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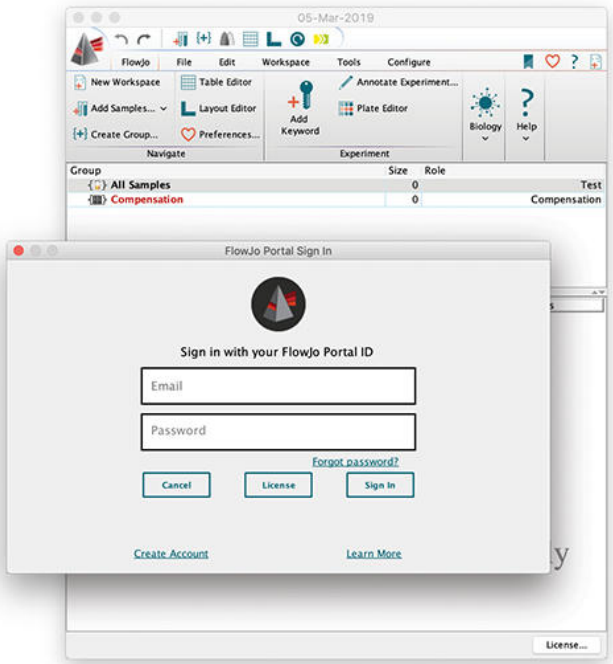

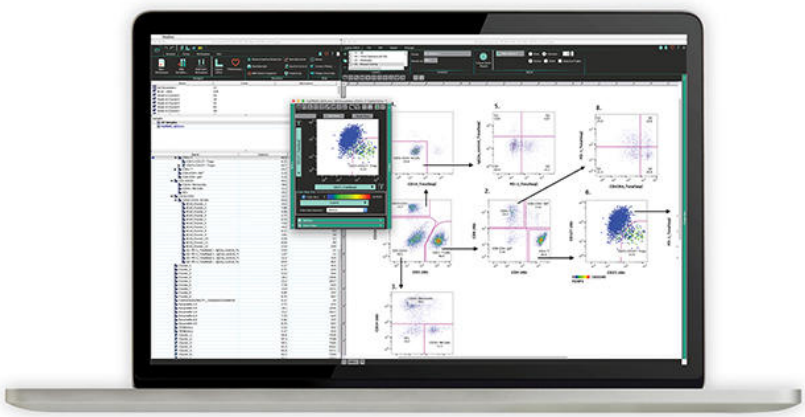

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Cytometry PART A
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Analysis by Flow Cytometry of Calcium Influx Kinetics in Peripheral Lymphocytes of Patients with Rheumatoid Arthritis

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Abstract

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.

Key terms

calcium influx; data analysis; kinetic flow cytometry; potassium channel; rheumatoid arthritis; T lymphocyte

The short-term activation of T lymphocytes, especially that of autoreactive T cells, plays a key role in initiating and maintaining most autoimmune reactions, including that observed in rheumatoid arthritis (RA). The transient increase of the cytoplasmic free calcium level ($[Ca^{2+}]_{cyt}$) in T lymphocytes is an indispensable part of this process. The engagement of the TCR/CD3 complex upon antigen presentation leads to the activation of several transmembrane signaling pathways which result in Ca^{2+} release from intracellular stores. This is followed by further Ca^{2+} entry from the extracellular space through the store-operated calcium release activated calcium (CRAC) channels. Voltage-gated Kv1.3 and calcium-dependent IKCa1 K^+ channels play a pivotal role in the maintenance of $[Ca^{2+}]_{cyt}$, since they sustain the electrochemical potential gradient needed for further Ca^{2+} entry by the efflux of K^+ from the cytoplasm (1,2). Specific inhibition of these channels results in diminished Ca^{2+} influx and a lower level of lymphocyte activation and proliferation (3,4).

