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DIFFERENTIAL CALCIUM SIGNALING AND Kv1.3 TRAFFICKING TO THE IMMUNOLOGICAL SYNAPSE IN SYSTEMIC LUPUS ERYTHEMATOSUS

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Abstract

Systemic lupus erythematosus (SLE) T cells exhibit several activation signaling anomalies including defective Ca^{2+} response and increased NF-AT nuclear translocation. The duration of the Ca^{2+} signal is critical in the activation of specific transcription factors and a sustained Ca^{2+} response activates NF-AT. Yet, the distribution of Ca^{2+} responses in SLE T cells is not known. Furthermore, the mechanisms responsible for Ca^{2+} alterations are not fully understood. Kv1.3 channels control Ca^{2+} homeostasis in T cells. We reported a defect in Kv1.3 trafficking to the immunological synapse (IS) of SLE T cells that might contribute to the Ca^{2+} defect. The present study compares single T cell quantitative Ca^{2+} responses upon formation of the IS in SLE, normal and rheumatoid arthritis (RA) donors. Also, we correlated cytosolic Ca^{2+} concentrations and Kv1.3 trafficking in the IS by two-photon microscopy. We found that sustained $[\text{Ca}^{2+}]_i$ elevations constitute the predominant response to antigen stimulation of SLE T cells. This defect is selective to SLE as it was not observed in RA T cells. Further, we observed that in normal T cells termination of Ca^{2+} influx is accompanied by Kv1.3 permanence in the IS, while Kv1.3 premature exit from the IS correlates with sustained Ca^{2+} responses in SLE T cells. Thus, we propose that Kv1.3 trafficking abnormalities contribute to the altered distribution in Ca^{2+} signaling in SLE T cells. Overall these defects may explain in part the T cell hyperactivity and dysfunction documented in SLE patients.

Keywords

human; T cells; autoimmunity; systemic lupus erythematosus

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1. INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic rheumatologic autoimmune disease characterized by overactive T lymphocytes [1]. The hyperactivity of SLE T cells has been linked to an exaggerated response to antigen stimulation including a more pronounced and more sustained increase in intracellular calcium levels ($[Ca^{2+}]_i$) following T cell receptor (TCR) ligation as compared to healthy T cells [2,3].

Regulated control of Ca^{2+} influx is essential for the activation and function of the adaptive immune response as Ca^{2+} is a key regulator of important transcription factors including nuclear factor of activated T cells (NF-AT) and nuclear factor- κ B (NF- κ B) [4–6]. It is well established that TCR stimulation induces heterogeneous Ca^{2+} responses varying in both amplitude and kinetics [7,8]. Some T cells respond with transient increases of $[Ca^{2+}]_i$, other with repetitive oscillations or sustained elevations. It is well established that this diversity serves to determine specificity of gene expression [7–11]. For instance, NF- κ B is activated by a short high amplitude $[Ca^{2+}]_i$ spike or infrequent oscillations. In contrast, NF-AT is activated by a Ca^{2+} signal of relatively low amplitude but of longer duration and also by frequent oscillations. These different requirements result from the different biochemical properties that control the activation/deactivation of these two transcription factors [7]. Specifically, NF-AT nuclear localization relies on phosphorylation/dephosphorylation mechanisms. These events occur within minutes, and as such frequent or sustained $[Ca^{2+}]_i$ input is required to maintain NF-AT in the nucleus. On the other hand, NF- κ B nuclear localization relies on degradation and re-synthesis of the inhibitory subunit associated with NF- κ B (I κ B). Since synthesis of new proteins takes tens of minutes, only a brief change in $[Ca^{2+}]_i$ is sufficient to maintain NF- κ B nuclear localization for a sustained period of time [7]. Interestingly, SLE T cells, which display abnormal Ca^{2+} signaling, are associated with increased NF-AT and diminished NF- κ B activity [12,13]. Importantly, the increased NF-AT activation is responsible for the overexpression of CD154 (CD40 ligand), which in turn supports B cell differentiation and autoantibody production leading to organ damage and manifestation of the disease [14,15]. Although Ca^{2+} plays such an important role in the fate and function of SLE T cells, limited information is available on the specific alterations of Ca^{2+} signaling in SLE T cells and the mechanisms underneath.

TCR-mediated Ca^{2+} response relies on the orchestrated function of ion channels and transporters. It is initiated by the depletion of Ca^{2+} from the endoplasmic reticulum which leads to influx of Ca^{2+} through the Ca^{2+} -release activated Ca^{2+} (CRAC) channels [16,17]. Ca^{2+} entry through CRAC channels is facilitated by membrane hyperpolarization, which increases the total driving force for Ca^{2+} entry, provided by Kv1.3 and KCa3.1 channels. In particular, Kv1.3 controls the membrane potential of resting human T cells and activated effector memory (T_{EM}) cells and its inhibition induces membrane depolarization and suppresses the Ca^{2+} response [18–20]. Thus anomalies associated with Kv1.3 could significantly contribute to abnormal Ca^{2+} signaling. We have reported a defect in Kv1.3 localization in the immunological synapse (IS) in SLE [21]. The IS, a highly organized signaling zone that forms at the point of contact between the T cell and an antigen presenting cell (APC), has been implicated in the regulation of Ca^{2+} signaling [22–24]. It has been speculated that localization of ion channels/transporters in the IS may modulate the development of the Ca^{2+} response [23,25,26]. We have recently shown that Kv1.3 localization in the IS prevents the development of an exaggerated Ca^{2+} response [27]. Kv1.3 channel localization in the IS is short-lived in SLE T cells, while in normal T cells Kv1.3 resides in the IS for a long time after conjugation with APCs [21]. We have hypothesized that prolonged Kv1.3 localization in the IS guarantees inhibition of the channel activity by signaling molecules recruited at this site and consequent down regulation of the Ca^{2+} response. Disruption of this regulatory mechanism in SLE affects Kv1.3 function and contributes to the alterations in Ca^{2+} homeostasis of these cells. In this study we have

