Published in final edited form as:

Mol Immunol. 2015 February ; 63(2): 540–549. doi:10.1016/j.molimm.2014.10.015.

Calcium mobilization is both required and sufficient for initiating chromatin decondensation during activation of peripheral T-cells

Megan D. Lee*, Kellie N. Bingham*, Taylor Y. Mitchell, Jenna L. Meredith, and Jason S. Rawlings

Department of Biology, Furman University, Greenville SC 29613, USA

Abstract

Antigen engagement of the T-cell receptor (TCR) induces a rapid and dramatic decondensation of chromatin that is necessary for T-cell activation. This decondensation makes T-cells competent to respond to Interleukin-2 providing a mechanism to ensure clonotypic proliferation during an immune response. Using murine T-cells, we investigated the mechanism by which TCR signaling can initiate chromatin decondensation, focusing on the role of calcium mobilization. During T-cell activation, calcium is first released from intracellular stores, followed by influx of extracellular calcium via store operated calcium entry. We show that mobilization of intracellular calcium is required for TCR-induced chromatin decondensation. However, the decondensation is not dependent on the activity of the downstream transcription factor NFAT. Furthermore, we show that the influx of extracellular calcium is dispensable for initiating chromatin decondensation. Finally, we show that mobilization of calcium from intracellular stores is sufficient to induce decondensation, independent of TCR engagement. Collectively, our data suggest that chromatin decondensation in peripheral T-cells is controlled by modulating intracellular calcium levels.

Keywords

T-cell activation; Chromatin decondensation; Calcium; NFAT

1. INTRODUCTION

Following maturation in the thymus, peripheral T-cells enter into a quiescent state characterized by a low metabolic profile, rudimentary organelles, and extremely condensed chromatin. These long-lived naïve T-cells circulate in the periphery and remain quiescent until activated by presentation of a T-cell receptor (TCR)-specific antigen. Engagement of the TCR causes dramatic changes including the rapid increase in metabolic rate, the

© 2014 Elsevier Ltd. All rights reserved.

Correspondence should be addressed to: Jason Rawlings, jason.rawlings@furman.edu, Department of Biology, 3300 Poinsett Hwy, Greenville, SC 29613, Phone (864) 294-3243.

*these authors contributed equally to this work

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

decondensation of nuclear material, the production of macromolecules, and the hallmark 'blasting' of the cytosol (Frauwrirth and Thompson, 2004; Jaehning et al., 1975; Morley et al., 1993; Paul, 2013; Rawlings et al., 2011). These changes are required for T-cell activation, clonotypic expansion, and the acquisition of effector functions required for a proper immune response.

Presentation of antigen to the TCR triggers multiple signaling pathways required for T-cell activation (reviewed in Lin and Weiss, 2001). Of particular importance is the activation of phospholipase C (PLC γ 1), which hydrolyzes phosphatidyl 4,5-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol triphosphate (IP₃). The primary action of DAG is to activate Protein Kinase C (PKC) which can then activate downstream signaling pathways, ultimately leading to the nuclear translocation of key transcription factors AP-1 and NF- κ B (reviewed in Isakov and Altman, 2002). Meanwhile, IP₃ engages the IP₃ receptor (IP₃R), releasing calcium from the endoplasmic reticulum (ER). Once these stores are depleted, the ER-bound calcium sensor Stim1 (Stromal interaction molecule 1) couples the ER to the cytosolic Ca²⁺ channel protein Orai1, allowing extracellular calcium to enter the cell via store-operated calcium entry (SOCE) (reviewed in Feske, 2007; Hogan et al., 2010). Mobilized intracellular calcium serves as a critically important second messenger for a wide range of biological processes (reviewed in Berridge et al., 2000). In T-cells, calcium signaling is required for activation, proliferation, and differentiation, primarily through the activity of NFAT (Nuclear Factor of Activated T-cells), a transcription factor that becomes activated as a consequence of increased intracellular calcium ([Ca²⁺]_i) (Macian, 2005). It has been shown that NFAT activation is necessary for the expression of genes required for proper T-cell activation (reviewed in Hogan et al., 2003).

