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Human Th1 and Th2 lymphocytes are distinguished by calcium flux regulation during the first 10 min of lymphocyte activation

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ABSTRACT

Preliminary data suggest different intracellular calcium handling of Th1 and Th2 lymphocytes that may contribute to distinct cytokine production patterns. In this study we explored the contribution of the main mechanisms in charge of the elevation and decrease of cytoplasmic free calcium levels, i.e., the endoplasmic calcium release, the calcium release activated calcium (CRAC) channel, the mitochondrial calcium uniporter (MCU), the sarco/endoplasmic reticulum calcium ATPase (SERCA), and the plasma membrane calcium ATPase (PMCA) during the first 10 min of activation in human Th1 and Th2 lymphocytes applying a kinetic flow cytometry approach. We isolated peripheral blood mononuclear cells from 10 healthy individuals. Cells were stained with CD4, CXCR3 and CCR4 cell surface markers to identify Th1 and Th2 cells, respectively and loaded with Fluo-3/AM calcium sensitive dye. Cells were activated with phytohemagglutinine and alterations of cytoplasmic free calcium levels were monitored for 10 min after specific inhibition of the above mechanisms. Our results revealed delicate differences in calcium flux kinetics of Th1 and Th2 lymphocytes. The lower activity of MCU, and therefore of CRAC channels, along with the higher activity of the SERCA pump account for the notion that Th2 cells go through a lower level of lymphocyte activation compared with Th1 cells upon identical activating stimuli. The observed differences in calcium flux of Th1 and Th2 cells may contribute to different calcium handling kinetics and, hence, to distinct cytokine production patterns by these subsets.

Introduction

The process of lymphocyte activation is closely linked to dynamic alterations of the cytoplasmic free calcium concentration ($[Ca^{2+}]_{cyt}$). The actual distribution of intracellular free calcium depends on finely tuned interactions of mechanisms responsible for its elevation and decrease. The main mechanisms in charge of the elevation of $[Ca^{2+}]_{cyt}$ consist of calcium release from the endoplasmic reticulum (ER) (Feske, 2007), calcium entry from the extracellular space through the calcium release activated calcium (CRAC) channels (Parekh, 2007), and mitochondrial calcium uptake (Fig. 1). Mitochondria have a dual role in shaping the calcium signal. In close proximity to CRAC channels, they sequester calcium locally during CRAC functioning using the mitochondrial calcium uniporter (MCU) located in the mitochondrial inner membrane. Therefore they prevent calcium-dependent negative feedback inhibition of CRAC channels and contribute to the maintenance of extracellular calcium influx (Gilabert and Parekh, 2000; Hoth et al., 1997; Quintana et al., 2006). However, in a later phase of lymphocyte activation, they may also take up and store large amounts of calcium from anywhere in the cytoplasm and, therefore, decrease $[Ca^{2+}]_{cyt}$ (Duchen, 2000).

The main mechanisms that specifically contribute to the control of elevated $[Ca^{2+}]_{cyt}$ through calcium clearance in lymphocytes are the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (Feske, 2007) and the plasma membrane Ca^{2+} ATPase (PMCA) (Di Leva et al.,

Abbreviations: AUC, area under the curve; $[Ca^{2+}]_{cyt}$, cytoplasmic free calcium concentration; CRAC, calcium release activated calcium channel; ER, endoplasmic reticulum; MCU, mitochondrial calcium uniporter; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinine; PMCA, plasma membrane Ca^{2+} ATPase; RR, ruthenium red; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; STIM1, stromal interaction molecule 1; TG, thapsigargin; t_{max}, time to reach maximum.

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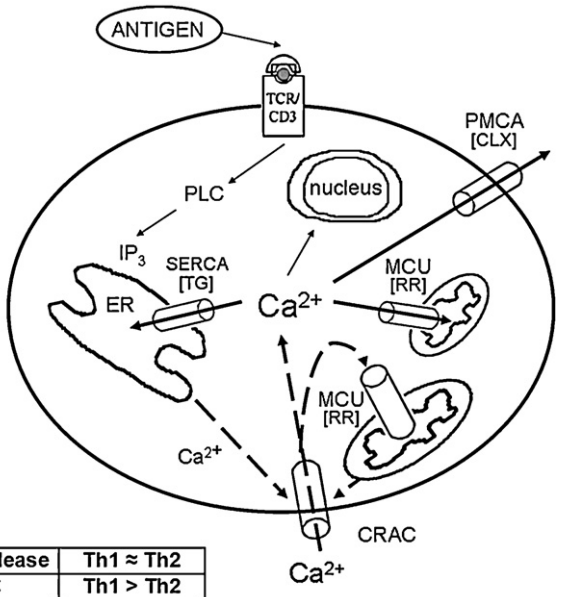

ER Ca^{2+} release	Th1 ≈ Th2
CRAC	Th1 > Th2
MCU	Th1 > Th2
SERCA	Th1 < Th2
PMCA	Th1 > Th2

Fig. 1. Simplified scheme of the regulation of cytoplasmic free calcium level. Interrupted arrows represent mechanisms responsible for the elevation, while bold arrows represent those responsible for the decrease of the cytoplasmic free calcium level. The applied inhibitors of the transporters are indicated in brackets. Mitochondria have a dual role in shaping the calcium signal: in close proximity to CRAC channels they sequester calcium locally, and therefore prevent calcium-dependent negative feedback inhibition of CRAC channels. In a later phase of lymphocyte activation, they may also take up and store calcium from anywhere in the cytoplasm, and therefore decrease the cytoplasmic free calcium level. The differential activity of mechanisms responsible for the elevation and decrease of cytoplasmic free calcium level in Th1 and Th2 cells is marked in the box. TCR: T-cell receptor, PLC: phospholipase-C, IP_3: inositol trisphosphate, ER: endoplasmic reticulum, CRAC: calcium release activated calcium channel, MCU: mitochondrial calcium uniporter, RR: ruthenium red, SERCA: sarco/endoplasmic reticulum calcium ATPase, TG: thapsigargin, PMCA: plasma membrane calcium ATPase, CLX: caloxin 2A1.