performed single cell quantitative Ca^{2+} measurements to define the abnormalities in $[\text{Ca}^{2+}]_i$ kinetics of SLE T cells. Moreover, we have performed live cell imaging experiments that allowed us to simultaneously record Kv1.3 movement to the IS and $[\text{Ca}^{2+}]_i$ in order to establish a correlation between abnormal Kv1.3 localization in the IS and altered Ca^{2+} homeostasis of SLE T cells.

2. MATERIALS AND METHODS

2.1 Human Subjects

All SLE patients included in this study fulfilled the American College of Rheumatology classification criteria for SLE [28,29]. Our study included 15 SLE patients, 3 male (M) and 12 female (F), 12 African American (AA) and 3 Caucasian (C), of whom 5 were on dialysis. As disease controls 5 RA patients, 5F, 1AA and 4C were included. These patients fulfilled the American College of Rheumatology classification criteria for RA. Also, 18 normal individuals were included in this study, 14C (6M and 8F), 1 Asian (F), 1 AA (F) and 2 unknown. Studies and informed consent forms were approved by the University of Cincinnati Institutional Review Board.

2.2 Cells

CD4^+ T cells were isolated from venous blood collected from consenting normal, SLE and RA donors using E-rosetting, as previously described [21]. Memory T cells were isolated from CD4^+ cells by negative selection by FACS. The purified CD4^+ cells were stained with CD45RA-FITC and CD8-APC (BD Biosciences, San Jose, CA), and sorted based on forward and side scatter and double-negative staining (FACSVantage flow cytometer, BD Biosciences, San Jose, CA). The purity of the memory population was determined to be approximately 98%. Memory T cells consist of central memory (T_{CM}) and effector memory (T_{EM}) cells with T_{CM} cells comprising ca. 4% of the memory population [21]. T cells and Epstein-Barr Virus (EBV) infected-B cells were cultured as previously described [21,30]. Human embryonic kidney (HEK 293) cells (American Tissue Culture Collection, Manassas, VA) were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 100 U/ml penicillin and 100 g/ml streptomycin.

2.3 T cell subset characterization by flow cytometry

Freshly obtained peripheral blood was stained with the following antibodies: CD3-FITC (BD Biosciences, Mountain View, CA) and CD4-PerCP (BD Biosciences, Mountain View, CA) followed by red cell lysis with FACSlyse solution (BD Biosciences, Mountain View, CA). The resultant white cell pellet was fixed in 1% paraformaldehyde (PFA) prior to flow cytometry analysis (FACSCalibur, Becton Dickinson, San Jose, CA). Distribution of T cell subsets was determined by FACS as previously described [21].

2.4 Molecular Biology

A GFP-tagged Kv1.3 construct was generated as described in the Supplemental Methods.

2.5 Cell Transfection

CD4^+ cells were transfected using the Amaxa Nucleofector technology (Amaxa Biosystems, Cologne, Germany) using 5×10^6 T cells, 5 μg of pEGFP-Kv1.3 or vector plasmid DNA and program U-14.

2.6 Measurement of Intracellular Calcium in Single Cells

CD4^+ cells loading with Fura-2/AM (Molecular probes, Eugene, OR) and recording were performed as previously described [27,30,31]. For activation with antigen presenting cells

(APC), EBV-infected B cells pre-pulsed with SEB (SEB-B cells) or beads coated with anti-CD3/anti-CD28 antibody (CD3/CD28 beads) were added to T cells bathed in 0.5 mM Ca^{2+} Ringer solution. The cells were then allowed to interact for 15 min before 1–2 μM ionomycin was added as a positive control. Visual inspection showed formation of APC (CD3/CD28)/T cell stable conjugates in the bath. Details of the method are reported in the Supplement.

2.7 Analysis of Ca^{2+} Responses

To determine the distribution of Ca^{2+} responses we categorized the cells as (i) Non-responding: no increase in Ca^{2+} after APC addition, (ii) Transient: a brief Ca^{2+} response that returns to baseline within the 15 min duration of the experiment, (iii) Continuous: a Ca^{2+} response that is followed by a sustained plateau within the 15 min duration of the experiment and (iv) Oscillatory: a Ca^{2+} response that includes three or more peaks in $[\text{Ca}^{2+}]_i$ during the experiments. The number of cells displaying a specific response was reported as % of total responding cells. For the analysis of oscillations we used home-written macros [27,32]. Because contact between APCs and T cells occurred at various time, time points prior to 50 sec before the first oscillation was observed were excluded. The data were fitted using a third order polynomial function. This eliminated low frequency components unrelated to Ca^{2+} oscillations. Next, the data were analyzed using the fast fourier transform (FFT) algorithm, which sums the squares of the values of a set of uniformly spaced points and normalizes by the number of data points. The FFT data were then used to determine the power spectral density (PSD), which describes how the power of the signal is distributed with frequency. The PSD function ultimately provides information regarding oscillation frequency and amplitude.