While TCR signaling regulates the activation of peripheral T-cells, the subsequent clonal proliferation required for a proper immune response is controlled by Interleukin-2 (IL-2). This cytokine utilizes the Jak (Janus kinase)/Stat (Signal transducer and activator of transcription) pathway in both paracrine and autocrine fashion to induce expression of genes required to drive clonal proliferation (Ihle et al., 1995; Moriggl et al., 1999; Rawlings et al., 2004). Importantly, peripheral T-cell proliferation is absolutely dependent on the two highly related Stat5 proteins (Stat5a and Stat5b; hereafter referred to as Stat5) as Stat5-deficient T-cells fail to proliferate in response to growth factors (Moriggl et al., 1999).

Regulation of IL-2 signaling is critically important for clonotypic expansion, as those T-cells for which TCR ligation has not occurred must be able to ignore the potent effects of this cytokine. Receptor presentation provides one mechanism for regulation. Naïve T-cells express the intermediate affinity IL-2 receptor, while activated T-cells express an additional receptor chain, IL-2R α , providing increased affinity for the ligand (Lin and Leonard, 1997). T-cells deficient for IL-2R α can proliferate as well as wild type cells so long as they are provided exogenous IL-2, suggesting that there are additional mechanisms downstream of the IL-2 receptor that regulate proliferation (Willerford et al., 1995). Interestingly, it has also been shown that Stat5 target genes are not expressed in naïve T-cells, even when provided exogenous IL-2, indicating that these control mechanisms must lie downstream of Stat5 activation (Gatzka et al., 2006).

Recently, we demonstrated that the status of chromatin dictates the outcome of IL-2/Stat5 signaling in peripheral T-cells. Naïve T-cells possess condensed chromatin such that phosphorylated, nuclear Stat5 dimers cannot engage target gene promoters. Upon TCR stimulation, chromatin rapidly decondenses permitting Stat5-DNA engagement and subsequent Stat5 target gene expression (Rawlings et al., 2011). The mechanism driving the TCR-dependent change in chromatin status and ultimately transcriptional competence is poorly understood, although our recent work indicates that activation-induced chromatin decondensation is not mediated by common epigenetic marks such as global changes in histone modification or DNA methylation (Rawlings et al., 2011).

Here we investigated the mechanism of TCR-induced chromatin decondensation, focusing specifically on the role of calcium mobilization in this process. We demonstrate that mobilization of calcium is required for proper chromatin decondensation during T-cell activation. However, inhibiting the activation of NFAT was not able to appreciably prevent decondensation, suggesting that calcium acts in an NFAT-independent manner. Interestingly, we found that extracellular calcium is dispensable for initial chromatin decondensation. Finally, we show that the mobilization of intracellular calcium is sufficient to induce chromatin decondensation in peripheral T-cells and can occur independently of TCR stimulation.

2. MATERIALS AND METHODS

2.1 Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocol was approved by the Furman University Institutional Animal Care and Use Committee (Permit number: A3242-01).

2.2 T-cell isolation and culture

Single cell suspensions were processed from spleens of 6-8 week old C57BL/6 mice (Jackson Laboratories). Purified naïve (CD25-) T-cells were obtained via negative selection from splenocytes using the mouse Pan T cell isolation kit II (Miltenyi Biotec) supplemented with biotinylated anti-CD25 antibodies (BD Biosciences). T-cell purity always exceeded 95% on the live cell gate. Splenocytes or purified T-cells were cultured in T-cell media consisting of RPMI 1640 media supplemented with 10% FBS, 10 mM Hepes (pH 7.0), 2mM L-glutamine, 1mM sodium pyruvate, 1X nonessential amino acids, penicillin, streptomycin (all from Life Technologies), and 50µM β-mercaptoethanol (Sigma). In some experiments, T-cell media was supplemented with 5mM EGTA (Fisher Bioreagents).

