the Ca<sup>2+</sup> signalling itself were impaired by prolonged TNF- $\alpha$  exposure [18]. In AS, only one study has been performed with a more robust analytical approach: Lee et al. did not observe a significant difference in intracellular Ca<sup>2+</sup> between AS patients and normal controls in activated peripheral blood mononuclear cells [19]. The inconsistency between their and our results is probably due to different methodology and cell types investigated [17].

### **HEALTHY PREGNANCY AND PREECLAMPSIA**

In healthy pregnancy (HP), the maternal immune system needs to acquire tolerance to protect the developing fetus from harmful immunological reactions. If this tolerance is impaired, a general maternal immune response may arise, resulting in systemic inflammation. This has been suggested to be a major factor in the pathogenesis of preeclampsia (PE) [20, 21]. This disorder is characterized by hypertension, proteinuria, edema and endothelial dysfunction usually evolving in the third trimester of pregnancy. We compared the activation-elicited Ca<sup>2+</sup> influx in major peripheral T lymphocyte subsets of HP and PE women to that in non pregnant women and tested the alteration of Ca<sup>2+</sup> influx upon treatment with specific inhibitors of the Kv1.3 and IKCa1 K<sup>+</sup> channels.

Our findings suggest that the Ca<sup>2+</sup> influx kinetics in activated T lymphocytes markedly differs in HP compared to non pregnant women: the decreased activation of the Th1 subset may partly be responsible for the well-established Th2/Th1 skewness in HP [22, 24]. Indeed, the maintained activation properties of Th1 lymphocytes in patients with PE may contribute to the lack of Th2 dominance associated with normal pregnancy. Similarly, CD8 cells in PE do not acquire suppressed activation kinetics either. [23] Thus, the decrease in their cytotoxic activity characteristic for HP is not present in PE. It is of particular interest that Ca<sup>2+</sup> influx of Th2 lymphocytes in HP was insensitive to K<sup>+</sup> channel inhibition, while Ca<sup>2+</sup> influx decreased significantly in non pregnant upon treatment with specific channel blockers. Of note, Th2 lymphocytes in PE presented with non pregnant-like characteristics and were sensitive to K<sup>+</sup> channel inhibition as well. While it is unclear whether the resistance or sensitivity of Th2 lymphocytes

to K<sup>+</sup> channel inhibition is reflected in

Th2 function, it is tempting to speculate that this may be an element contributing to the Th2 shift present in HP, but absent in PE. This hypothesis may be supported by reports suggesting that the shape of Ca<sup>2+</sup> influx influenced by K<sup>+</sup> channel functions may determine the cytokine production profile of helper T lymphocytes. Interestingly, other differences were also observed between HP and PE. While Ca<sup>2+</sup> influx in CD8 and Th1 lymphocytes was resistant to K<sup>+</sup> channel inhibition in PE, that of HP lymphocytes was sensitive. Again, while it is unclear whether the resistance of Th1 lymphocytes to K<sup>+</sup> channel inhibition is reflected in their function, the insensitivity of the Th1 subset in PE may be linked to the Th1 skewness. Comparing the inhibitory properties of lymphocyte K<sup>+</sup> channels in non pregnant women and PE, we found that – similarly to the Ca<sup>2+</sup> influx kinetics – they are comparable in all investigated subsets, except for the CD8 lymphocytes. Our results indicate marked differences of Ca<sup>2+</sup> influx kinetics and sensitivity to lymphocyte K<sup>+</sup> channel inhibition in major lymphocyte subsets between non pregnant, HP and PE lymphocytes. It is of interest that these properties in PE are more comparable to non pregnant than to HP. These results suggest that there is a characteristic pattern for Ca<sup>2+</sup> influx and its sensitivity to K<sup>+</sup> channel inhibition in HP that is missing in PE. This raises the notion that lymphocyte Ca<sup>2+</sup> handling upon activation may have a role in the characteristic immune status of healthy pregnancy [24].

## NEONATES

Decreased functionality of neonatal T cells is a widely recognized experimental and clinical phenomenon. Reduced functioning is well characterized by a lower level of cytokine production compared with adult T cells [25, 26]. Several factors might be responsible for the decreased cytokine expression compared with that of adult lymphocytes. Among many others, propositions include naivety of neonatal lymphocytes. The majority of these cells is naive (CD45RA) lymphocytes in contrast to adults, where effector (CD45RO) cells dominate [26].

Another possible factor might be the impairment of mechanisms regulating short-term activation of lymphocytes compared with adults. Kv1.3 and IKCa1 lymphocyte K<sup>+</sup> channels are essential components of these mechanisms. We hypothesized that short-term T lymphocyte activation properties are different in neonates compared with adults. The aim of our study was to characterize the Ca<sup>2+</sup> influx kinetics upon activation in major T lymphocyte subsets in the neonate and its sensitivity to the specific inhibition of Kv1.3 and IKCa1 lymphocyte K<sup>+</sup> channels, important regulators of Ca<sup>2+</sup> influx.

