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Fine-Tuning of Regulatory T Cell Function: The Role of Calcium Signals and Naive Regulatory T Cells for Regulatory T Cell Deficiency in Multiple Sclerosis

Alexander Schwarz,* Marijana Schumacher,* Daniel Pfaff,* Kai Schumacher,* Sven Jarius,* Bettina Balint,* Heinz Wiendl, † Jürgen Haas,* and Brigitte Wildemann*

The suppressor function of regulatory T cells (Tregs) is impaired in multiple sclerosis (MS), but the mechanisms underlying this deficiency are not fully understood. As Tregs counteract the sustained elevation of intracellular calcium, which is indispensable for full activation of conventional T cells (Tcons), we hypothesized that interference with this pathway might prompt MS-related Treg dysfunction. Using single-cell live imaging, we observed that Tregs rapidly reduce Ca2+ influx and downstream signals in Tcons upon cell contact, yet differ in their potency to efficiently suppress several target cells at the same time. Strikingly, individual Tregs harboring a CD4+CD25+FOXP3+CD45RA– naive phenotype suppressed significantly more adjacent Tcons than did CD4+CD25+FOXP3+CD45RA– memory Tregs. Some constituents even completely failed to dampen Tcon Ca2+ influx and were contained exclusively in the memory subset. In accordance with their more powerful suppressive performance, the Ca2+ signature was considerably enhanced in naive Tregs in response to TCR triggering, compared with the memory counterparts. MS Tregs became defective because they lack naive subtypes and are disproportionately enriched in memory cells that have lost their inherent downregulatory activity. The Journal of Immunology, 2013, 190: 4965–4970.

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D4+CD25+FOXP3+ regulatory T cells (Tregs) are key players in maintaining immunological self-tolerance, and a functional Treg deficiency is associated with various autoimmune diseases, including multiple sclerosis (MS) (1, 2). The ability of Tregs derived from patients with MS to down-regulate both proliferation and cytokine secretion of activated conventional T cells (Tcons) in vitro is impaired, and this dysfunctional state is linked to a possibly thymic-dependent imbalance between naive and more short-lived memory cells that compose the peripheral Treg population (3–5). In addition, other abnormalities in Tregs that constitute functionally heterogeneous sub- sets are likely to contribute to the Treg defect in MS (6). Thus, recent findings demonstrate that less suppressive Tregs capable of producing IFN-γ are significantly increased in the peripheral blood of patients with MS (7) and that certain subtypes of patient-derived memory Tregs exhibit a downscaled suppressive performance in response to distinct T cell–activating stimuli (8). Notably, the defective Treg state is ameliorated under disease-modifying treatment, indicating that pharmacological modulation of Tregs might prove to be a promising strategy for restoring immunological homeostasis in MS (7, 9, 10). Although the molecular mechanisms that confer inhibition and reconstitution have been poorly understood, insights from an elegant, recently published study have provided compelling evidence that Tregs counteract the sustained elevation of intracellular free Ca2+ ions in target cells and thus interfere with a fundamental requirement for almost all aspects of Tcon activation (11). In that study, cocultured cell populations were used to demonstrate rapid suppression of Tcon Ca2+ signaling by Tregs, followed by downregulation of the Ca2+-dependent transcription factor NFAT1 and NF-κB as important downstream events. However, using this approach, the authors could not elucidate in detail how Tregs interact with individual surrounding Tcons, and it remains unknown whether cells composing the Treg subset differ in their overall or individual ability to suppress Ca2+ influx in target cells.

In this article, we hypothesized that a difference in the ability to downregulate Ca2+ influx in effector T cells might affect the suppressive performance of total Tregs and might be involved in the Treg defect that can be detected in patients with MS. We established an in-house, single-cell live imaging assay by which we could directly monitor Ca2+ signatures in a multitude of individual cells exhibiting either a CD4+CD25+FOXP3+ Treg or a CD4+CD25–FOXP3– Tcon phenotype and closely monitor the contact-dependent impact of a single Treg on Ca2+ signaling in one or more adjacent Tcons. Because we and others have shown

*Division of Molecular Neuroimmunology, Department of Neurology, University Hospital Heidelberg, 69120 Heidelberg, Germany; and †Department of Neurology, University Hospital Munster, 48149 Munster, Germany

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Address correspondence and reprint requests to Dr. Brigitte Wildemann, Division of Molecular Neuroimmunology, Department of Neurology, University Hospital Heidelberg, INF 350, D-69120 Heidelberg, Germany. E-mail address: brigitte.wildemann@med.uni-heidelberg.de

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Abbreviations used in this article: CIS, clinically isolated syndrome suggestive of multiple sclerosis; HC, healthy control donor; MS, multiple sclerosis; Tcon, conventional T cell; Treg, regulatory T cell.