2.8 Measurement of intracellular Ca^{2+} by flow cytometry

CD3⁺ T cells were loaded with 2 $\mu\text{g}/\text{mL}$ Indo-1/AM in the presence of Cell Loading Media (CLM: HBSS, containing Ca^{2+} and Mg^{2+} , and 1% FBS) for 30 minutes at 30°C. Cells were then washed, resuspended in CLM, and measured on either a Becton-Dickinson FACS Vantage or FACS Aria flow cytometer. Flow rate was maintained at 300–400 events/second during collection. To establish a baseline, events were collected for 30 seconds prior to addition of the agonist. Agonists were as follows: anti-CD3 and anti-CD28 antibody at a final concentration of 2 $\mu\text{g}/\text{mL}$ followed by 0.2 mg/mL goat-anti-mouse Immunoglobulin. Ionomycin was used as a positive control at a concentration of 2 $\mu\text{g}/\text{mL}$. Data analysis was performed using the kinetics package of FlowJo software (Tree Star, Inc).

2.9 T cell Activation and Immunocytochemistry

The pEGFP-Kv1.3 transfected T cells were stimulated with SEB-B cells loaded with Far Red Tracker DDAO dyes (Molecular Probes). Experiments were conducted as previously described and reported in more detail in the Supplemental methods [21, 26].

2.10 Simultaneous intracellular Ca^{2+} and Kv1.3 trafficking measurements by Two-Photon Time-Lapse Microscopy

Fura2-loaded pEGFP-Kv1.3 transfected CD4⁺ T cells were plated onto poly-L-lysine coated coverslips and placed in a microscope Attofluor chamber (Molecular Probes) enclosed by a dark environmental chamber (Solent Scientific Ltd, Segensworth, UK) at 37°C. Cells were imaged using a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Microimaging Inc) and a Plan-Apochromat 63X water immersion objective. Z-stacks of 1 μm intervals were captured every 30–60 sec. For green fluorescence excitation an Ar laser was used. For fura-2 imaging the Ca^{2+} free form was excited at 800 nm using the two-photon microscope laser (Ti-Sa). For these experiments we used Fura-2 as a non-ratiometric Ca^{2+} dye [33]. We recorded only the Ca^{2+} free excitation at 800 nm. This was due to the fact that at lower excitation wavelengths (700–780 nm) both the Ca^{2+} bound and free Fura-2 forms are excited, albeit to different

degrees. Consequently, Fura-2 was excited at 800 nm, which avoids excitation of the Ca^{2+} bound Fura-2 [33]. This is important as, although both Ca^{2+} bound and Ca^{2+} free Fura-2 have different excitation wavelengths, they both emit at approximately 510 nm. Of note, since the Ca^{2+} free Fura-2 emission is recorded, a decrease in fluorescence is indicative of an increase in $[\text{Ca}^{2+}]_i$. Also, at this wavelength, our clone was not excited (Supplemental Fig. 1). The “Multi Track” option was used to avoid cross-talk between channels. Images were processed using the Metamorph software [34,35]. Since optical sectioning was required to obtain all the green signal, the most representative section and its corresponding DIC image were isolated [34]. To analyze $[\text{Ca}^{2+}]_i$, a region was drawn around the cell of interest and the fluorescence was quantified. Subsequently, F_0-F/F_0 was used to obtain a pseudoratio where F is the fluorescence intensity and F_0 is the averaged prestimulus fluorescence [35]. To calculate the time of Kv1.3 arrival into the IS we approximate the minimum time of recruitment to 35 s. This approximation was made necessary by the fact that readings were taken with 35 s intervals. Thus when recruitment was observed already in the frame immediately following the image with the initial T/APC interaction (within 1 min from contact with the APC), since we did not know when during the 35 s interval the channels enter the IS, we arbitrarily assigned a time of 35 s.

2.11 Electrophysiology

Kv1.3 currents were induced in whole-cell configuration by depolarizing voltage steps from -80 mV holding potential (HP) to $+50$ mV applied every 30 s [21,36]. The composition of the external and pipette solutions are reported in the Supplemental Methods.

2.12 Statistical Analysis

All data are presented as means $\pm$ SEM. Statistical analyses were performed using Student's t-test (paired or unpaired); $p \leq 0.05$ was defined as significant.

3. RESULTS

3.1 Sustained $[\text{Ca}^{2+}]_i$ elevations constitute the predominant response to antigen stimulation of SLE T cells

Single cell quantitative Ca^{2+} studies were performed to investigate the Ca^{2+} response of SLE T cells and compare it to those in cells from normal donors and RA patients. The characteristics and drug regimen of the SLE and RA patients included in our studies are reported in Table I. Overall eleven SLE patients (Table I, patients 1–11) were included in these studies: age 41.4 ± 3.4 years; range 25–54 years. As controls we studied twelve normal subjects (age 41.6 ± 3.6 years; range 25–59 years) and five RA patients (age 53.8 ± 5.6 years; range 33–64 years). The normal and SLE donors were age matched as Ca^{2+} alterations related to age have been previously documented [37]. In addition, although our RA patients were not age matched, their age was not statistically different from that of SLE patients ($p=0.09$) and normal donors ($p=0.10$). The SLE patients enrolled displayed a significant reduction in CD4:CD8 ratio (0.8 ± 0.1 , $n=11$) as compared to healthy donors (1.7 ± 0.1 , $n=4$, $p=0.006$), attributed to a significant decrease in CD4^+ (% CD4^+ T cells 29.7 ± 3.2 , $n=11$, in SLE vs 56.4 ± 10.6 , $n=5$, in normal $p=0.006$) and an increase in CD8^+ cells (% CD8^+ T cells 41.7 ± 4.3 , $n=11$, for SLE and 21.8 ± 5.6 , $n=5$, for normal, $p=0.02$) and in agreement with previous reports [21,38,39]. We have also measured the percentage of naïve, T_{EM} and T_{CM} in the SLE patients ($n=11$), and in a fraction of controls ($n=5$). Consistently with our previous report, we have observed no change in T_{CM} or naïve T cells, while there was a significant decrease in $\text{CD8 } T_{EM}$ (from 58.5 ± 5.2 % in normal to 33.8 ± 4.5 % in SLE, $p=0.01$) and a tendency to an increase in $\text{CD4 } T_{EM}$ (59 ± 7.6 % in normal and 66.1 ± 2.9 % in SLE; $p=0.3$), although it is not significant due to the limited number of samples [21].