2008) pumps, while mitochondrial calcium uptake has a minor role, typically restricted to extremely high $[Ca^{2+}]_{cyt}$ levels (Duchen, 2000) (Fig. 1).

The steps of lymphocyte activation begin with a transient increase of $[Ca^{2+}]_{cyt}$ in both Th1 and Th2 cells. The signal transduction pathway culminates in the activation of a number of transcription factors (Crabtree and Clipstone, 1994). Th1 and Th2 cells produce a different set of cytokines in spite of the fact that their activation begins with identical stimuli. Since the expression of certain cytokine genes is distinctly influenced according to the characteristics of calcium influx kinetics, the differences in calcium handling of the Th1 and Th2 subset may remarkably contribute to alterations of cytokine production (Dolmetsch et al., 1998). Fanger et al. previously described that murine Th1 and Th2 lymphocytes are distinguished by differences in the kinetics of calcium handling (Fanger et al., 2000). They showed that mechanisms regulating calcium influx, such as the activity of potassium channels, are different in the two subsets: calcium-dependent IKCa1 channels were less active in Th2 cells. These observations obtained by patch-clamp analysis were reinforced by our previous investigations using flow cytometry in human lymphocytes (Toldi et al., 2010).

Fanger et al. further described that Th2 cells diminish $[Ca^{2+}]_{cyt}$ more rapidly than Th1 cells do. They concluded that the combination of a faster calcium clearance mechanism and lower calcium-activated potassium currents account for the lower calcium response in Th2 cells and that the mechanisms governing calcium response kinetics of lymphocytes are likely to differ in these two T helper subpopulations. However, their experiments provided no further detail on the differences of calcium clearance in Th1 and Th2 cells, and on the specific role of mechanisms responsible for the elevation and decrease of $[Ca^{2+}]_{cyt}$.

In this study we applied a kinetic flow cytometry approach to gather data regarding the differences between calcium handling of Th1 and Th2 lymphocytes. We investigated the contribution of the ER calcium release, the CRAC channel, and, using specific inhibitors, the MCU (inhibited by ruthenium red (RR) (Hajnóczky et al., 2006; Matlib et al., 1998; Xu et al., 1999)), the SERCA pump (inhibited by thapsigargin (TG) (Thastrup et al., 1990)) and the PMCA pump (inhibited by caloxin 2A1 (Chaudhary et al., 2001; Szewczyk et al., 2008)) to the regulation of $[Ca^{2+}]_{cyt}$ during the early period (first 10 min) of T lymphocyte activation and found significant differences between these subsets.

Materials and methods

Peripheral blood samples were taken from 10 healthy donors (5 women and 5 men, age: 24 [23–25] years (median [range])). Informed consent was obtained from all subjects, and our study was approved by the ethical committee of the institution. Peripheral blood mononuclear cells (PBMCs) were separated by a standard density gradient centrifugation (Ficoll Paque, Amersham Biosciences AB, Uppsala, Sweden, 27 min, 400 g, 22 °C) from 9 ml of freshly drawn peripheral venous blood collected in lithium heparin treated tubes (BD Vacutainer, BD Biosciences, San Jose, CA, USA). This cell suspension was washed twice in phosphate buffered saline (PBS). From then on, cells were kept throughout staining with fluorescent markers, treatment with inhibitors and measurement on flow cytometer either in a modified RPMI medium (Sigma–Aldrich, St. Louis, MO, USA) or in calcium-free PBS. The calcium concentration of the modified RPMI medium was set to 2 mM by the addition of crystalline $CaCl_2$.

The population of lymphocytes was gated from PBMCs according to Forward Scatter Characteristics and Side Scatter Characteristics. For surface marker staining, PBMCs were incubated for 30 min in dark at room temperature with the following conjugated anti-human monoclonal antibodies: anti-CD4 APC-Cy7 (excitation wavelength: 650 nm, emission wavelength: 785 nm, BioLegend, San Diego, CA, USA), anti-CXCR3 APC (for the identification of the Th1 subset, excitation wavelength: 650 nm, emission wavelength: 660 nm, PharMingen, San Diego, CA, USA), and anti-CCR4 PerCP (for the identification of the Th2 subset, excitation wavelength: 482 nm, emission wavelength: 678 nm, BioLegend) according to the manufacturers' instructions. To monitor intracellular calcium levels, PBMCs were loaded with calcium-sensitive Fluo-3/AM dye (excitation wavelength: 506 nm, emission wavelength: 526 nm) supplemented with Pluronic F-127 according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). Cells were incubated for 20 min in dark at 30 °C, and then washed. After washing, cells were kept at room temperature in dark.