2.3 T-cell activation assays

For all experiments, cells were seeded at 2×10^6 cells/mL. Cells were stimulated with 1µg/mL anti-CD3 antibodies (2C11; BD Biosciences), 10µg/mL PMA (Sigma), 250ng/mL Ionomycin (Sigma), or 1µM thapsigargin (Molecular Probes). In some experiments, cells were pretreated with 100nM FK506, 1µg/mL cyclosporin A (all from Sigma), 10µM

BAPTA-AM (Molecular Probes), or DMSO (Fisher Bioreagents) for thirty minutes and then activated with 1 μ g/mL anti-CD3 antibodies.

2.4 Western blot

Histone solubility was evaluated by detection of histone H3 (Rawlings et al., 2011). Whole-cell extracts of purified T-cells were prepared by lysing cells in NP40 buffer (1% NP40, 50mM Tris pH7.5, 100mM NaCl, 50mM NaF, 40mM β -Glycerophosphate, 1mM Sodium Orthovanadate, 5mM EDTA) supplemented with Complete Mini protease inhibitors and PhosSTOP inhibitors (Roche) for 30 minutes at 4°C. Cell pellets were removed by centrifugation, proteins were quantified via BCA assay (ThermoFisher), and equal volume of Laemmli sample buffer (BioRad) was added.

Proteins (40 μ g) were resolved via SDS-PAGE, transferred to nitrocellulose membranes and blocked in TBST (10mM Tris pH 8.0, 150mM NaCl, 0.1% Tween 20) containing 5% (w/v) non-fat dry milk. Histone H3 (Abcam, ab1791) or actin antibodies (Sigma, A2066) were added and the blots were incubated overnight at 4°C followed by 1 hour at room temperature. The blots were then washed in TBST and incubated with anti-rabbit IgG HRP-linked antibody (Cell Signaling Technologies) for 1 hour. Proteins were visualized via Opti-4CN detection (Bio-Rad) or by ECL detection (ThermoFisher).

2.5 Flow Cytometry

Histone accessibility was determined by analysis of H3K4me1 staining. Previously, we demonstrated that the ability of H3K4me1 antibodies to bind to target epitopes accurately reflects the chromatin condensation state in peripheral T-cells. Specifically, in naïve peripheral T-cells this antibody cannot access its epitopes as well as in activated T-cells because the chromatin in naïve cells is more condensed. Importantly, there is no global net change in methylation of H3K4, thus H3K4me1 staining can be used to determine the chromatin condensation status (Rawlings et al., 2011). Briefly, cells were washed with PBS (ThermoFisher) and dead cells were stained using the LIVE/DEAD Fixable red dead cell stain kit (Life Technologies) following the manufacturer's instructions. Intracellular staining of H3K4me1 (Abcam, ab8895) was performed in combination with surface staining for CD4 and CD8 T-cell markers (eBioscience). After surface staining, cells were washed twice with PBS and fixed in 4% paraformaldehyde in PBS. Cells were again washed twice with PBS and then permeabilized/blocked in PBS + 2% FBS, + 0.02% Triton, + 0.2 μ L/mL normal rabbit serum (Sigma). Cells were then incubated with PE-coupled H3K4me1 antibodies for one hour. The Lynx rapid RPE kit (Abcam) was used to conjugate PE to the H3K4me1 antibody following the manufacturer's protocol. Following two washes with PBS+2%FBS, cells were analyzed with a FACSsort (BD Biosciences) and FlowJo software (TreeStar). At least 10,000 events were collected from the lymphocyte gate.

2.6 Statistical methods

Error bars indicate standard error of the means. A Student's t-test was used to analyze results for statistical significance.