Quantitative Ca^{2+} imaging studies were performed to examine and compare the kinetics of single cell $[\text{Ca}^{2+}]_i$ upon exposure to APCs (SEB-B cells) in T cells from SLE (patients #1–8 of Table I), normal individuals and RA (patients #1–5 of Table I). All the experiments reported herein, including those that required transfection, were performed within 24 hr from isolation of the cells from the blood to avoid the change to a normal phenotype that occurs when they are maintained in culture for more than 24 hr [38]. Stimulation with APCs triggers a variety of Ca^{2+} responses including transient (Fig. 1A, top), sustained (Fig. 1A, middle) and oscillatory (Fig. 1A, bottom) $[\text{Ca}^{2+}]_i$ in more than 25% of the T cell population. Interestingly, we found that a significantly higher percentage of SLE T cells display sustained (continuous) responses to antigen stimulation, while transient responses were more pronounced in healthy individuals and RA patients (Fig. 1B). No significant differences were observed in the number of cells showing oscillatory responses. Similar differences in Ca^{2+} response between normal and SLE T cells were observed with beads coated with anti-CD3 and anti-CD28 antibodies (Fig. 1C). CD3/CD28 beads have been used as surrogate artificial APCs as they trigger a variety of activation responses similar to actual APCs without allogeneic response [21,30,40]. There was no statistical difference between the percentage of normal cells that responded with transient, sustained and oscillatory responses upon SEB-pulsed B cell exposure and those exposed to CD3/CD28 beads. Furthermore, in agreement with the data obtained with SEB-pulsed B cells, the majority of SLE T cells responded to CD3/CD28 beads with sustained $[\text{Ca}^{2+}]_i$.

Overall, the ratio of sustained over transient responses (S/T ratio) triggered by APCs in SLE T cells was 3- and 7- fold higher than that of normal and RA T cells, respectively (Fig 1D). This abnormal pattern was independent of disease severity. We analyzed the S/T ratio with respect to SLEDAI by regression analysis and found that there was no correlation among the two ($r=-0.13$, $p=0.76$). It also occurs independently of immunosuppressive therapy as 2 SLE patients who were not under immunosuppressive therapy (allowing $<10\text{mg}$ prednisone/day) displayed this defect (patients #6 and 8 of Table I had S/T ratios of 2 and 11, respectively), and it was not observed in RA patients, also undergoing similar immunosuppressive therapy.

These results were obtained in a mixed population of resting T cells (composed of naïve, T_{EM} and T_{CM} cells) thus raising the question as to whether specific Ca^{2+} patterns are exclusively associated with specific T cell subtypes. Since SLE T cells have a higher percentage of $\text{CD4}^+ \text{T}_{\text{EM}}$ as compared to normal donors it is possible that the observed differences may just reflect the difference in T cell population makeup [21]. We thus isolated $\text{T}_{\text{EM}} \text{CD4}^+$ cells from normal donors and compared their Ca^{2+} response to that of the mixed CD4^+ population. We found no unique Ca^{2+} response associated with T_{EM} cells; instead the S/T ratio of NL T_{EM} cells was comparable to the mixed CD4^+ population (Fig. 1D; $n=3$, $p=0.2$).

Overall, these experiments demonstrate that in SLE T cells there is a shift in the shape of the Ca^{2+} response to a more sustained $[\text{Ca}^{2+}]_i$ as compared to normal donors. Moreover, this appears to be unique to SLE T cells as it was not observed in RA patients.

3.2 SLE T cells display lower $[\text{Ca}^{2+}]_i$ oscillation frequencies

Since the frequency of oscillation has also been reported to affect gene expression, we compared this feature in normal and SLE T cells [9]. The frequency characteristics were analyzed using power spectral density (PSD) analysis as described in the materials and methods section. The PSD analysis allowed us to evaluate the shape, amplitude and periodicity of oscillations. For each cell the frequency was determined by using the PSD maximum value. Power spectra for a normal and SLE donor with the corresponding original trace are shown in Fig. 2. The median response for SLE T cells was 4.6 mHz (periodicity = 217 sec), while for normal T cells it was 5.7 mHz (periodicity = 175 sec). The oscillation pattern in RA T cells closely mirrored that of normal T cells with a median frequency response of 5.7 mHz. The detailed distribution for normal and SLE T cells is shown in the frequency histogram in Fig.

2. Overall, the majority of SLE T cells displayed longer periodicity (lower frequency) of oscillations than normal and RA T cells. Interestingly, 33% of normal T cells have a periodicity of < 100 s while only 11% of SLE T cells have this feature.