PBMCs separated from a blood sample were divided into four vials with equal quantity of cells (approximately 600,000 cells/vial). Approximately 60,000 Th1 cells and 25,000 Th2 cells were present in a vial. One vial was treated with RR (1.25 mM) for 10 min before measurement (Sigma–Aldrich, St. Louis, MO, USA). Another vial was treated with TG (750 nM) immediately before measurement (Sigma–Aldrich). The third vial was supplemented with caloxin 2A1 (750 μM) for 5 min before measurement (AnaSpec, Fremont, CA, USA). The fourth vial was used as control and was treated with no inhibitor. Measurements were initiated immediately following the administration of 20 μg of phytohemagglutinine (PHA) to each vial as an unspecific activating stimulus. Flow cytometry measurements were conducted on a BD FACSAria flow cytometer

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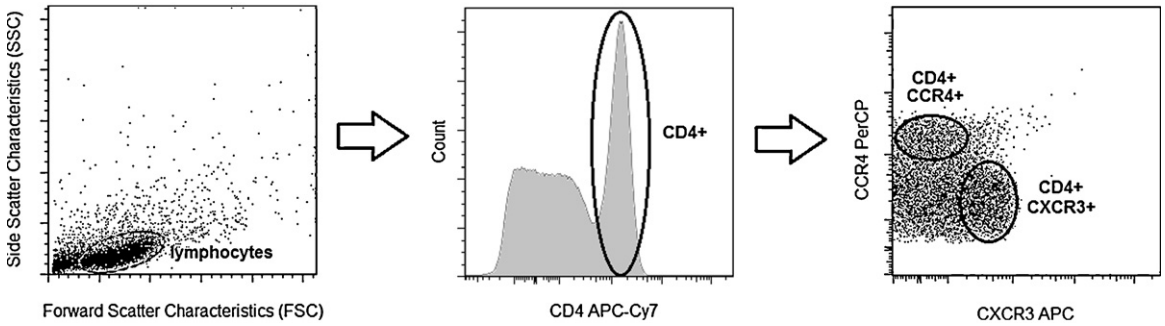

Fig. 2. Gating strategy for the detection of the investigated lymphocyte subsets. APC: allophycocianin, PerCP: peridinin chlorophyll protein.

(BD Biosciences). Cell fluorescence data were continuously measured and recorded for 10 min (average cell acquisition rate was 1000 cells/s).

After gating either the CD4+ CXCR3+ (Th1) or the CD4+ CCR4+ (Th2) subset (the gating strategy is shown in Fig. 2), the flow cytometry recording was split into 100 equal time intervals, the medians of the Fluo-3/AM fluorescence intensity values were calculated in each interval, and the built-in lowess smoothing method (using smoother span parameter $f = 0.3$) of the R statistical programming environment (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria) was applied to calculate the smoothed median curve describing the kinetic process. We determined the following parameters from the curve: the Area Under the Curve (AUC), the steepest slope of the ascending phase of the curve (Slope), the Maximum value (Max) and the Time to reach maximum value (t_{max}) (Fig. 3). The Slope value represents the point when the ratio of mechanisms responsible for the elevation and decrease of $[Ca^{2+}]_{cyt}$ reaches the maximal level during the measurement. The Max value represents the point when $[Ca^{2+}]_{cyt}$ reaches the highest level during the measurement. The t_{max} value represents the time point when the Max value is reached. AUC values characterize the overall cytoplasmic calcium exposure of cells, reflecting the magnitude of the calcium response in general. AUC values correspond to $[Ca^{2+}]_{cyt}$, which further correspond to the level of lymphocyte activation (Panyi, 2005).

Data are expressed as median and range. Comparisons between two sample populations within the same subset were made with Wilcoxon tests. Comparisons between different subsets were made with Mann-Whitney tests. p-Values less than 0.05 were considered significant. Statistics were calculated using the R software (R Development Core Team).

Results

Calcium flux kinetics of Th1 and Th2 lymphocytes. In our study, CD4+ CXCR3+ cells were regarded as the Th1 subset, while CD4+ CCR4+ cells were regarded as the Th2 subset. First, we compared the kinetics of calcium response in the Th1 and Th2 lymphocyte subsets following PHA stimulation. AUC, Slope and Max values were lower in the Th2 lymphocyte subset upon activation (Table 1). However, the t_{max} value did not significantly differ in the two subsets.

The individual contribution of ER calcium release and calcium entry through CRAC channels to the elevation of $[Ca^{2+}]_{cyt}$ *during lymphocyte activation*. To determine the contribution of ER, we measured calcium flux kinetics in an extracellular milieu containing no calcium (thus preventing CRAC channel functionality). We then calculated the contribution of CRAC by subtracting the parameter values of this set of measurements from the values of measurements performed in an extracellular milieu with 2 mM calcium concentration. Subsequently, we determined the ratio of contribution of the two sources for $[Ca^{2+}]_{cyt}$ elevation (CRAC/ER ratio) in case of each investigated parameter. Results are shown in Table 1.

Effects of RR treatment on calcium flux kinetics of lymphocytes. RR decreased the AUC values in both investigated lymphocyte subsets (Fig. 4 and Table 2). Furthermore, the Slope and Max values were lowered in the Th1 subset, while the t_{max} value was elevated in the Th2 subset upon RR treatment. This finding suggests that RR plays a role in shaping both the magnitude and kinetics of calcium influx.