This observation offers a unique opportunity for targeted pharmacological intervention with improved specificity from existing drugs against disease-causing

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autoreactive lymphocytes. According to Beeton et al., disease-associated autoreactive T cells are mainly CCR7− CD45RA− effector memory T cells (TEM cells) with elevated Kv1.3 channel expression, in comparison with naive and central memory T cells (TCM cells) that express low levels of Kv1.3 channels. Consequently, Kv1.3 inhibitors primarily suppress the activation and proliferation of the autoantigen-specific TEM cells, while sparing other classes of T cells (5). Beeton et al. also evaluated the therapeutic effects of Kv1.3 inhibitors in a rat model of RA. The animals treated with the specific Kv1.3 channel blockers showed significantly less joint deviations and significant improvement in radiological and histopathological findings (6). In addition, no clinical side-effects or signs of toxicity were identified during the trial. However, our earlier trial on human samples from patients with RA showed that Th2 and particularly CD8 cells are inhibited more dominantly than Th1 and CD4 cells. Thus the inhibition of Kv1.3 channels does not seem to be specific enough in peripheral human RA lymphocytes, since anti-inflammatory Th2 cells are also affected to a noteworthy extent (7).

Flow cytometry is a suitable method for the sequential determination of $[Ca^{2+}]_{cyt}$ in millions of stimulated lymphocytes over a time period. It also enables the comparison of Ca^{2+} influx kinetics in more than one lymphocyte subsets of the same sample simultaneously. However, until recently, there had been an unmet need for a method that can reliably compare kinetic flow cytometry data. A number of approaches have been available as part of various flow cytometry software for characterizing kinetic measurements. These approaches calculate median fluorescence of the dye of interest and apply a smoothing method in order to describe given parameters of the recording, such as the area under the curve (AUC) value (8).

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 (9).

In this study, we used flow cytometry to characterize Ca^{2+} influx kinetics upon activation in major T cell subsets (CD4, Th1, Th2, CD8) isolated from patients with recently diagnosed and established RA and their sensitivity to the specific inhibition of Kv1.3 and IKCa1 lymphocyte K^+ channels. For the evaluation of kinetic flow cytometry data, we utilized FacsKin, our novel algorithm (9), and the kinetic module of FlowJo, a currently available flow cytometry software. We hypothesized that FacsKin enables the discovery of more differences between Ca^{2+} influx kinetics of the investigated RA groups and provides a more precise characterization of kinetic measurements than currently available methods.

Materials and Methods

Sample Collection

Peripheral blood samples were drawn from nine patients with recently diagnosed RA who had not received anti-rheumatic treatment, including non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDs), and glucocorticoids until the time of blood sampling, as well as from six patients with established RA who had been treated with methotrexate monotherapy and low dose methyl-prednisolone for years. Clinical parameters of study participants are summarized in Table 1. Patients with RA were diagnosed according to the latest ACR/EULAR criteria (10). No co-morbidities were detected in patients based on routine laboratory investigations and physical examination that could have influenced our investigation. Written informed consent was obtained from all subjects, and our study was reviewed and approved by an independent ethical committee of the university. Laboratory studies and interpretations were performed on coded samples lacking personal and diagnostic identifiers. The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

Sample Preparation

Peripheral blood mononuclear cells (PBMCs) were isolated by a standard density gradient centrifugation (Ficoll Paque, Amersham Biosciences AB, Uppsala, Sweden, 25 min, 400*g*, room temperature) from 9 ml of freshly drawn peripheral venous blood collected in lithium heparin treated tubes (BD Vacutainer, BD Biosciences, San Jose, CA). The cell suspension was washed twice in phosphate buffered saline. Cells were kept in a modified RPMI medium (Sigma-Aldrich, St. Louis, MO) throughout the following steps of the procedure. The Ca^{2+} concentration of this medium was set to 2 mM by the addition of crystalline $CaCl_2$.