3.3 The magnitude of $[Ca^{2+}]_i$ in the three populations is comparable among SLE and normal T cells

Another feature of the Ca^{2+} response that affects gene expression is the amplitude of the actual $[Ca^{2+}]_i$ achieved in a given response. Although ratiometric Ca^{2+} studies have been performed in SLE T cells using soluble antibody [3,41], at present, there exist no quantitative measurements of actual $[Ca^{2+}]_i$ in SLE T cells. Here we looked at the baseline and peak $[Ca^{2+}]_i$ in sustained, transient and oscillatory responses described above. In cells that exhibited a sustained response we also included the $[Ca^{2+}]_i$ magnitude after 5 min from the initial Ca^{2+} onset to investigate $[Ca^{2+}]_i$ decay. There were no differences in resting/baseline $[Ca^{2+}]_i$ among the different groups (Table II). The resting $[Ca^{2+}]_i$ ranged between 100–160 nM as previously reported for human T cells [25]. Exposure to APCs triggered an increase in $[Ca^{2+}]_i$ of 300–600 nM. There were no differences in SLE peak $[Ca^{2+}]_i$ in all the categorical responses. However, RA T cells showed a trend for diminished $[Ca^{2+}]_i$ upon stimulation as previously described ($p=0.08$ vs normal T cells) [42]. No differences were also observed for the steady-state $[Ca^{2+}]_i$ of the continuous responses (measured 5 min after the $[Ca^{2+}]_i$ onset): 498 ± 93 nM in normal individuals, 339 ± 92 nM in SLE ($p=0.2$ vs normal and $p=0.6$ vs RA) and 273 ± 59 nM in RA ($p=0.1$ vs normal). Further analysis showed no differences in the duration of the transient Ca^{2+} response, that is, the time from initial $[Ca^{2+}]_i$ rise to signal termination (normal: 209 ± 14 sec, $n=120$; SLE: 229 ± 16 sec, $n=100$; $p=0.33$). However, the duration of the transient response in T cells from RA patients was significantly lower as compared to SLE patients, but no with normal controls (RA: 184 ± 15 sec, $n=116$; $p=0.04$ RA vs SLE and $p=0.22$ RA vs normal).

These results contrast in part with what has been reported in the literature by other investigators [3,43]. Flow cytometry studies with soluble anti-CD3 antibody have reported that SLE T cells have either similar [3] or higher [43] baseline Ca^{2+} and higher peak and steady-state (5–15 min from peak Ca^{2+}) increases in Ca^{2+} levels [3,43]. To reconcile our findings with those in the literature, we performed flow cytometry experiments with soluble anti-CD3/anti-CD28 antibody in a separate group of SLE patients (5F, 2C and 3 AA; patients 6 and 12–15 of Table I) and 7 NL (3M and 4F; 5C, 1AA and 1 Asian) (Fig. 3). The average ages of the SLE patients and NL were not statistically different from each other and from those of the individuals enrolled in the microscopy studies: 39.2 ± 4.6 years ($n=5$) for SLE patients and 39.7 ± 5.9 years ($n=6$) for NL. Although we have only a limited number of experiments and the differences are not statistically significant, consistent with other investigators, we observed a tendency to a higher peak Ca^{2+} response in SLE compared to normal individuals in flow cytometry experiments with soluble antibodies (Fig. 3A). There was no difference in the baseline or steady-state Ca^{2+} response (Fig. 3B). We then calculated the average Ca^{2+} response derived from the single cell data of this manuscript (average of all cells) to compare with the flow cytometry results. Contrary to the flow studies, we saw no differences in peak Ca^{2+} levels (Fig. 3). This comparison indicates that focal stimulation and formation of an IS triggers a different Ca^{2+} response than soluble antibody, suggesting that different ionic mechanisms are engaged depending on the different types of TCR stimulation. Overall, focal stimulation, which most closely mimics the antigen stimulation occurring in nature, reveals significant differences in the kinetics of $[Ca^{2+}]_i$ in SLE T cells, with a predominance of T cells exhibiting sustained $[Ca^{2+}]_i$. Furthermore, we found that oscillating SLE T cells, although equally represented in SLE, normal and RA donors, oscillate with lower frequency. These alterations were not accompanied by differences in the magnitude of $[Ca^{2+}]_i$ between SLE and normal T cells in

sustained, transient or oscillatory responses. These data suggest that SLE T cells present with specific abnormalities in Ca^{2+} handling mechanisms.

3.4 Kv1.3 channel activity influences the development of the Ca^{2+} response

The mechanisms underlying individual Ca^{2+} responses are not yet fully understood. We have previously shown that Kv1.3 inhibition depresses the increase in Ca^{2+} induced by CD3/CD28 beads in approximately 50% of normal T lymphocytes [30]. We observed similar inhibition of the Ca^{2+} response in SLE T cells stimulated with SEB-B cells (Fig. 4). Application of the Kv1.3 specific blocker ShK-Dap²² determines the rapid return of $[\text{Ca}^{2+}]_i$ to baseline. This finding confirms that modulation of Kv1.3 activity can shape the development of the Ca^{2+} response. Thus, it is possible that alterations in Kv1.3 function underlie the sustained Ca^{2+} response of SLE T cells. We have recently reported that Kv1.3 localization in the IS is shorter in SLE T cells compared to normal T cells [21]. Thus, we proceeded to investigate whether a correlation exists between defective Kv1.3 localization to the IS and the predominance of sustained Ca^{2+} responses of SLE T cells.