Our results indicate that mitochondrial calcium uptake is present in both the initial and the peak phase of early lymphocyte activation in Th1 cells (represented by the alteration of both the Slope and Max values upon MCU inhibition), while it plays a role in $[Ca^{2+}]_{cyt}$ regulation only once the peak of calcium influx is reached

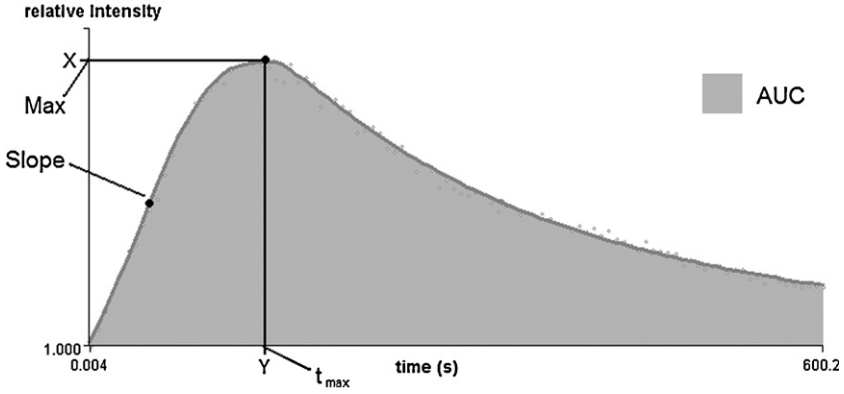

Fig. 3. Parameter values calculated for each recording. The vertical axis represents the relative fluorescent intensity of Fluo-3/AM. One unit of the area under the curve value is defined as one relative intensity value in 1 s, where relative intensity values are the rate of actual intensity values divided by intensity values at 0 s. Max: maximum value (X), t_{max}: time to reach maximum value (Y), and AUC: area under the curve.

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Table 1
Parameter values (AUC: area under the curve in units (U), Slope, Max: maximum value in relative parameter value (rpv), t_{max}: time to reach maximum value in seconds (s)) of experiments performed in the presence ('ER + CRAC contribution'), or in the absence of extracellular calcium ('ER contribution') in lymphocytes obtained from 10 healthy individuals. To determine the 'CRAC contribution', parameter values of 'ER contribution' were subtracted from parameter values of 'ER + CRAC contribution'. Subsequently, the ratio of contribution of the two sources ('CRAC/ER ratio') was determined. Data are expressed as median and range. CD4+ CXCR3+: Th1 subset, CD4+ CCR4+: Th2 subset.

Subset	Parameter	ER + CRAC contribution	ER contribution	CRAC contribution	CRAC/ER ratio
Th1 lymphocytes (CD4+ CXCR3+)	AUC (U)	460 [201–779]	98 [65–218]	362 [136–561]	3.7
	Slope	0.006 [0.002–0.011]	0.002 [0.001–0.003]	0.004 [0.001–0.008]	2
	Max (rpv)	2.130 [1.486–2.636]	1.238 [1.199–1.459]	1.892 [1.287–2.177]	3.7
	t_{max} (s)	473 [281–600]	358 [267–600]	115 [14–600]	0.3
Th2 lymphocytes (CD4+ CCR4+)	AUC (U)	312* [211–454]	93 [58–114]	219 [153–340]	2.4
	Slope	0.003* [0.002–0.005]	0.001 [0.001–0.002]	0.002 [0.001–0.003]	2
	Max (rpv)	1.732* [1.492–2.209]	1.262 [1.226–1.435]	1.470 [1.266–1.774	1.8
	t_{max} (s)	425 [285–600]	314 [235–600]	111 [50–600]	0.4

* $p < 0.05$ vs. the corresponding parameter of the CD4+ CXCR3+ subset.