3. RESULTS

3.1 The DAG and IP₃ pathways can decondense chromatin

Activation via the TCR induces a rapid decondensation of chromatin allowing T-cells to gain the ability to proliferate in response to IL-2 (Figure 1 and Rawlings et al., 2011). We wanted to determine the signaling mechanisms downstream of the TCR responsible for initiating the decondensation of nuclear material. Following activation via the TCR, PLC γ 1 hydrolyzes PIP₂ into DAG and IP₃, which in turn activate multiple signaling pathways required for T-cell activation. The activity of these signaling molecules can be mimicked by phorbol 12-myristate 13-acetate (PMA) and ionomycin, respectively. Stimulation with a combination of PMA and ionomycin induces the activation, proliferation, and differentiation of T-cells, bypassing the need to stimulate the TCR directly (Nobrega et al., 1986). To investigate the signaling mechanism(s) by which TCR engagement causes chromatin to decondense, we stimulated splenocytes with PMA, ionomycin, or PMA in combination with ionomycin and assessed chromatin accessibility in T-cells by flow cytometry (Figure 1A and 1B). We found that both PMA and ionomycin are able to independently (or in combination) induce the decondensation of chromatin in both CD4⁺ and CD8⁺ peripheral T-cells. These findings were substantiated by assessing histone solubility via western blot on sorted peripheral T-cells (Figure 1C). Together, these data suggest that activation of either the IP₃ or the DAG pathways can decondense chromatin in T-cells.

3.2 Intracellular calcium is required for proper chromatin decondensation

Since the mobilization of calcium occurs rapidly following TCR stimulation prior to the time-frame when chromatin begins decondensing, we decided to investigate the role of [Ca²⁺]_i in the decondensation process. First, we wanted to determine if mobilization of intracellular calcium is required for proper TCR-mediated chromatin decondensation. To address this, we chelated intracellular calcium with BAPTA-AM, a cell permeable calcium chelator that has been shown to inhibit calcium flux and proliferation in T-cells (Gelfand et al., 1988; Saxena and Dikshit, 2005; Tsien, 1980). Specifically, it has been shown to eliminate the transient change in [Ca²⁺]_i caused by release of calcium from stores during activation (Gelfand et al., 1988). Splenocytes were treated with BAPTA-AM and histone accessibility of T-cells was measured in response to TCR stimulation via anti-CD3 antibodies. Surprisingly, the simple addition of BAPTA-AM to the culture caused chromatin to become more condensed than basal levels in both CD4⁺ and CD8⁺ cells (Figure 2A and 2C, 0 hour time points), suggesting that perturbation of calcium homeostasis is sufficient to affect the chromatin condensation state of T-cells. Furthermore, the chelation of intracellular calcium significantly prevented the rapid decondensation of chromatin in response to TCR stimulation (Figure 2B and 2D, 1 hour time points). We did not detect any appreciable level of chromatin decondensation until after three hours of TCR stimulation. At three hours, the fold change in decondensation was equivalent to controls (Figure 2B and 2D); however, the chromatin was still significantly more condensed (Figure 2A and 2C). The fact that BAPTA-AM was unable to maintain the inhibition for three hours is not surprising because it is known that the entry of extracellular calcium (e.g. via SOCE) exceeds the buffering capacity of BAPTA-AM (Gelfand et al., 1988). Taken together, all of the above suggests that

increasing $[Ca^{2+}]_i$ is required to initiate the rapid decondensation of chromatin during T-cell activation.