3.5 Short-lived Kv1.3 localization in the IS in SLE T cells is associated with sustained Ca^{2+} signaling

Two-photon time-lapse microscopy experiments were conducted in T cells transfected with a pEGFP-tagged Kv1.3 construct. This construct induces the expression of Kv1.3 channels functionally similar to wild-types and conserving the ability of moving into the IS (Supplemental Fig. S2). We simultaneously visualized pEGFP-Kv1.3 movement to the IS and the $[\text{Ca}^{2+}]_i$ upon encounter with SEB-B cells in 7 T/APC conjugates from five healthy donors and 4 SLE patients (patients # 8–11 of Table I) (Fig. 5). We observed significant differences in the kinetics of Kv1.3 movement in and out the IS. While in normal T cells Kv1.3 relocated to the IS within 3.4 ± 0.9 min ($n=7$) from contact with an APC and herein resided for the remaining of the experiment (45.5 ± 11.6 min), faster kinetics were observed in SLE. Kv1.3 recruitment to the IS in SLE T cells required 1.25 ± 0.4 min ($n=7$, $p=0.05$). Furthermore, with the exception of one cell, Kv1.3 recruitment was short lived and Kv1.3 remained in the IS only for 10.2 ± 1.9 min ($n=6$; $p=0.02$ vs normal) before moving out of the IS where it remained for the duration of the experiment (37.4 ± 3.2 min). These data are in agreement with our previous finding showing alterations of native Kv1.3 mobility in SLE T cells [21]. In regards to the Ca^{2+} response, in normal T cells we observed that in 43% of conjugates Kv1.3 localization in the IS was associated with a decrease in Ca^{2+} response and $[\text{Ca}^{2+}]_i$ returned back to baseline within 10–50 min (Fig. 5A). In contrast, in 100% of SLE T cells that displayed the short-lived Kv1.3 localization in the IS, $[\text{Ca}^{2+}]_i$ remained elevated for the duration of the experiment (Fig. 5B). These experiments demonstrate that Kv1.3 channels remain in the IS when $[\text{Ca}^{2+}]_i$ returns to baseline, supporting the hypothesis that the channels' dwelling in the IS facilitates their regulation and consequent termination of the Ca^{2+} response in a subset of T cells. In SLE T cells Kv1.3 channels move out of the IS faster and $[\text{Ca}^{2+}]_i$ remains elevated. These data suggest that the early removal of Kv1.3 channel from the IS results in the disruption of the channel regulation and contributes to the development of the abnormal long lasting Ca^{2+} response.

4. DISCUSSION

The current studies provide evidence of abnormalities in the kinetics of Ca^{2+} signaling in SLE T cells. Furthermore, we found a correlation between the Ca^{2+} defect and altered Kv1.3 localization into the IS. Overall these defects may explain, in part, the T cell hyperactivity and dysfunction documented in SLE patients.

Numerous signaling anomalies have been reported in SLE T cells following TCR- engagement [44,45]. Among these, a more pronounced and sustained $[\text{Ca}^{2+}]_i$ response has been detected

in both SLE CD4⁺ and CD8⁺ T cell lineages by non-quantitative flow cytometry [3,41,43]. This technique does not allow analysis of Ca²⁺ signaling in single cells. This information is critical as specificity of gene expression relies on the shape and magnitude of the Ca²⁺ response [9,11,46]. As a result, we have conducted detailed single cell quantitative [Ca²⁺]_i studies in SLE patients and compared the distribution of Ca²⁺ responses to that of normal individuals and RA patients. The latter was chosen as disease control as it is an autoimmune rheumatic disease, but contrary to SLE, it is associated with a reduced Ca²⁺ response [42,47]. In our studies the Ca²⁺ response was triggered by exposure to APCs. We chose this method of stimulation because it closely resembles the “in vivo” activation process [48].

As previously described, a variety of Ca²⁺ responses are elicited in individual healthy T cells by TCR stimulation [26,30,49,50]. This heterogeneity was also observed in SLE, but in SLE T cells sustained increases in [Ca²⁺]_i constitute the dominant response while transient increases are significantly diminished. Despite the alterations in the [Ca²⁺]_i shape we report no differences in the magnitude of [Ca²⁺]_i for each categorical response in SLE as compared to normal T cells. However, RA T cells did present with a tendency for diminished [Ca²⁺]_i as previously described although the limited number of RA patients we tested did not allow definitive conclusions [42]. Previous flow cytometry studies have shown different Ca²⁺ responses in SLE depending on the stimulus used. PHA stimulation induced normal, or even decreased, peak [Ca²⁺]_i [51,52]. On the contrary, soluble anti-CD3 antibody (alone or with anti-CD28) pointed to enhanced peak [Ca²⁺]_i [3,43]. We have compared the results obtained by microscopy with focal stimulation with those obtained with soluble antibody in flow cytometry experiments (Fig. 3). In agreement with previous studies, our results confirm that the peak Ca²⁺ response to diffuse antigen stimulation is enhanced in SLE patients [3,43]. This is not observed with focal stimulation. Experimental differences may account for this discrepancy. Flow cytometry studies were performed using soluble antibodies while we used focal stimulation which implies formation of an IS. This structure is known to be important for regulation in Ca²⁺ signaling affecting both amplitude and duration of the Ca²⁺ response. [23,24]. We have recently reported that Kv1.3 accumulate in the IS and the channel localization at this site inhibits Ca²⁺ signaling [21,27]. This is evidence that in the IS the channel is downregulated. These findings suggest that the ionic and signaling events that take place when an IS is formed are quite different than those triggered by diffuse TCR stimulation. These observations support our conclusions that the differences between our and others' findings may be due to the type of stimulation used (focal or diffuse). Still, we can not exclude that because our technique only allows to analyze a limited number of cells as compared to flow cytometry, small differences in [Ca²⁺]_i might have gone undetected.