Ca<sup>2+</sup> influx following PHA activation of T lymphocytes markedly differs in the neonate from that in the adult. Upon treatment of lymphocytes with selective inhibitors of the Kv1.3 and IKCa1 channels (MGTX and TRAM, respectively), Ca<sup>2+</sup> influx reduction was not demonstrated in neonatal lymphocytes only in CD8 subsets. The finding that neonatal lymphocytes are less sensitive to the specific inhibition of K<sup>+</sup> channels compared with adults may be due to altered functionality or a lower expression of these channels. Therefore, we measured the expression of Kv1.3 K<sup>+</sup> channels on the investigated lymphocytes. Instead of lower values, we found increased expression of Kv1.3 channels on neonatal CD4, CD8 and Th2 lymphocytes compared with adults. Thus, the option that the decreased sensitivity of lymphocytes to K<sup>+</sup> channel inhibition is due to lower channel expression should be excluded. Based on the lower sensitivity of lymphocyte Ca<sup>2+</sup> influx to inhibition at higher channel expression values, it is reasonable to postulate that neonatal Kv1.3 channels are functionally altered. Signs of altered sensitivity to K<sup>+</sup> channel inhibition were present in all major lymphocyte subsets investigated (i.e. Th1, Th2, CD4 and CD8 cells). The only subset in which the short-term activation was inhibited significantly by specific blockers of both Kv1.3 and IKCa1 channels was CD8 lymphocytes. However, even in this case, the level of inhibition did not reach the extent described in adults in spite of high Kv1.3 expression values. This suggests that our observations are generally characteristic of all lymphocyte subpopulations studied. Furthermore, an interesting observation is that the decreased Ca<sup>2+</sup> influx found in the CD8 subset in neonates after MGTX and TRAM treatment is coupled with a massive elevation in the expression of Kv1.3 channels by this subset. However, no correlation was detected between Ca<sup>2+</sup> influx parameters and channel expression data; thus, a causative relation between the two findings is unlikely.

Our results may partly explain why neonatal lymphocytes are less responsive to activating stimuli and, hence, exert a lower intensity of immune response. We demonstrated that short-term activation and associated intracellular Ca<sup>2+</sup> influx kinetics are lower in neonatal lymphocytes compared with adults. This phenomenon is associated with the altered function of lymphocyte K<sup>+</sup> channels. Our results improve the understanding of the mechanisms that prevent neonatal T lymphocytes from adequate activation upon activating stimuli. They show that the functional impairment of lymphocyte K<sup>+</sup> channels may be of importance in those mechanisms [27].

## SUMMARY

Based on our results, a number of dominant features were identified that were present in most of the investigated autoimmune diseases. First, the time when the peak of Ca<sup>2+</sup> influx was reached decreased in autoimmune patients compared to healthy

individuals, indicating that these cells are in a state of sustained reactivity due to the ongoing autoimmune reaction. Earlier studies were limited to the investigation of K<sup>+</sup> channels in naive and memory lymphocytes. As an extension of these findings to other significant T lymphocyte subsets, in Th1 cells in patients suffering from an autoimmune disease we detected a different pattern of sensitivity to the inhibition of lymphocyte K<sup>+</sup> channels compared to healthy individuals: a greater decrease of Ca<sup>2+</sup> influx upon the inhibition of the Kv1.3 channel than that of the IKCa1 channel was observed. On the contrary, in healthy individuals the IKCa1 channels had a more effective inhibition profile compared to the Kv1.3 channels. This finding is of special interest, since Th1 cells are regarded as key players in the mediation of pro-inflammatory responses.

However, the selectivity of the investigated inhibitors (MGTX and TRAM) was limited in our experiments, as they did not only affect a single subset, as previously suggested. Although in earlier observations the inhibition of Kv1.3 channels specifically blocked the function of TEM cells, our investigations extending to other significant T lymphocyte subsets demonstrated that the inhibitory effect is present not only in disease-associated CD8 and Th1 cells, but also in the anti-inflammatory Th2 subset. The induced decrease in their function could lead to unexpected potential side-effects *in vivo* and also in a setback of therapy, since Th2 cells are responsible for anti-inflammatory responses.

The advantage of our newly developed method compared with the single-cell techniques is that it enables characterizing the progress of lymphocyte activation in complex cellular systems, since the interaction between the different cell subsets is also taken into account. Substances produced during lymphocyte activation (e.g. cytokines, chemokines) modulate the function of other cell subsets. Thus cell activation does not only affect one cell type but indirectly takes an effect on other cells in the sample. Accordingly, different lymphocyte subsets might be regarded as a network, where cells are in connection with the ones surrounding them. Furthermore, not only K<sup>+</sup> channel blockers, but other molecules and drugs might be screened. Investigating other lymphocyte subsets of interest is also possible with our method depending on the type of surface markers used for discriminating the cell types. Furthermore, our method may play a key role in several other fields of basic and clinical research where the characterization of different intracellular kinetic processes are needed that can be identified with fluorescent reagents (e.g. the generation of reactive oxygen radicals, alterations of membrane potential, etc). Further details of our algorithm and its application can be found at [www.facskin.com](http://www.facskin.com).

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