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previously that homeostatic changes in the Treg compartment affect total Treg function (3–5). Tregs were further separated according to surface expression of CD45RA in naive and memory Treg subtypes. We used this experimental approach to screen for differences in the mode of suppression between cells isolated from peripheral blood of healthy donors versus those obtained from MS patients. The recorded Ca2+ signaling components were correlated with the subordinate nuclear translocation of NFAT that propagates the Ca2+ signal to the nucleus to initiate gene transcription, as, for example, for IFN-γ or IL-2 (12). These readouts were complemented by assessing proliferative responses and cytokine release of TCR-triggered Tcons.

Materials and Methods

Human samples

Peripheral blood samples (40–50 ml) were obtained from 19 healthy control donors (HC, mean age: 33.6 y; range: 21–55 y) and from 13 patients with relapsing-remitting MS (n = 9) or a clinically isolated syndrome (CIS, n = 4) according to the revised McDonald criteria (13); means of age, 35.1 y (range: 22–56 y); previous relapses, 1.8 (range: 1–6); mean Expanded Disability Status Scale, 2.7 (range: 0–6.5); and disease duration range, 1–5 y. Ten patients had clinically active disease, and three patients were in clinical remission. None of the patients had been treated with corticosteroids within ± 3 mo prior to the time of blood sampling or had received immunomodulatory agents in the past. The protocol was approved by the University Hospital Heidelberg ethics committee, and all individuals gave written informed consent.

Cells

PBMCs were isolated by density-gradient centrifugation with Ficoll-Hypaque (Biochrom AG, Berlin, Germany). Tregs were isolated using a Regulatory CD4+CD25+ T Cell Kit (Invitrogen). The remaining Treg-depleted PBMCs were retained and, if required, Tcons were additionally isolated from PBMCs by a CD4+ T Cell Negative Isolation Kit (Invitrogen). Prestimulated Tregs or Tcons were induced with soluble anti-CD3 (1 μg/ml, clone OKT3; eBioscience) and anti-CD28 (0.5 μg/ml, clone CD28.2; eBioscience) mAbs, followed by 24 h of incubation at 37°C and 5% CO2 in 96-well plates in 200 μl culture medium supplemented with penicillin-streptomycin and 5% FCS in 96-well plates in 200 μl culture medium (RPMI 1640, supplemented with penicillin-streptomycin and 5% FCS). The same conditions were chosen for coculture experiments with 24 h of incubation of Tregs and PBMCs (ratio: 1:2). For calcium imaging, cells were loaded with Fura-2 AM (Life Technologies) and supplemented with 0.1% FCS (PAA) and reduced HEPES (Invitrogen) in culture medium (supplemented with HEPES) at room temperature for 25 min and washed with fresh medium. Cells were either immediately used or stored at 4°C for ≥ 3 h.

Live-cell imaging

In coculture assays with prestimulated Tregs, IL-2 and IFN-γ mRNA production in Tcons is considerably suppressed within hours after TCR stimulation (14). As IL-2 and IFN-γ transcription critically depends on the effectual activation of the preceding Ca2+/NFAT pathway, we established a live-cell imaging protocol to determine the impact of prestimulated Tregs on Ca2+ signaling and subordinate nuclear translocation of NFAT in Tcons. The setting of the live-cell imaging system is schematically shown in Supplemental Fig. 1.