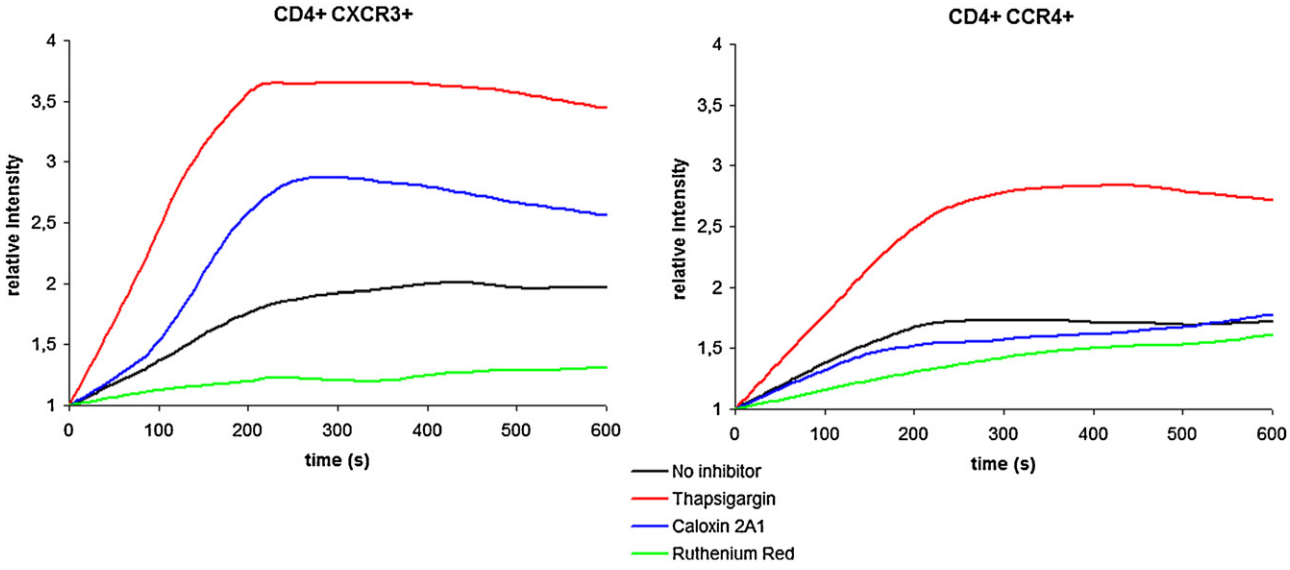

Fig. 4. Representative calcium responses upon treatment with inhibitors. Records of samples from one donor. The vertical axis represents the relative fluorescent intensity of Fluo-3/AM. CD4+ CXCR3+: Th1 subset, and CD4+ CCR4+: Th2 subset.

Table 2
Effects of thapsigargin, caloxin 2A1 and ruthenium red on parameter values (AUC: area under the curve in units (U), Slope, Max: maximum value in relative parameter value (rpv), t_{max}: time to reach maximum value in seconds (s)) of calcium influx kinetics in lymphocytes obtained from 10 healthy individuals in the presence of extracellular calcium. Data are expressed as median and range. Inhibitor treated samples were compared with samples with no inhibitor treatment. CD4+ CXCR3+: Th1 subset, CD4+ CCR4+: Th2 subset, ns: not significant, $p < 0.05$.

Subset	Parameter	No inhibitor	Thapsigargin		Caloxin 2A1		Ruthenium red	
CD4+ CXCR3+	AUC (U)	460 [201-779]	1105 [691-2411]	+140%	504 [373-828]	+10%	123 [63-544]	−73%
	Slope	0.006 [0.002–0.011]	0.011 [0.007–0.021]	+83% (+0.005)	0.008 [0.003–0.018]	ns	0.002 [0.001–0.009]	−66%(−0.004)
	Max (rpv)	2.130 [1.486–2.636]	3.599 [2.525–7.602]	+69%	2.645 [1.890–3.168]	+24%	1.321 [1.155–2.236]	−38%
	t_{max} (s)	473 [218–600]	516 [225–600]	ns	316 [194–600]	ns	515 [145–600]	ns
CD4+ CCR4+	AUC (U)	312 [211–454]	889 [602–2057]	+184%	323 [190–537]	ns	180 [122–450]	−42%
	Slope	0.003 [0.002–0.005]	0.008 [0.006–0.022]	+166%(+0.005)	0.003 [0.002–0.006]	ns	0.002 [0.001–0.005]	ns
	Max (rpv)	1.732 [1.492–2.209]	3.017 [2.266–6.737]	+74%	1.748 [1.600–2.412]	ns	1.601 [1.310–2.092]	ns
	t_{max} (s)	425 [285–600]	491 [309–600]	ns	582 [328–600]	ns	600 [486–600]	+41%

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in Th2 cells (represented by the alteration of solely the t_{max} value upon MCU inhibition).

Effects of TG treatment on calcium flux kinetics of lymphocytes. TG elevated AUC, Slope and Max values in both Th1 and Th2 cells compared with the corresponding populations without inhibitor treatment (Fig. 4 and Table 2). These results demonstrate that TG shapes both the magnitude and kinetics of calcium clearance in Th1 and Th2 cells.

The increase of the Slope value upon TG treatment was two-fold higher in Th2 than in Th1 cells. This indicates that the initiation of ER calcium uptake is more rapid in Th2 cells. Further calcium uptake, however, in the following phase of calcium influx (around the peak) is similar in Th1 and Th2 cells, since the application of TG elevates the Max value to a similar extent in the two subsets.

Effects of caloxin 2A1 treatment on calcium flux kinetics of lymphocytes. We demonstrated alterations in calcium flux properties induced by caloxin 2A1 in the Th1 lymphocyte subset. The Max value was elevated by 24% upon incubation of the samples with caloxin 2A1 compared with samples without inhibitor treatment (Fig. 4 and Table 2). These results indicate that caloxin 2A1 induces alterations in the peak phase of the calcium response instead of the ascending slope.

Interestingly, Th2 cells are unaffected by caloxin 2A1 treatment. The PMCA pump plays a role in $[Ca^{2+}]_{cyt}$ clearance only in Th1 cells during the first 10 min of lymphocyte activation, and is restricted to the phase when the peak of calcium influx is reached.

The applied concentrations of the inhibitors were set based on literary data (Chaudhary et al., 2001; Hajnóczky et al., 2006; Matlib et al., 1998; Szewczyk et al., 2008; Thastrup et al., 1990; Xu et al., 1999) and on preliminary control measurements regarding inhibitory efficacy.

Discussion

Dissimilar $[Ca^{2+}]_{cyt}$ patterns upon lymphocyte activation were suggested to influence cytokine production in Th1 and Th2 cells differently, since the distinct variations of calcium signal are associated with the activation of a particular set of transcription factors (Abbott et al., 1998; Barve et al., 1994; Wenner et al., 1997).