PBMCs were divided into three vials with equal cell numbers and one extra vial for the assessment of Kv1.3 channel expression. In order to differentiate T lymphocyte subsets, PBMCs were incubated with the following conjugated anti-human monoclonal antibodies for 30 min in dark at room temperature according to the manufacturer's instructions (all from BD Pharmingen, San Diego, CA): anti-CD4 PE-Cy7 (clone SK3), anti-CD8 APC-Cy7 (clone SK1), anti-CXCR3 APC (clone 1C6/CXCR3), and anti-CCR4 PE (clone 1G1). The extra vial was in addition incubated with anti-Kv1.3

Table 1. Clinical characteristics of study participants

CHARACTERISTICS	RECENTLY DIAGNOSED RA PATIENTS, $N = 9$	RA PATIENTS WITH ESTABLISHED DISEASE, $N = 6$
Age (years)	58 [44–61]	61 [59–72]
Gender (male/female)	4/5	2/4
RA duration	3 [2–4.5] months	7.5[a] [5–9.5] years
CRP (mg/L)	30.6 [16.1–63.0]	7.4[a] [5.7–17.8]
ESR (mm/h)	50 [44–74]	20[a] [16–37]

Data are expressed as median [interquartile range] for continuous variables and as number for categorical variables.

CRP, C reactive protein; ESR, erythrocyte sedimentation rate, RA, rheumatoid arthritis.

[a] $P < 0.05$ vs. patients with recently diagnosed RA.

channel FITC (Sigma-Aldrich). After washing, PBMCs were loaded with Ca^{2+} sensitive Fluo-3 and Fura Red dyes supplemented with Pluronic F-127 for 20 min in dark at 30°C according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA). Cells were washed once before measurement and kept at 30°C in dark until measurement.

One vial was used as control. Another vial was treated with margatoxin (MGTX, 60 nM), a selective blocker of the Kv1.3 channel (Sigma-Aldrich) for 15 min before measurement. The third vial was treated with a triarylmethane compound, TRAM-34 (60 nM), a specific inhibitor of the IKCa1 channel (Sigma-Aldrich) for 10 min before measurement. PBMCs were activated by the addition of 20 μg phytohemagglutinin (PHA) and recording was initiated directly afterwards.

Flow Cytometric Analysis

Flow cytometry measurements were conducted on a BD FACSAria flow cytometer (BD Biosciences) and data were processed using the FACSDiva software (v 6.0). The original instrument settings have not been altered regarding flow cell (fixed-alignment cuvette flow cell) and fluidics, light sources, excitation optics, optical filters, detectors, and paths.

Cell-bound fluorescent labels were excited with a 488-nm Coherent Sapphire solid state (14 mW) and a 633-nm JDS Uniphase HeNe air-cooled (14 mW) laser. PE fluorescence was detected through a 575/26 nm BP filter, PE-Cy7 fluorescence through a 780/60 nm BP filter, APC fluorescence through a 660/20 nm BP filter, APC-Cy7 fluorescence through a 780/60 nm BP filter, Fluo-3 and FITC fluorescence through a 530/30 nm BP filter, and Fura Red fluorescence through a 695/40 nm BP filter. The fluorescence signal data were recorded with logarithmical amplification. Compensation was performed computationally post acquisition, based on single stained control settings. The flow cytometer was set up before experiments using seven-color beads (BD FACS, BD Biosciences). Cell fluorescence data were measured and recorded for 10 min in a kinetic manner (average cell acquisition rate was 1,000 cells per sec).

Lymphocytes were separated from PBMCs and cell debris by setting a polygonal gate in the forward scatter (FSC)/side scatter (SSC) plot. CD4 and CD8 lymphocytes were identified and selected in the CD4/CD8 plot by their CD4 positivity and lack of CD8 expression, as well as by their CD8 positivity and lack of CD4 expression, respectively. CD4 cells were further analyzed in a CXCR3/CCR4 plot. Th1 lymphocytes were regarded as CD4+ CXCR3+ CCR4-cells, while Th2 lymphocytes were regarded as CD4+ CXCR3− CCR4+ cells. $[Ca^{2+}]_{cyt}$ was calculated based on a fix ratio of Fluo-3 and Fura Red dyes (25%) after gating of the individual lymphocyte subsets plotted against time (Fig. 1A).