Overall our findings indicate that the majority of SLE T cells, and not normal and RA T cells, respond to focal stimulation with sustained [Ca²⁺]_i. This may explain the increased NF-AT nuclear localization of SLE T cells as compared to normal donors and patients with other autoimmune diseases [12]. Previous reports showed that a sustained [Ca²⁺]_i response promotes NF-AT activation [10,11]. Instead, the Ca²⁺ defect does not appear to have any correlation with decreased NF-κB activation reported in SLE T cells [13]. Indeed, abnormal NF-κB activity in T lymphocytes from SLE patients has been shown to be associated with decreased p65-RelA expression and this defect is independent of membrane ionic events [13].

The mechanisms that mediate the predominance of a sustained Ca²⁺ response in SLE T cells are still not fully known. It is well established that conditions that reduce Ca²⁺ influx, such as depolarization, immediately terminate [Ca²⁺]_i rise [16]. In support of this we show that blockade of Kv1.3 terminates the Ca²⁺ response triggered by focal stimulation. Furthermore, recent studies showed that blockade of Kv1.3 accumulation in the IS results in an exaggerated Ca²⁺ response indicating that the IS is a site of Kv1.3 downregulation [27]. Thus, it is possible that in a percentage of normal T cells Kv1.3 localization in the IS results in inhibition of the

channel activity and consequent termination of the Ca^{2+} response. In a previous study we showed that in SLE the channels are removed from the IS significantly faster than in normal T cells [21]. Thus, the premature exit of the channel may affect its regulation and as such promote the enhanced Ca^{2+} pattern we observed. We have observed in the two-photon experiments that in normal T cells prolonged Kv1.3 localization in the IS is accompanied by an increase in $[\text{Ca}^{2+}]_i$. Interestingly, when $[\text{Ca}^{2+}]_i$ returned to baseline Kv1.3 was still localized in the IS, advocating a role for Kv1.3 in the termination of the Ca^{2+} response. This correlation was observed only in ca. 40% of the cell conjugates studied. The technical complexity of these types of experiments did not allow screening of a large population of cells. Nevertheless, such responsiveness to Kv1.3 blockade limited only to a subpopulation of normal T cells was previously observed and reported by us and it suggests that different mechanisms regulate the development of the Ca^{2+} response in human T cell subsets [30]. On the contrary, sustained Ca^{2+} signaling in SLE T cells highly correlates with short-lived Kv1.3 localization in the IS. In all the SLE T/B cell conjugates that exhibited a transient Kv1.3 localization in the IS $[\text{Ca}^{2+}]_i$ failed to return to baseline.

Overall the data we presented herein indicate that a defect in Kv1.3 membrane mobility underlies the abnormal Ca^{2+} signaling of SLE T cells. Furthermore, these data suggest that the IS is an important site for Kv1.3 regulation thus introducing a novel regulatory mechanism in which spatio-temporal modulation of Kv1.3 activity controls the physiological development of Ca^{2+} response in T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Differential and unique pattern of Ca^{2+} response in SLE T cells
A. Representative Ca^{2+} responses induced in T cells by exposure to SEB-B cells. Each panel shows representative measurements of cytoplasmic $[\text{Ca}^{2+}]_i$ in individual cells. Resting human T cells were loaded with the ratiometric dye Fura-2 and stimulated with SEB- pulsed EBV- infected B cells for 15 min. The points of introduction of the B cells and ionomycin (iono, 1–2 μM) into the bath are indicated by arrows. Contact with a SEB-pulsed B cell triggered differential responses in T cells including transient (top), sustained (middle) and oscillatory Ca^{2+} responses. These data were from one SLE patient. Similar responses were triggered in normal and RA T cells. B. Detailed analysis of $[\text{Ca}^{2+}]_i$ response distribution in normal, SLE and RA T cells. Number of T cells displaying a transient, sustained or oscillatory response are reported as normalized for the total number of T cells that show an increase in $[\text{Ca}^{2+}]_i$ upon SEB- pulsed EBV- B cell exposure. The data are the average of 8 healthy individuals, 8 age-matched SLE and 5 RA patients; 20–70 responding cells/donor. * $p < 0.05$ vs normal; ** $p < 0.005$ vs normal; $\#$ $p < 0.05$ vs RA; $\neq$ $p < 0.005$ vs RA; $\$$ $p < 0.001$ vs transient SLE and $\&$ $p < 0.001$ vs oscillatory SLE. C. Distribution of Ca^{2+} responses to CD3/CD28 beads in NL and SLE T cells. The percentage of T cells from normal individual ($n=3$) and 1 SLE patient that displayed a categorical Ca^{2+} response to anti-CD3/CD28 beads is reported. D. Comparisons of S/T ratios. The S/T ratio for SLE, NL and RA mixed CD4 cells was derived from the

experiments reported in B. These values were compared to the S/T ratio of T_{EM} and $CD4^+$ cells from 3 NL individuals. There was no statistical differences between CD4 and T_{EM} of NL individuals ($n=3$; $p=0.2$), but there were statistical differences between SLE and RA or NL. * $p<0.05$ vs normal; $\neq p<0.05$ vs RA.

Figure 2. Power spectra of APC-induced $[Ca^{2+}]_i$ oscillations

The frequency of oscillations was determined using PSD in single cells in normal (A) (n=6 donors, 42 cells) and SLE (B) (n=6 donors, 47 cells) T cells. For specific criteria for determining oscillatory activity refer to materials and methods section. A trace representative of the median response frequency is shown in the top panels for normal (A) and SLE (B) donors. Directly below is the corresponding PSD analysis for that cell. The lower panels show the distribution of oscillation frequencies for normal and SLE donors. Results are presented as percentage of cells, within the oscillating population, displaying a particular frequency.