With this setup, isolated Tregs can be reunited with PBMCs or Tcons on site that had been prestimulated for 24 h with anti-CD3 and anti-CD28 mAbs were allowed to adhere to a poly-L-lysine-coated (0.1 mg/ml; Sigma-Aldrich) glass coverslip at room temperature in a sandwiched, self-made chamber, which permits solution exchanges within < 1 s. Subsequently, PBMCs or Tcons were inserted into the chamber; they descended to the bottom and occupied the interspace between Tregs, thereby forming random cell–cell contacts (Supplemental Fig. 2, Supplemental Video 1, Supplemental Video 2). Excitation and emission wavelengths were removed by a single flood with buffer solution after 6 min. TCR triggering with anti-CD3 mAb followed 2 min later. The measurement chamber was then placed on a Nikon Eclipse Ti inverted microscope equipped with a 20× S Fluor objective (numerical aperture: 0.75) and additional 1.5× intermediate enlargement, resulting in a 30× optical magnification. Experiments were performed in HBSS medium with Ca2+ and Mg2+, but no phenol red (Life Technologies), and supplemented with 0.1% FCS (PAA) and reduced l-glutathione, 1 mg/l (Roth). Cells were alternately illuminated (Intensilight; Nikon) at 340 nm and 387 nm with an automatic filter wheel exchange (λ 10-2; Sutter). The emission signals at 468–550 nm were recorded with a charge-coupled device camera (ORCA-AG, Hamamatsu).

Data analysis

For data analysis, single-cell signals were extracted from the raw data with NIS-Elements AR (Nikon). Data were processed to determine characteristic signaling patterns individually, using Igor Pro (WaveMetrics). Statistical analysis was performed with Excel (Microsoft) and IBM SPSS Statistics software, version 20.0 (SPSS, Chicago, IL). A nonparametric test (two-tailed Mann–Whitney U test) was applied for statistical analysis of Ca2+ data. Fisher’s exact test was used to examine the significance of categorical data. A p value < 0.05 was considered significant.

Calcification of cell populations and NFAT localization

To classify the different cell populations and the subcellular distribution of NFAT, cells were immediately fixed (Fixation & Permeabilization Buffer; eBioscience) on the microscope stage after the experiment, permeabilized, and stained with fluorescent mAbs obtained from BD Pharmingen (anti-human CD3 Pacific blue, anti-human CD4 PerCP), Dako (anti-human CD25, PE), eBioscience (anti-human FOXP3-APC-F647), Santa Cruz Biotechnology (anti-human NFATc1-APC488), and BD Pharmingen (CD45RA APC-H7). Nuclei were stained with DAPI (Sigma-Aldrich). For epifluorescence, microscopy standard filter sets (AHF; Analysetechnik) were used (Supplemental Fig. 3).
Results

Tregs rapidly reduce Ca\(^{2+}\) influx and downstream signals in Tcons upon cell contact

We used single-cell live imaging to analyze the influence of Tregs on cell contact–dependent Ca\(^{2+}\) signaling in Tcons (Fig. 1). Tcons derived from HC (n = 11) were TCR triggered in the presence of prestimulated syngeneic Tregs (2:1 ratio), and Ca\(^{2+}\) influx was comparatively assessed in individual Tcons with (n = 361) or without (n = 499) close contact with Tregs (as defined by overlapping CD4 surface-staining signals). We found that intimate contact with Tregs was essential to suppress Ca\(^{2+}\) signaling and Ca\(^{2+}\)-driven nuclear import of NFAT in Tcons. In contrast, calcium signaling was either not downregulated ([Ca\(^{2+}\)]\(_{max}\), [Ca\(^{2+}\)]\(_{auc}\)) or only marginally downregulated ([Ca\(^{2+}\)]\(_{osc}\), [Ca\(^{2+}\)]\(_{t-osc}\)) in Tcons that did not have direct contact with Tregs (as shown, for example, in Fig. 2; data given in Fig. 3).

Ca\(^{2+}\) signals in Tcons not having contact with Tregs did not significantly differ from Tcons that were stimulated in the absence of Tregs. We did not observe any effects on Ca\(^{2+}\) signals when Tcons were TCR triggered in the presence of non-prestimulated Tregs or if prestimulated Tcons were used instead of prestimulated Tregs (not depicted).

We next aimed to verify whether the experimental conditions were appropriate for inhibiting functional consequences of T cell activation. PBMCs were TCR stimulated alone or in the presence of HC Tregs (n = 4). Tregs efficiently suppressed IL-2 and IFN-\(\gamma\) (Fig. 3C) production by Tcons as early as 24 h after stimulation, as determined by serial assessment of suppression supernatants.