For evaluation of the acquired kinetic data, we compared FacsKin (v 0.5.16, Fig. 1B), a specific software developed at our laboratory (available at www.facskin.com along with a detailed description of the evaluation process) as well as the kinetic module of FlowJo (v 7.6.3, Fig. 1C). Analysis with FacsKin is based on the calculation of a double-logistic function for each Ca^{2+} influx recording. This function is used to characterize measurements that have an increasing and a

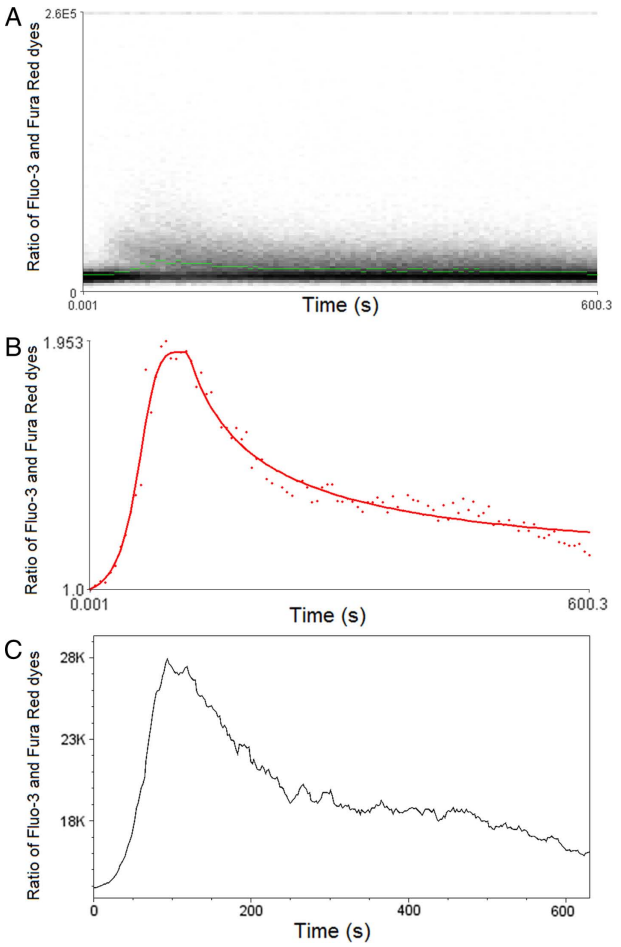

Figure 1. **A**: The ratio of Fluo-3 and Fura Red dyes after gating of the individual lymphocyte subsets plotted against time. **B**: Double-logistic function representing Ca^{2+} influx kinetics calculated by FacsKin. **C**: Ca^{2+} influx kinetics calculated by FlowJo based on median fluorescence data and smoothing. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

decreasing intensity as time passes. The software calculates parameter values describing each function, such as the AUC, Maximum (Max), Time to reach maximum (t_{max}), and Slope values (Fig. 2). The AUC value describes the full amount of $[Ca^{2+}]_{cyt}$ during the whole recorded period of Ca^{2+} influx

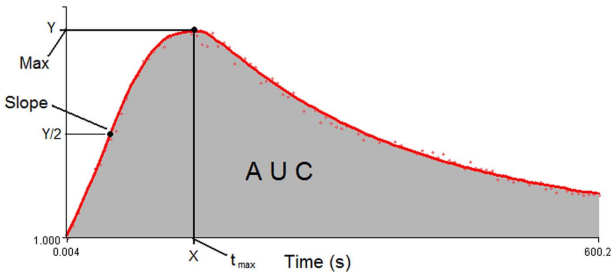

Figure 2. Parameter values describing each function calculated by FacsKin. AUC, area under the curve; Max, Maximum; t_{max}, Time to reach maximum. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

and thus corresponds to the sum of $[Ca^{2+}]_{cyt}$ increase, which is consistent with the level of lymphocyte activation (1). One unit (U) of the AUC value is defined as one relative intensity value in 1 sec, where relative intensity values are the rate of actual intensity values divided by intensity values at zero second. The Maximum value represents the peak of the Ca^{2+} influx curve upon lymphocyte activation, thus it reflects the maximal amount of $[Ca^{2+}]_{cyt}$ along the course of activation. The Time to reach maximum value represents how soon the Max value is reached. The Slope value reflects how rapidly the peak of Ca^{2+} influx is reached (7,11,12). Since AUC is regarded as the best single parameter describing the magnitude of Ca^{2+} influx, this parameter was also calculated using the kinetic module of FlowJo (v 7.6.3).