3.3 Activation-induced chromatin decondensation occurs independently of NFAT activation

In T-cells, $[Ca^{2+}]_i$ exerts its effects primarily via the activation of Calmodulin, which in turn activates the phosphatase Calcineurin. Nuclear Factor of Activated T-cells (NFAT) is a latent cytosolic transcription factor, that when dephosphorylated by Calcineurin, translocates to the nucleus where it can induce the expression of genes required for T-cell activation, including cytokine genes (reviewed in Hogan et al., 2003). This calcium signaling pathway can be inhibited with the classical Calcineurin inhibitors FK506 and cyclosporin A (CsA) (Flanagan et al., 1991; Fruman et al., 1992). These inhibitors have been shown to prevent the nuclear translocation of NFAT, inhibit cytokine gene expression, and ultimately inhibit T-cell proliferation (Aiello et al., 1986; Emmel et al., 1989; Nobrega et al., 1986; Sawada et al., 1987). To determine if the calcium-mediated chromatin decondensation we observed is dependent on the activation of NFAT, splenocytes were pre-treated with FK506 or CsA and then T-cells were activated with anti-CD3 antibodies. Inhibition of NFAT activation had a relatively minor effect (Figure 3). Although the difference in chromatin decondensation was significantly different at one hour using either inhibitor (Figure 3A and 3C), the difference was relatively minor compared to what was seen when intracellular calcium was chelated (Figure 2). By three hours, there was no significant difference between the inhibited cells and controls. Furthermore the rate of decondensation in response to TCR stimulation was no different than controls throughout the timecourse (Figure 3B and 3D). These data suggest that NFAT may have only a minor, transient role in the initial decondensation of chromatin during T-cell activation.

3.4 Extracellular calcium is not required for the induction of chromatin decondensation

Calcium is mobilized from two sources during T-cell activation. First a transient spike in $[Ca^{2+}]_i$ as intracellular stores are depleted, followed by a sustained influx of extracellular calcium via SOCE. Since extracellular calcium is responsible for the sustained increase in $[Ca^{2+}]_i$, we wanted to determine if extracellular calcium is required for the initiation of TCR-mediated chromatin decondensation. To investigate this possibility, we chelated extracellular calcium with EGTA. This reagent has been used extensively to chelate extracellular calcium in the study of various aspects of T-cell activation (Diamantstein and Ulmer, 1975; Greene et al., 1976; Shapiro et al., 1985; Weiss et al., 1984) and has been shown to completely prevent the influx of extracellular calcium caused by TCR stimulation (June et al., 1986). Splenocytes cultured in media containing EGTA were stimulated with anti-CD3 antibodies and histone accessibility was assessed by flow cytometry. In both CD4⁺ and CD8⁺ T-cells, we observed an increase in histone accessibility (Figure 4A and 4B). Similar results were obtained when we stimulated cells with ionomycin under the same conditions (Figure 4A and 4B). This finding is consistent with the observation that ionomycin does not increase $[Ca^{2+}]_i$ via action on the plasma membrane, rather it stimulates the mobilization of calcium from stores (Morgan and Jacob, 1994). All of the above results were validated by assessing histone solubility in purified peripheral T-cells via western blot (Figure 4C). Stimulation of T-cells in media containing EGTA with either anti-CD3

antibodies or ionomycin resulted in an increase in histone solubility. Together, these findings suggest that extracellular calcium is not required for the initiation of chromatin decondensation.

3.5 Mobilization of intracellular calcium is sufficient to induce chromatin decondensation

The initial transient increase in $[Ca^{2+}]_i$ following TCR engagement can be attributed to the activity of IP_3 , which binds to IP_3R on the endoplasmic reticulum (ER), releasing calcium from stores into the cytosol. Given that chromatin begins decondensing rapidly following TCR engagement, it is possible that the initial ER-dependent increase in $[Ca^{2+}]_i$ is sufficient to initiate chromatin decondensation. This is consistent with our observation that ionomycin can induce chromatin decondensation in the absence of extracellular calcium (Figure 4). To test this possibility directly, we induced the release of calcium from stores with thapsigargin. Thapsigargin is a Ca^{2+} -ATPase inhibitor that blocks Ca^{2+} influx into the ER, resulting in a net efflux of Ca^{2+} into the cytosol (Lytton et al., 1991; Zweifach and Lewis, 1993). Thapsigargin has been shown to induce Ca^{2+} flux in peripheral T-cells and in combination with PMA can induce T-cell proliferation in the absence of TCR stimulation (Gouy et al., 1990; Kankaanranta et al., 1996). Splenocytes were treated with thapsigargin and chromatin status was determined by flow cytometry. Release of calcium from intracellular stores via thapsigargin treatment caused chromatin to decondense in both CD4+ and CD8+ cells (Figure 5A). However, in this scenario it is possible that this decondensation could be due to entry of extracellular calcium caused indirectly by thapsigargin (via SOCE) and not a direct effect of mobilizing intracellular calcium. To eliminate this possibility, we chelated extracellular calcium with EGTA and then stimulated cells with thapsigargin, thus any increase in $[Ca^{2+}]_i$ can be solely attributed to release from intracellular stores (Sarkadi et al., 1991). Under these conditions, we still observed chromatin decondensation (Figure 5B and 5C). Similar results were seen when we assessed histone solubility in sorted peripheral T-cells using the same conditions (Figure 5D). Together, these data suggest that mobilization of just the stores of intracellular calcium is sufficient to induce chromatin decondensation. Importantly, these data also indicate that calcium mobilization is sufficient to induce decondensation independently of TCR stimulation.