Both Treg-mediated inhibition of Tcon Ca\(^{2+}\) signaling and calcium influx in Tregs upon TCR triggering are reduced in MS

To test the hypothesis that the functional Treg defect in MS is linked to an impaired interference with Ca\(^{2+}\) signaling in target
Tcons, we used single Tcons and Tregs obtained from MS patients ($n = 9$) and HCs ($n = 9$) to determine whether cell contact-dependent Ca$^{2+}$ influx was reduced in Tcons. In total, 1539 cell clusters composed of one single Treg and one to six surrounding Tcons were assessed. On average, HC-derived Tregs downregulated the maximum Ca$^{2+}$ influx in the sum of Tcons recorded by 61.9 ± 23.4%. When using cells from MS patients, the mean suppression of [Ca$^{2+}$]$_{\text{max}}$ mediated by Tregs was clearly reduced (38.0 ± 15.4%, $p = 0.02$; Fig. 4A).

To elucidate this difference in more detail, we next determined the number of Tcons that were suppressed by a single Treg. A single Tcon was classified as “suppressed” when its maximum Ca$^{2+}$ influx was reduced by ≥ 15% upon forming cell contact with one Treg. When using HC-derived cells, almost three fourths (72.6 ± 4.7%) of Tcons displaying close contact with the index Treg became rapidly suppressed (Fig. 4B), thereby demonstrating the ability of Tregs to simultaneously inhibit more than one target cell at the same time. The number of adjacent Tcons per single Treg had no impact on Treg inhibition rates and did not differ between study cohorts (HC: mean, 2.8 ± 0.6 Tcons per Treg; MS: mean, 3.2 ± 0.5 Tcons per Treg; $p = 0.23$). Strikingly, MS Tregs suppressed significantly fewer surrounding Tcons, and a reduction in [Ca$^{2+}$]$_{\text{max}}$ could be detected only in 63.8 ± 10.3% Tcons ($p = 0.04$; Fig. 4B). Notably, a small contingent of Tregs completely failed to reduce [Ca$^{2+}$]$_{\text{max}}$ in any surrounding Tcons, and this fraction of “nonsuppressors” was significantly higher among MS Tregs (mean: 24.4 ± 11.8%) than HC Tregs (mean: 13.6 ± 5.1%; $p = 0.05$; Fig. 4C). As a contrasting observation, half of HC Tregs (53.0 ± 8.7%), but only 31.6 ± 13.7% of MS Tregs, categorically diminished [Ca$^{2+}$]$_{\text{max}}$ in every adjacent Tcon ($p = 0.02$).

When percentages of “nonsuppressors” were plotted against the percentages of mean [Ca$^{2+}$]$_{\text{max}}$ obtained from intraindividual Tcons, we found a clear, negative correlation between overall Treg suppressive capacities and the prevalence of “nonsuppressors” ($r^2 = 0.44$; Fig. 4D).

To compare activation-induced Ca$^{2+}$ influx in Tregs and Tcons from the two study cohorts, we separately assessed monocultures of TCR-triggered Tregs ($n = 600$) and Tcons ($n = 800$) (obtained from 9 MS patients, 4 CIS patients, and 13 HCs). We found that Ca$^{2+}$ signals were significantly downscaled in MS Tcons, whereas the difference in Tcon Ca$^{2+}$ signaling between study groups was not significant (Fig. 4E).

Parallel data from in vitro proliferation assays and live imaging of single cells obtained from the same donor were available from six patients and six HCs. As expected, HC Tregs suppressed the proliferative immune responses of Tcons much better than did MS Tregs (Fig. 4F).

**CD45RA$^+$** naive Tregs exhibit superior suppressive capacities compared with memory Tregs and are reduced in MS

We next determined inhibitory potencies of naive and memory Treg subtypes at the single-cell level. We assessed Ca$^{2+}$ signaling patterns, together with intracellular expression of FOXP3 and surface expression of CD4, CD25, and CD45RA, in 555 individual
Tregs (obtained from five HCs and five patients). We found that CD4+CD25+FOXP3+CD45RA+ Tregs suppressed significantly more adjacent Tcons than did CD4+CD25+FOXP3+CD45RA- memory Tregs (74.5 ± 21.1% versus 62.0 ± 25.3%; p < 0.001; Fig. 5A). Of note, nonsuppressive cells were exclusively present in the memory fraction, whereas three fourths of cells in the naive fraction showed high suppressive activity, with 50–100% suppressed Tcons (Fig. 5B). As another prominent feature, Ca2+ signatures recorded in TCR-triggered naive Tregs revealed considerably higher values of Ca2+ influx than did those in memory Tregs (Fig. 5C). Furthermore, naive Tregs exhibited lower FOXP3 expression levels than did Tregs harboring a CD45RA- memory phenotype (Fig. 5D). Whether memory Tregs or naive Tregs were obtained from HC donors or MS patients had no influence on individual suppressive capacities, calcium signatures, or FOXP3 expression levels. In contrast, MS Tregs showed markedly lower numbers of CD45RA+ cells than did HC Tregs (HC: 37.1 ± 9.2% versus MS: 25.3 ± 11.9%; p < 0.05; Fig. 5E). This finding was confirmed by parallel flow cytometric data (available from eight patients and six HCs), revealing reduced frequencies of CD45RA+ naive and CD31+CD45RA+ recent thymic emigrant cells among circulating MS Tregs (naive Tregs: MS, 26.7 ± 10.4%, versus HC, 34.0 ± 6.6%; p = 0.01; recent thymic emigrant Tregs: MS, 2.6 ± 1.9%, versus HC, 4.9 ± 2.8%; p = 0.04).