Statistical Analysis

Data are expressed as median and interquartile range. Comparisons between the two patient groups were made with Mann–Whitney tests. Comparisons between paired values (samples with or without treatment with specific K^+ channel blockers) in the same patient were made with Wilcoxon tests. *P* values less than 0.05 were considered significant. The study was not powered for the multitude of statistical tests performed, thus some significances could occur by chance alone. Statistics were calculated using GraphPad Prism v 5.0 (GraphPad Software, San Diego, CA).

Results

Clinical Data

The age and gender distribution of participants were similar in both RA study groups. Inflammatory parameters (C reactive protein values and erythrocyte sedimentation rate) were higher in patients with recently diagnosed RA than in patients suffering from established RA (Table 1).

Lymphocyte Calcium Influx Kinetics Evaluated with FacsKin

The calculated parameter values (AUC, Max, t_{max}, and Slope) were compared upon lymphocyte activation between patients with recently diagnosed RA and established RA. The parameters are shown in Supporting Information Table 1. Slope values showed no alteration between the study groups in the investigated lymphocyte subsets, and therefore are not included in the table. In the Th2 subset, the t_{max} value of Ca^{2+} influx was elevated in patients with established RA compared to patients with recently diagnosed RA. Meanwhile, we registered a significantly lower t_{max} value within the CD8 subset in patients with established RA than in patients with recently diagnosed RA. We found no difference in the parameters of calcium influx in the CD4 and Th1 subsets. t_{max} values were higher in lymphocytes isolated from patients with RA and are reached more rapidly in the CD4+ subsets compared to healthy controls.

The Effects of K^+ Channel Inhibitors on Lymphocyte Calcium Influx Evaluated with FacsKin

We evaluated the effects of specific inhibitors of the Kv1.3 and IKCa1 channels (MGTX and TRAM, respectively) on parameter values of Ca^{2+} influx in both RA study groups. Results are shown in Figure 3 and Supporting Information Table 1. Slope values showed no alteration in the investigated lymphocyte subsets upon inhibitor treatment, and therefore are not included in the table. In healthy individuals, the