In this study, we assessed the functionality of the ER calcium release, the CRAC channel, the MCU, the SERCA pump and the PMCA pump in order to evaluate the individual contribution of these mechanisms regulating calcium flux properties during the first 10 min of T lymphocyte activation. Our results indicate characteristic differences in the kinetics of calcium flux between Th1 and Th2 cells.

The role of ER calcium release and calcium entry through CRAC channels in the elevation of $[Ca^{2+}]_{cyt}$ *during lymphocyte activation*. To differentiate between the individual contribution of ER calcium release and calcium entry via CRAC channels to the elevation of $[Ca^{2+}]_{cyt}$ during lymphocyte activation, we performed experiments both in the presence and in the absence of extracellular calcium. When calcium was absent from the extracellular milieu, the contribution of CRAC channels to the elevation of $[Ca^{2+}]_{cyt}$ was ruled out. Calcium release from the ER was, however, elicited in the same way. Therefore, the differences seen between the two sets of recordings reflect the contribution of CRAC channels to the elevation of $[Ca^{2+}]_{cyt}$ upon lymphocyte activation.

Stromal interaction molecule 1 (STIM1) acts as the ER-resident calcium sensor in the signal-transduction mechanism of store operated calcium entry. The calcium release-activated calcium modulator 1 molecule (Orai1) is the pore-forming subunit of CRAC channels. Following TCR engagement and ER calcium store depletion, calcium unbinds from STIM1's low-affinity luminal EF-hand domain. This triggers STIM1 to oligomerize and to translocate to the plasma membrane where it activates Orai1 to form the functional CRAC channel (Vig and Kinet, 2009).

Our results indicate that calcium entering the cell from the extracellular space is generally of major importance in the elevation of $[Ca^{2+}]_{cyt}$ compared with the ER release in lymphocytes. However, a difference can be observed in the dominance of CRAC channels between Th1 and Th2 cells. Based on our results, the amount of calcium released from the ER was similar in both subsets. Nevertheless, the CRAC/ER ratio was higher for both the AUC and Max values in Th1 cells. We could not detect such a difference in case of the Slope value, and the CRAC/ER ratio was also similar in the two subsets for the t_{max} value. The higher contribution of CRAC channels in the Th1 subset is closely related to the regulation of CRAC channels by MCU.

The role of MCU in calcium flux regulation of Th1 and Th2 lymphocytes. Based on our measurements, mitochondrial calcium uptake is also likely to have different properties in Th1 and Th2 cells. Mitochondria seem to be responsible for the uptake of a larger amount of $[Ca^{2+}]_{cyt}$ in Th1 than in Th2 cells, since the specific inhibition of the mitochondrial calcium uniporter channel (MCU) affects the AUC value to a greater extent in the Th1 subset. Since mitochondrial calcium uptake regulates CRAC channel function, it is almost self-explanatory why the CRAC/ER ratio is higher in Th1 cells in the light of these findings. Since mitochondria sequester a larger amount of calcium in the proximity of CRAC channels in Th1 cells, the calcium induced negative feedback is lower, and therefore CRAC channels function more actively in this subset than in Th2 cells. This is a contributing factor to the generally lower level of calcium flux upon activation in the Th2 subset.

As already referred to in the introduction, mitochondria have a dual role in shaping the calcium signal: besides their regulatory role on CRAC channel activity, they may also take up and store calcium to decrease $[Ca^{2+}]_{cyt}$, especially at extremely high $[Ca^{2+}]_{cyt}$ levels. However, since the AUC value and other investigated parameters did not increase, but rather decreased upon RR treatment, we could not demonstrate this effect in our experiments during the first 10 min of T lymphocyte activation.

RR is also one of the most potent inhibitors of ER calcium release (used at a concentration above 20 μM) through the inhibition of ryanodine receptors. In contrast to IP_3, which elicits a transient $[Ca^{2+}]_{cyt}$ peak, ryanodine receptor-mediated endoplasmic calcium release results in a long-term calcium signal (Dolmetsch et al., 1998). This property of RR may contribute to its effect that decreases AUC besides its inhibitory action on mitochondrial calcium uptake and the regulation of CRAC (Dammermann and Guse, 2005; Langhorst et al., 2004).

The role of the SERCA and PMCA pumps in calcium clearance of Th1 and Th2 lymphocytes. Previously, Fanger et al. (2000) observed that $[Ca^{2+}]_{cyt}$ increases during lymphocyte activation to a lower extent in Th2 compared to Th1 lymphocytes. Our earlier data obtained in human samples do support this finding (Toldi et al., 2010). The present results also indicate that AUC, Slope and Max values are significantly lower in Th2 cells compared with Th1 cells upon the application of the same stimulus to activate T lymphocytes. Fanger and colleagues argue that the most likely cause for a more rapid calcium clearance by Th2 cells is the differential activity of the PMCA. Our current results do support this notion as seen from the difference between the two subsets upon caloxin 2A1 treatment. Furthermore, other findings suggest that PMCA activity is responsible for the removal of the majority of calcium from the cytoplasm in lymphocytes (Donnadieu et al., 1992).