**Discussion**

We hypothesized that interference with Ca2+ signaling in Tcons as a pivotal mechanism used by Tregs to confer suppression of Tcon activation might differ among Treg subsets and might contribute to the Treg defect that has been reported for MS and other autoimmune diseases (15). Our data show that normal human Tregs rapidly reduced TCR-triggered Ca2+ signaling and activation of NFAT in Tcons, thereby prompting the inhibition of Tcon immune activity, as reflected by substantially diminished IL-2 and IFN-γ release and dampened proliferation. These findings are well in line with the results of a recent study that used cocultured, purified T cell populations instead of single cells to demonstrate rapid suppression of Tcon Ca2+ signaling by Tregs, followed by down-regulation of NFAT1 and NF-kB (11). The fast kinetics of cytokine suppression observed in our study is also consistent with the results of a previous study showing that Tregs inhibit the induction of Th1 cytokine mRNA in Tcons as early as 1 h after TCR activation (14). Calcium influx suppression was most pronounced when Tcons were in close contact with Tregs, supporting the model of contact dependency that was originally deduced from proliferation assays (16, 17) and reinforced by the more recent demonstration of gap junction formation between Tregs and Tcons (18).

Next, we comparatively assessed the impact of Tregs on Tcon Ca2+ signaling, using cells derived from HCs and from MS patients to elucidate in more detail how Tregs confer suppression at the single-cell level. Notably, although Tregs consistently prompted downregulation of Ca2+ influx in the large majority of neighboring Tcons in the HC group, the number of Tcons exhibiting a clear decrease in Ca2+ signals when located in direct cell contact with a single Treg was significantly decreased in MS, thus explaining the diminished performance of MS Tregs to downregulate mean calcium signals recorded from Tcons. This finding was paralleled by a marked increase in the percentage of cells among patient-derived Tregs that completely failed to suppress Ca2+ signaling in adjacent Tcons. These abnormalities are in line with an earlier report demonstrating that only a minority of Treg clones derived from MS patients exhibit regulatory activity (19). That and other previous studies have shown that the reduced inhibitory capacity of Tregs in MS is based on a defective performance of Treg or Treg subsets (7), and these investigations did not find evidence of an abnormal resistance of Tcons toward Treg-mediated suppressive signals (19–21). In line with this knowledge, we did not detect a higher activation state of TCR-triggered MS Tcons with respect to Ca2+ influx in single cells and proliferation. Thus, our data show that loss of inhibitory function in MS is not evenly distributed within the Treg population as a whole.

When we screened for distinguishing features between suppressive and nonsuppressive cells, we found that CD45RA+ naive cells were enriched in the fraction of Tregs classified as strongly suppressive. Consistent with the reduced proportions of strongly suppressive cells, MS-derived Tregs contained significantly fewer naive cells than did HC Tregs, which is well in line with earlier reports on disturbed T cell and Treg homeostasis in MS (3–5, 9, 10, 22, 23). Naive Tregs featured lower FOXP3 expression, naive Tcell contact with a single Treg was significantly decreased in MS, thus explaining the diminished performance of MS Tregs to downregulate mean calcium signals recorded from Tcons. This finding was paralleled by a marked increase in the percentage of cells among patient-derived Tregs that completely failed to suppress Ca2+ signaling in adjacent Tcons. These abnormalities are in line with an earlier report demonstrating that only a minority of Treg clones derived from MS patients exhibit regulatory activity (19). That and other previous studies have shown that the reduced inhibitory capacity of Tregs in MS is based on a defective performance of Treg or Treg subsets (7), and these investigations did not find evidence of an abnormal resistance of Tcons toward Treg-mediated suppressive signals (19–21). In line with this knowledge, we did not detect a higher activation state of TCR-triggered MS Tcons with respect to Ca2+ influx in single cells and proliferation. Thus, our data show that loss of inhibitory function in MS is not evenly distributed within the Treg population as a whole.