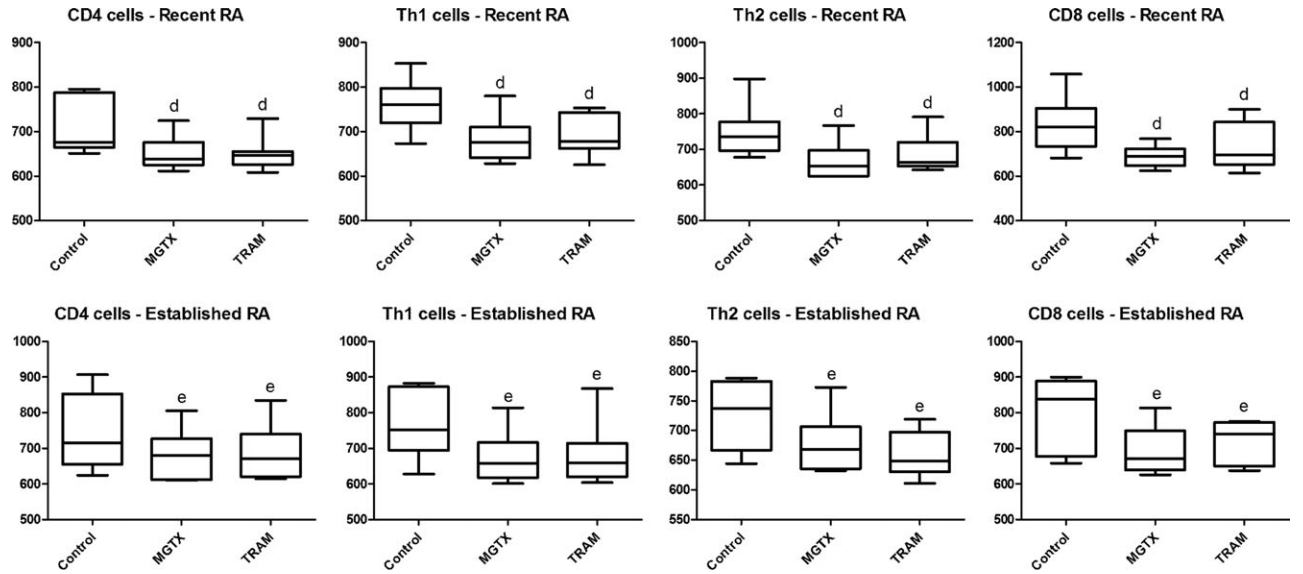

Figure 3. Analysis of results using FacsKin. Box-plots representing the effects of MGTX and triarylmethane (TRAM) application on AUC values of Ca^{2+} influx kinetics in lymphocytes obtained from nine patients with recently diagnosed RA and six RA patients with established disease. Horizontal line—median, box—interquartile range, whiskers—range. RA, rheumatoid arthritis. [d]MGTX and TRAM treated samples were compared with samples with no inhibitor application within lymphocytes isolated from patients with recently diagnosed RA, $P < 0.05$, [e]MGTX and TRAM treated samples were compared with samples with no inhibitor application within lymphocytes isolated from patients with RA with established disease, $P < 0.05$.

inhibition of the IKCa1 channel decreased calcium influx in Th2 and CD4 cells to a lower extent than in Th1 and CD8 cells. The AUC value was decreased by both MGTX and TRAM in all subsets in both RA study groups. Both MGTX and TRAM decreased the Max value in all subsets of patients with recently diagnosed RA, but only in the Th1 subset in patients with established RA. The t_{max} value was only decreased by TRAM in the CD8 subset of patients with recently diagnosed RA. On the contrary, the t_{max} value of the CD8 subset in samples isolated from patients with established RA showed an increasing tendency upon TRAM treatment. The extent of the alterations in parameter values was characteristic for the investigated study groups and the inhibited K^+ channels in each subset, as detailed in the Discussion.

Lymphocyte Calcium Influx Kinetics Evaluated with FlowJo

Since the AUC value is considered to be the most representative single value for Ca^{2+} influx characteristics, AUC values were compared upon lymphocyte activation between patients with recently diagnosed RA and patients with established RA using the kinetic module of FlowJo (Supporting Information Table 2). No differences were detected between the two study groups in AUC values.

The Effects of K^+ Channel Inhibitors on Lymphocyte Calcium Influx Evaluated with FlowJo

We also evaluated the effects of specific Kv1.3 and IKCa1 channel inhibitors (MGTX and TRAM, respectively) on AUC values of lymphocyte Ca^{2+} influx in both RA study groups using FlowJo. Results are presented in Figure 4 and Supporting Information Table 2. AUC values were decreased by MGTX in the CD4 and Th2 subsets of patients with recently diagnosed RA. Upon TRAM treatment, the CD4, Th1, and Th2 subsets of patients with recently diagnosed RA showed a decrease in AUC values. Neither TRAM nor MGTX treatment had a detectable effect on lymphocyte Ca^{2+} influx of patients with established RA.

Kv1.3 Channel Expression in the Investigated Lymphocyte Subsets

We compared the median fluorescence intensity of the antibody against Kv1.3 channels in all investigated lymphocyte subsets between the two study groups. No significant difference could be observed in the median fluorescence intensity values (Table 2).