4. DISCUSSION

The rapid decondensation of chromatin that precedes the ‘blasting’ of the cytosol is a hallmark of T-cell activation. Here, we investigated the mechanism by which TCR signaling decondenses chromatin, focusing on the role of calcium mobilization. We determined that mobilization of calcium is required for the initiation of chromatin decondensation, demonstrated by the ability of the intracellular calcium chelator BAPTA-AM to significantly impede TCR-induced decondensation (Figure 2). However, we noted that BAPTA-AM was unable to maintain inhibition of chromatin decondensation, as we observed chromatin decondensing at a similar rate to controls at three hours after TCR stimulation (Figure 2B and 2D). Presumably, this is due to the fact that while BAPTA-AM can chelate calcium released from stores, it cannot buffer the entry of extracellular calcium (Gelfand et al., 1988). Additionally, it should be noted that activation of the DAG pathway also induces chromatin decondensation (Figure 1), suggesting that there may be additional TCR-induced,

calcium-independent mechanisms that produced the decondensation we observed at three hours following TCR stimulation.

The influx of extracellular calcium via SOCE provides the sustained increase in $[Ca^{2+}]_i$ observed during T-cell activation. Extracellular calcium is required for T-cell activation, differentiation, and proliferation (Esser et al., 1998; Gelfand et al., 1988; Harris et al., 1988). Here, we demonstrate that it is dispensable for initiating chromatin decondensation induced by the TCR (Figure 4). In agreement with this, the mobilization of calcium from intracellular stores (even in the absence of extracellular calcium) is sufficient to initiate decondensation (Figure 5). Under these conditions it is known that there is a very minor, transient increase in $[Ca^{2+}]_i$ as stores are emptied that returns to baseline within minutes (Donnadieu et al., 1992; Gelfand et al., 1988; Sarkadi et al., 1991), yet we observed that chromatin continued to decondense for at least 6 hours (Figure 5). Taken together, these observations suggest that the mobilization of calcium could be a triggering event for the decondensation of chromatin and not likely involved in maintaining the decondensed state.

Here we demonstrate that simply increasing $[Ca^{2+}]_i$ is sufficient to induce chromatin decondensation, raising the possibility that lowering $[Ca^{2+}]_i$ could have the opposite effect. Surprisingly, when calcium homeostasis is perturbed with BAPTA-AM we noted that chromatin appears to become even more condensed than what is seen in naïve T-cells (Figure 2A and 2C, 0 hour time point), suggesting that the simple depletion of $[Ca^{2+}]_i$ can cause chromatin to condense. This higher degree of condensation is reminiscent of what has been observed in CD4⁺CD8⁻ and CD4⁺CD8⁺ thymocytes relative to quiescent peripheral CD4⁺ and CD8⁺ T-cells, respectively (Rawlings et al., 2011). Together, these findings suggest that the basal state of chromatin condensation in quiescent peripheral T-cells does not represent the most condensed configuration. Importantly, our findings suggest that even subtle changes in $[Ca^{2+}]_i$ can have significant effects on the condensation state of chromatin. These observations are also consistent with the hypothesis that chromatin condensation could be tied directly to intracellular calcium levels, providing a rapid means to modulate chromatin status. This mechanism could potentially contribute to the differential responses to TCR signals seen throughout T-cell development, activation, and differentiation.