The harmonized function of the SERCA pump and the PMCA pump in calcium clearance of Th1 and Th2 lymphocytes. The individual contribution of the SERCA and PMCA pumps is delicately orchestrated in T lymphocytes in time and space in order to adequately shape

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$[Ca^{2+}]_{cyt}$ level kinetics upon activation. The SERCA pump has an important role already in the resting state of the cells as it counterbalances calcium leakage from the ER. Therefore, this pump functions right from the initiation of lymphocyte activation and the elevation of $[Ca^{2+}]_{cyt}$ in both investigated lymphocyte subsets. This is represented by the fact that its inhibition influences the Slope value. The regulatory role of ER calcium uptake in counterbalancing $[Ca^{2+}]_{cyt}$ elevation is more accentuated in Th2 cells, and this mechanism is activated more rapidly in this subset at the initial increase of $[Ca^{2+}]_{cyt}$. Furthermore, the rise in the AUC value was higher upon TG treatment in the Th2 subset, thus contributing to the clearance of a greater amount of $[Ca^{2+}]_{cyt}$ as previously noted by Fanger et al. (2000).

The higher activity of the SERCA pump, along with the lower activity of mitochondrial calcium reuptake, and therefore of the CRAC channels account for the notion that Th2 cells go through a generally lower level of lymphocyte activation (and altered cytokine production pattern) compared with Th1 cells upon identical activating stimuli (Crabtree and Clipstone, 1994) (Fig. 1).

In contrast to the SERCA pump, the PMCA pump in Th1 cells has a role in the shaping of $[Ca^{2+}]_{cyt}$ level kinetics from a stage when $[Ca^{2+}]_{cyt}$ is already elevated, and not right from the initiation of calcium influx, thus ensuring the reconstitution of the original $[Ca^{2+}]_{cyt}$ level. This is represented in our results by the fact that the inhibition of these mechanisms affects the Max values but not the Slope value in this subset. The main regulator of the PMCA pump is the elevated level of $[Ca^{2+}]_{cyt}$ (Di Leva et al., 2008). As already discussed, calcium reuptake by ER is activated more rapidly in Th2 cells, thus $[Ca^{2+}]_{cyt}$ will not increase to the extent observed in Th1 cells (Fig. 4). Therefore, it seems that $[Ca^{2+}]_{cyt}$ will not be elevated sufficiently in the Th2 subset to activate the PMCA pump during the investigated first 10 min of activation. In contrast, in Th1 cells, due to the lower extent of ER reuptake, $[Ca^{2+}]_{cyt}$ will increase sufficiently to activate the PMCA pump. Thus, the contribution of ER to $[Ca^{2+}]_{cyt}$ clearance is more significant at the initiation of calcium response upon lymphocyte activation. This mechanism is more accentuated in the Th2 subset. The initial calcium uptake of the ER has an important regulatory effect on the function of the PMCA pump. This pump rather contributes to $[Ca^{2+}]_{cyt}$ clearance in the peak phase of calcium flux.

We faced some limitations evaluating our results compared with previous studies in the field. First, due to the large number of comparisons, the nominal level of the probability of type 1 error is far beyond 5 per cent, therefore our results should be regarded as exploratory ones. We report here nominal *p*-values without adjustment for multiplicity. The study was not powered for the multitude of statistical tests we performed, thus some significances could occur by chance alone. Second, we did not perform any patch-clamp measurements regarded as the golden standard for investigation of ion channels. The use of patch-clamp technique is limited by the fact that it describes the significance of ion channels solely in a single cell setting, and is not suitable to investigate the functionality of these channels in a complex cellular milieu that contains different types of interacting immune cells. Therefore, we consider that the novel method we used provides new insight into the mechanisms of calcium clearance in lymphocytes from a functional, rather than a descriptional perspective. Thus, our method has the advantage over patch-clamp that it preserves physiological cell–cell interactions during the measurements.

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 handling. 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 (Annunziato et al., 1998, 1999; Yamamoto et al., 2000).

Delicate differences exist in the activity of mechanisms regulating calcium flux in the Th1 and Th2 lymphocyte subsets. Differential calcium signalling and distinct kinetics of the alterations of $[Ca^{2+}]_{cyt}$ may have an important contributing role to the production of dissimilar cytokines by these two subsets. The distinct properties of the elicited calcium signal influence the expression of cytokine genes to a great extent. Further research needs to be carried out to determine the exact characteristics of calcium flux kinetics required for the cellular production of particular types of cytokines. These results would probably also contribute to the identification of novel methods of immunomodulation targeting mechanisms of calcium clearance during the course of lymphocyte activation in Th1 and Th2 cells. This approach may potentially hold beneficial therapeutic effects for the treatment of immune-mediated disease, such as autoimmune disorders.

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References

Abbott, K.L., Friday, B.B., Thaloor, D., Murphy, T.J., Pavlath, G.K., 1998. Activation and cellular localization of the cyclosporine A-sensitive transcription factor NF-AT in skeletal muscle cells. Mol. Biol. Cell 9, 2905–2916.

Annunziato, F., Galli, G., Cosmi, L., Romagnani, P., Manetti, R., Maggi, E., Romagnani, S., 1998. Molecules associated with human Th1 or Th2 cells. Eur. Cytokine Netw. 9, 12–16.

Annunziato, F., Cosmi, L., Galli, G., Beltrame, C., Romagnani, P., Manetti, R., Romagnani, S., Maggi, E., 1999. Assessment of chemokine receptor expression by human Th1 and Th2 cells in vitro and in vivo. J. Leukoc. Biol. 65, 691–699.

Barve, S.S., Cohen, D.A., De Benedetti, A., Rhoads, R.E., Kaplan, A.M., 1994. Mechanism of differential regulation of IL-2 in murine Th1 and Th2 T cell subsets. J. Immunol. 152, 1171–1181.