Table 2. Median fluorescence values of the anti-Kv1.3 FITC antibody in lymphocytes isolated from 9 recently diagnosed RA patients and 6 RA patients with established disease

	RECENTLY DIAGNOSED RA PATIENTS, $N = 9$	RA PATIENTS WITH ESTABLISHED DISEASE, $N = 6$
CD4	54 [39–64]	63 [21–99]
Th1	53 [36–69]	31 [14–58]
Th2	47 [27–95]	25 [10–53]
CD8	63 [19–93]	32 [13–50]

Data are expressed as median [interquartile range].
RA, rheumatoid arthritis.

Discussion

In our investigation, we aimed to compare peripheral T lymphocyte Ca^{2+} influx kinetics upon activation in two different subgroups of RA, and to demonstrate the differences in analysis of kinetic flow cytometry data when using two different algorithms.

After analyzing data with FacsKin, significant differences could be observed in Ca^{2+} influx kinetics between patients with recently diagnosed RA and patients with established RA (Supporting Information Table 1). Our results revealed that the t_{max} value of the Th2 subset in patients with established RA is reached slower compared to patients with recently diagnosed RA, indicating that Th2 cells respond slower to activating stimuli as the disease duration increases. Th2 cells mainly regulate anti-inflammatory responses, thus this finding possibly indicates the remission of immunoregulatory process along with the duration of the disease. This is in contrast with our earlier findings in RA, which presented an elevated Th2 response in the early stages of RA compared to healthy controls (Supporting Information Table 1) (7), presumably to control the ongoing inflammation. On the contrary, we measured lower t_{max} values in the CD8 subset of patients with established RA, which may indicate an enhanced direct cytotoxic response, raising the notion that the increasing duration of the disease results in an even greater imbalance between the pro- and anti-inflammatory responses. The peak of calcium influx in lymphocytes isolated from patients with RA is reached more rapidly in the CD4+ subsets compared to healthy individuals, indicating that they respond more quickly to stimulation, probably due to the ongoing autoimmune response.

Upon treatment with specific inhibitors of the Kv1.3 and IKCa1 channels (MGTX and TRAM, respectively), AUC values decreased in all subsets in both RA study groups. However, the sensitivity of the two study groups was different to lymphocyte K^+ channel inhibition. Blocking the Kv1.3 channel decreased Ca^{2+} influx in the CD4, Th2, and CD8 subsets of patients with established RA to a lower extent than in patients with recently diagnosed RA. However, Th1 cells were more sensitive to the inhibition of Kv1.3 channels in patients with established RA. In patients with recently diagnosed RA, Max values decreased in all subsets upon treatment with MGTX, while in patients with established RA, the Max value decreased in the Th1 subset only. Similarly, upon the inhibition of the IKCa1 channel, Th1 cells of patients with established RA showed a higher level of decrease of the AUC and Max values than those of patients with recently diagnosed RA.

The above results could either be due to the altered expression or different functionality of Kv1.3 channels. Therefore, we measured the cell surface expression of these channels on the investigated lymphocyte subsets in a semi-quantitative manner using a specific antibody. However, we could not detect a difference in the expression of Kv1.3 channels between