Figure 3. Comparison between Ca^{2+} measurements by flow cytometry with soluble anti-CD3 antibody and by fluorescent microscopy with APCs

Flow cytometry experiments were conducted with soluble anti-CD3/CD28 antibody and Indo-1 as Ca^{2+} sensitive dye in freshly-isolated resting $\text{CD}3^{+}$ cells from normal individuals (NL) and SLE patients. The microscopy average data are derived from the Fura-2 experiments presented in this manuscript. This corresponds to the average of all type of responding cells (sustained+transient+oscillatory). A. Changes in Ca^{2+} levels in NL and SLE patients measured by flow cytometry (left) and by microscopy (right). Increases in Ca^{2+} levels induced by TCR stimulation are reported as relative to baseline Ca^{2+} levels. The data are the average of 5 SLE patients and 7 NL for the flow cytometry experiments and 8 SLE patients and 8 NL in the microscopy studies. B. Baseline Ca^{2+} levels in NL and SLE patients measured by flow cytometry (left) and by microscopy (right). The Ca^{2+} levels in the flow cytometry experiments are reported as average fluorescence ratio (FR). Actual $[\text{Ca}^{2+}]_i$ were measured by microscopy.

Figure 4. Effect of Kv1.3 blockade on the Ca^{2+} response to antigen presentation in SLE T cells [Ca^{2+}]_i (expressed as 340/380 ratio) was measured by Fura-2 in SLE T cells. Arrows indicate time of SEB EBV-B cell, ShK-Dap²² (10 nM) and ionomycin (Iono, 1–2 μM) addition. SEB-B cells triggered Ca^{2+} responses were inhibited by the selective Kv1.3 blocker ShK-Dap²² (38/56 cells).

Figure 5. Time-lapse imaging of Kv1.3 channel trafficking and $[Ca^{2+}]_i$

The time of each panel is indicated on the top of the DIC image and corresponds to the actual time from the beginning of the experiment. For sake of space only a few time points are shown. From top to bottom we are showing the following corresponding images: DIC, Fura-2 (displayed in pseudocolor scale, right. A white line marks the contour of the APC), Kv1.3 (green), linescan analyses of Kv1.3 fluorescent intensity (FI) over the whole cell membrane (4 um on the x axis correspond to the center of the IS) and development of Ca^{2+} response over time ($[Ca^{2+}]_i$). Since optical sectioning was applied to assure detection of all the green fluorescent signal, the most representative section and its corresponding DIC image were isolated. To analyze $[Ca^{2+}]_i$, a region was drawn around the T cell and the fluorescence was

quantified. Subsequently, $F_0 - F/F_0$ was used to obtain a pseudoratio where F is the fluorescence intensity and F_0 is the averaged prestimulus fluorescence. Although an increase in $[Ca^{2+}]_i$ is accompanied by an actual decrease in fluorescence, the estimated ratio inverts the data. Therefore, in the depicted graph, an increase in $[Ca^{2+}]_i$ is represented by a rise in the curve.

A. Normal T cell interacting with an APC. Initially the Kv1.3 channels are evenly distributed around the membrane (1:48 min) and upon contact with B cell (2:12 min) redistribute to the T/B interface (IS). At 8:51 min there is a decrease in fluorescence of Fura-2 indicating an increase in $[Ca^{2+}]_i$. Kv1.3 remains in the IS for the duration of the experiment whilst at 27:04 min the Ca^{2+} signal starts to be recovered reaching baseline at 53:01 min. B. SLE T cell interacting with an APC. Kv1.3 channels accumulate at the IS immediately upon contact with an APC (16:56), but ca. 12 min later the channels already exit the IS. A steady-state increase in $[Ca^{2+}]_i$ persists throughout the experiment. The continuous line indicates a time period when the image went out of focus. Although we could still follow the cell, we could not accurately analyze the images. Each result is representative of 7 T/B conjugates in 5 normal donors and 7 T/B conjugates in 4 SLE patients. Scale bar = 5 μ m. Snapshot sequences for normal and SLE T cells correspond to Supplemental Movies S1 and S2, respectively.

Table 1

Details of SLE and RA patients

Pt #	SLEDAI	MEDICATIONS
SLE patients		
1	1	HCQ + MMF + Pred. *
2	19	Pred.
3	6	HCQ + Pred. *
4	0	Pred.
5	2	HCQ + Pred.
6	2	Pred. *
7	6	HCQ + Pred. * + MMF
8	3	Pred. *
9	0	HCQ + Pred. * + MMF
10	4	Pred. * + HCQ + Cytoxan
11	12	Pred.
12	10	HCQ + Pred. + MMF
13	5	HCQ + Pred. *
14	nd	MMF
15	nd	HCQ + MMF
RA patients		
1		None
2		Pred. *
3		HCQ + MTX + Pred. *
4		MTX + Pred. *
5		MTX

HCQ=Hydroxychloroquine (Plaquenil); MMF=Mycophenolate Mofetil (CellCept); MTX=Methotrexate; Pred=Prednisone;
* ≤10mg; nd=not determined

Table II

Levels of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in normal, SLE and RA T cells

	NL		SLE		RA	
	Baseline	Peak	Baseline	Peak	Baseline	Peak
Sustained	164 ± 31	631 ± 113 (162)	133 ± 50	450 ± 105 (200)	119 ± 47	342 ± 62 (42)
Transient	128 ± 29	421 ± 77 (112)	121 ± 51	432 ± 117 (72)	109 ± 48	295 ± 62 (87)
Oscillatory	120 ± 28	467 ± 74 (68)	118 ± 55	438 ± 103 (64)	114 ± 42	324 ± 50 (56)

The $[\text{Ca}^{2+}]_i$ is reported in nM.