Chaudhary, J., Walia, M., Matharu, J., Escher, E., Grover, A.K., 2001. Caloxin: a novel plasma membrane Ca2+ pump inhibitor. Am. J. Physiol. Cell. Physiol. 280, 1027–1030.

Crabtree, G.R., Clipstone, N.A., 1994. Signal transmission between the plasma membrane and nucleus of T lymphocytes. Annu. Rev. Biochem. 63, 1045–1083.

Dammermann, W., Guse, A.H., 2005. Functional ryanodine receptor expression is required for NAADP-mediated local Ca2+ signaling in T-lymphocytes. J. Biol. Chem. 280, 21394–21399.

Di Leva, F., Domi, T., Fedrizzi, L., Lim, D., Carafoli, E., 2008. The plasma membrane Ca2+ ATPase of animal cells: structure, function and regulation. Arch. Biochem. Biophys. 476, 65–74.

Dolmetsch, R.E., Xu, K., Lewis, R.S., 1998. Calcium oscillations increase the efficiency and specificity of gene expression. Nature 392, 933–936.

Donnadieu, E., Bismuth, G., Trautmann, A., 1992. Calcium fluxes in T lymphocytes. J. Biol. Chem. 267, 25864–25872.

Duchen, M.R., 2000. Mitochondria and calcium: from cell signalling to cell death. J. Physiol. 529, 57–68.

Fanger, C.M., Neben, A.L., Cahalan, M.D., 2000. Differential Ca2+ influx, KCa channel activity, and Ca2+ clearance distinguish Th1 and Th2 lymphocytes. J. Immunol. 164, 1153–1160.

Feske, S., 2007. Calcium signalling in lymphocyte activation and disease. Nat. Rev. Immunol. 7, 690–702.

Gilabert, J.A., Parekh, A.B., 2000. Respiring mitochondria determine the pattern of activation and inactivation of the store-operated Ca(2+) current I(CRAC). EMBO J. 19, 6401–6407.

Hajnóczky, G., Csordás, G., Das, S., Garcia-Perez, C., Saotome, M., Sinha Roy, S., Yi, M., 2006. Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca2+ uptake in apoptosis. Cell Calcium 40, 553–560.

Hoth, M., Fanger, C.M., Lewis, R.S., 1997. Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. J. Cell Biol. 137, 633–648.

Langhorst, M.F., Schwarzmann, N., Guse, A.H., 2004. Ca2+ release via ryanodine receptors and Ca2+ entry: major mechanisms in NAADP-mediated Ca2+ signaling in T-lymphocytes. Cell. Signal. 16, 1283–1289.

Matlib, M.A., Zhou, Z., Knight, S., Ahmed, S., Choi, K.M., Krause-Bauer, J., Phillips, R., Altschuld, R., Katsube, Y., Sperelakis, N., Bers, D.M., 1998. Oxygen-bridged

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dinuclear ruthenium amine complex specifically inhibits Ca2+ uptake into mitochondria in vitro and in situ in single cardiac myocytes. J. Biol. Chem. 273, 10223–10231.
Parekh, A.B., 2007. Functional consequences of activating store-operated CRAC channels. Cell Calcium 42, 111–121.
Panyi, G., 2005. Biophysical and pharmacological aspects of K+ channels in T lymphocytes. Eur. Biophys. J. 34, 515–529.
Quintana, A., Schwarz, E.C., Schwindling, C., Lipp, P., Kaestner, L., Hoth, M., 2006. Sustained activity of calcium release-activated calcium channels requires translocation of mitochondria to the plasma membrane. J. Biol. Chem. 281, 40302–40309.
Szewczyk, M.M., Pande, J., Grover, A.K., 2008. Caloxins: a novel class of selective plasma membrane Ca2+ pump inhibitors obtained using biotechnology. Pflugers Arch. 456, 255–266.
Thastrup, O., Cullen, P.J., Drøbak, B.K., Hanley, M.R., Dawson, A.P., 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase. Proc. Natl. Acad. Sci. U.S.A. 87, 2466–2470.
Toldi, G., Treszl, A., Pongor, V., Gyarmati, B., Tulassay, T., Vasarhelyi, B., 2010. T-lymphocyte calcium influx characteristics and their modulation by Kv1.3 and IKCa1 channel inhibitors in the neonate. Int. Immunol. 22, 769–774.
Vig, M., Kinet, J.P., 2009. Calcium signaling in immune cells. Nat. Immunol. 10, 21–27.
Wenner, C.A., Szabo, S.J., Murphy, K.M., 1997. Identification of IL-4 promoter elements conferring Th2-restricted expression during T helper cell subset development. J. Immunol. 158, 765–773.
Xu, L., Tripathy, A., Pasek, D.A., Meissner, G., 1999. Ruthenium red modifies the cardiac and skeletal muscle Ca(2+) release channels (ryanodine receptors) by multiple mechanisms. J. Biol. Chem. 274, 32680–32691.
Yamamoto, J., Adachi, Y., Onoue, Y., Adachi, Y.S., Okabe, Y., Itazawa, T., Toyoda, M., Seki, T., Morohashi, M., Matsushima, K., Miyawaki, T., 2000. Differential expression of the chemokine receptors by the Th1- and Th2-type effector populations within circulating CD4+ T cells. J. Leukoc. Biol. 68, 568–574.

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