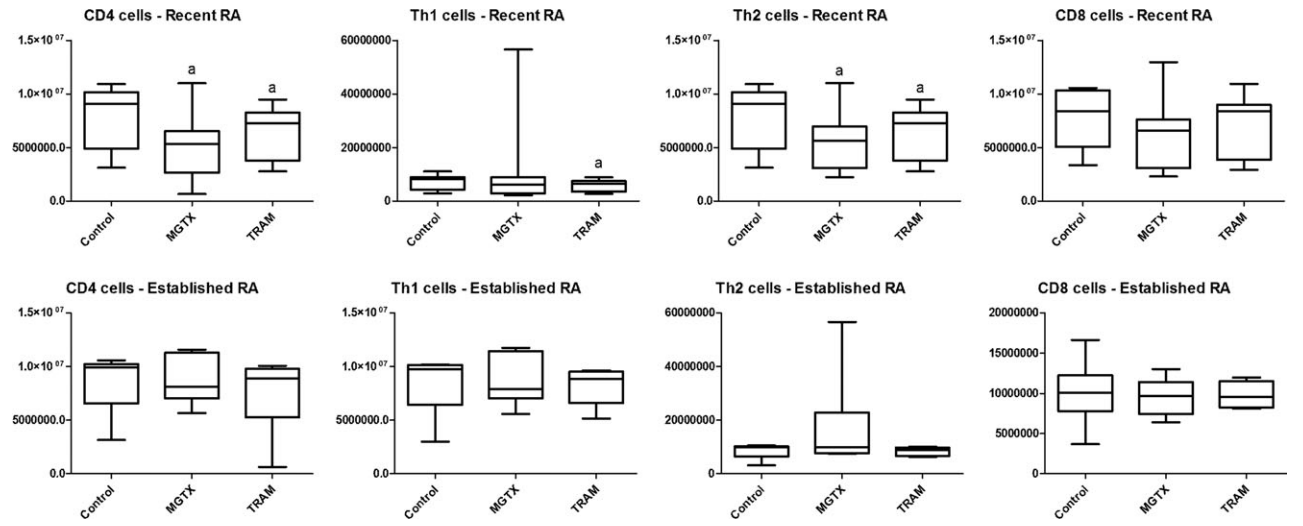

Figure 4. Analysis of results using FlowJo. Box-plots representing the effects of MGTX and triarylmethane (TRAM) application on AUC values of Ca^{2+} influx kinetics in lymphocytes obtained from nine patients with recently diagnosed RA and six RA patients with established disease. Horizontal line—median, box—interquartile range, whiskers—range. RA, rheumatoid arthritis. [a]MGTX and TRAM treated samples were compared with samples with no inhibitor application within lymphocytes isolated from patients with recently diagnosed RA, $P < 0.05$.

the two study groups. Hence, we presume that functional alterations are responsible for the altered sensitivity to Kv1.3 channel inhibition in patients with established RA. Of note, due to the lack of commercially available antibodies against IKCa1, we could not investigate its expression in a similar manner.

Our findings indicate that 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. Based on our results, it is difficult to tell whether this difference is due to the primary course of the disease or whether it is influenced by pharmacological treatment.

We also compared the AUC values, the most representative parameter for kinetic measurements, using the kinetic module of FlowJo (Supporting Information Table 2). However, the above difference between the sensitivity of recently diagnosed and established RA lymphocytes to K^+ channel inhibition could not be demonstrated based on results obtained with this approach. In fact, we could not observe any effect of the applied inhibitors in the established RA group when data were analyzed with FlowJo. A plausible explanation for this might be that FlowJo is based on a smoothing method of median fluorescence values performed by moving average calculation. However, due to the non-normal distribution of flow cytometry data (13), techniques that assume normality (such as those calculating average or standard deviation) lead to potentially incorrect smoothing and artifacts in the kinetic parameter (8).

With our method of function fitting and selecting the best fitting function for the kinetics of the studied biological mechanism instead of smoothing, more statistically significant differences of K^+ channel inhibition between the recently diagnosed and established RA groups could be demonstrated. The use of our algorithm largely improves the evaluation of kinetic flow cytometry compared to the current, non-standardized approaches that largely depend on the observers' personal experience, revealing more detailed information and delicate differences of the investigated kinetic process (8,9).

A limitation of our study is the use of cell surface chemokine receptors instead of intracellular cytokine staining for the identification of Th1 and Th2 cells. The option to use intracellular cytokines as markers of the T helper subsets in our current experiment had to be excluded, since permeabilization of the cell membrane to stain intracellular cytokines would have prevented us from studying calcium influx. However, literary data and our experience indicate that the applied cell surface chemokine receptor markers are sufficient for the identification of the investigated T helper subsets (14,15).

In conclusion, following evaluation with FacsKin, we were able to describe numerous differences in Ca^{2+} influx kinetics of major peripheral blood T lymphocyte subsets of patients with recently diagnosed RA and patients with established RA which could not be detected using currently available approaches employing smoothing methods. We found that 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. These data demonstrate that the function fitting algorithm of FacsKin is suitable to provide a common basis for evaluating and comparing flow cytometry kinetic data.

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