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sustained functional fitness of this subtype. In agreement with this hypothesis, naïve Tregs, unlike memory Tregs, are insensitive to apoptotic elimination, remain proliferative ex vivo, and maintain full inhibitory activity after expansion (24–26).

Taken together, our data support the idea that average, Treg-mediated suppression rates, as measured by conventional in vitro proliferation assays, directly depend on the numbers of suppressed Tcons, which in turn are determined by the prevalence of highly suppressive naïve Tregs. Consequently, the homeostatic press shift toward functionally exhausted memory Tregs, which in turn are determined by the prevalence of naïve Tregs, unlike memory Tregs, are insensitive to sustained functional fitness of this subtype. In agreement with this hypothesis, naïve CD4(+)CD25(+)FOXP3(+)CD31(+) T-cells in patients with multiple sclerosis.

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Disclosures
The authors have no financial conflicts of interest.

References
Supporting Information

Figure S1

Schematic calcium life imaging experiment.

Lymphocytes adhere on a poly-L-lysine-coated glass cover slip in a sandwiched chamber, which permits solution exchanges within less than 1 sec. Excess nonadherent cells can be removed by a single flush with buffer solution. For measurement the chamber can be placed on an inverted microscope equipped with a 30X S Fluor object lens. Cells are alternately illuminated at 340 and 387 nm and emission signals at 468 - 550 nm recorded with a CCD camera.
Figure S2

Ca²⁺ imaging in classified Tcon and Treg.

Full-screen image of Fura-2 emission at 387 nm before (a) and after (b) adding PBMC to Treg. (c) Color-coded image detail of the same experiment at a single measurement point. Warmer colors (yellow/red) indicate high [Ca²⁺] while colder colors (blue/pink) indicate low [Ca²⁺]. (d) Corresponding image section with fixed and stained cells after the live-cell imaging experiment: red anti-FOXP3, blue anti-CD4, green anti-CD25.
Figure S3

Subcellular localization of NFAT.

(a) Resting PBMC were fixed and stained with anti-NFAT (green) and DAPI for the nuclei (blue). No or only minor overlay of NFAT and DAPI signals indicate the cytoplasmatic localization of NFAT in resting cells. (b) PBMC were stimulated with TG to induce sustained elevation of [Ca^{2+}]\_i and fixed after 20 minutes. Nuclear translocation of NFAT results in an overlay of the anti-NFAT and DAPI fluorescence signals.
**Video S1**

**Inhibition of Ca\(^{2+}\) signals in Tcon by prestimulated Treg.**

Color-coded live-cell imaging experiment with prestimulated Treg seeded on the cover slip of the live-cell imaging chamber followed by insertion of PBMC and T-cell stimulation with anti-CD3 mAb. Color scale covers 25 - 500 nM of [Ca\(^{2+}\)]\(_i\) concentration with colder colors (blue/pink) indicating low [Ca\(^{2+}\)]\(_i\) and warmer colors (yellow/red) indicating high [Ca\(^{2+}\)]\(_i\). The anti-CD3 stimulus induces no or only marginal elevation of [Ca\(^{2+}\)]\(_i\) in the majority of Tcon (while prestimulated Treg predominantly generate oscillatory Ca\(^{2+}\) signals). Respective annotations highlight the different stages of the ongoing experiment. A chronometer shows the elapsed time. Tcon and Treg are marked with orange (Tcon) and green (Treg) arrows. The classification of Tcon and Treg is illustrated by cross-fading of the postexperimental immunofluorescence staining at the end of the video (red anti-FOXP3, blue anti-CD4).
**Video S2**

*Effective anti-CD3 mAb induced Ca\(^{2+}\) signaling in Tcon in the absence of Treg.*

PBMC are seeded on the cover slip of the live-cell imaging chamber without Treg and stimulated with anti-CD3 mAb. Contrary to the experimental settings visualized in Video S1, anti-CD3 mAb induces substantial Ca\(^{2+}\) signals in the majority of Tcon. Data presentation corresponds to Video S1.