Once mobilized, calcium binds and activates Calmodulin, which in turn activates Calcineurin ultimately leading to the dephosphorylation and nuclear localization of NFAT (Gwack et al., 2007). Activation of NFAT is critical for the expression of genes required for proper T-cell activation and proliferation. However, inhibition of NFAT activation using the classical Calcineurin inhibitors CsA and FK506 had only a minor effect on decondensation (Figure 3) and did not account for the difference in chromatin decondensation seen when intracellular calcium is chelated (Figure 2), suggesting that this signaling pathway may not play an important role in this process. Studies have shown that NFAT activation requires a sustained increase in $[Ca^{2+}]_i$, such as that produced by SOCE and suggests that the initial spike of $[Ca^{2+}]_i$ caused by release from stores may be insufficient for optimal NFAT activation (Dolmetsch et al., 1997; Loh et al., 1996). Our findings are consistent with these studies. Importantly, it must be noted that our data do not preclude a role for NFAT in regulating later stages of chromatin decondensation.

If not NFAT, then the question remains: how does calcium mobilization induce the decondensation of chromatin? It is possible that calcium could regulate the activity of a chromatin remodeling complex, such as the SWI/SNF-like BAF (Brahma-related gene/Brahma associated factor) complex. The BAF complex has been implicated in regulating diverse aspects of T-cell biology including signaling via the pre-TCR during thymocyte development and regulating gene expression in peripheral T-cells (reviewed in Chi, 2004). It has been shown that the BAF complex becomes tightly associated with chromatin of isolated nuclei rapidly following T-cell activation in a PIP₂-dependent process (Zhao et al., 1998). In the same study, it was shown that the addition of calcium to these preparations was sufficient to induce BAF-chromatin interactions. In a follow-up study, actin was also implicated as a subunit of the BAF complex, raising the possibility that assembly or function of this complex could be regulated in a calcium-dependent fashion (Janmey, 1994). Alternatively, chromatin condensation in peripheral T-cells could be controlled via the Condensin II complex. Previously, we demonstrated that this complex is required for proper chromatin condensation during thymocyte development via analysis of mice harboring a mutation in the Kleisin- β subunit (Gosling et al., 2007; Rawlings et al., 2011). Condensin II belongs to a family of protein complexes that also include Cohesins and the Dosage Compensation Complex. These complexes all share a heterodimer of Structural Maintenance of Chromosome (SMC) proteins as well as accessory proteins, including a Kleisin protein (Wood et al., 2010). In addition to its roles in condensing chromatin during mitosis and meiosis, Condensin II has been implicated in chromatin condensation in a number of contexts during interphase (reviewed in Aragon et al., 2013; Hirano, 2012). While no direct link between $[Ca^{2+}]_i$ and Condensin II function has been established, the calcium protease Calpain-1 has been shown to cleave the α -kleisin subunit of the related Cohesion complex, promoting sister chromatid separation (Nasmyth and Haering, 2009; Panigrahi et al., 2011). Regulation of chromatin condensation via calcium by any of the above mechanisms is attractive because calcium is abundant in the cell and is rapidly mobilized in the time-frame in which we observed the initiation of chromatin decondensation following TCR stimulation.

There is significant interplay between the IP₃ and DAG pathways during T-cell activation, which raises the possibility that these pathways could work cooperatively or antagonistically to decondense chromatin. In support of this, we noted in our initial experiments that DAG signaling can also decondense chromatin (Figure 1). It has been shown that several PKC isoforms (α , β , and γ) expressed in T-cells require calcium for their localization to the plasma membrane where they can then be activated by DAG (Tan and Parker, 2003). Additionally, several PKC isoforms have been shown to work synergistically with calcium signaling in regulating diverse aspects of T-cell biology including activation, cell growth, and apoptosis (Altman et al., 1992; Grumont et al., 2004; Villunger et al., 1999). On the other hand, the DAG and IP₃ pathways could work antagonistically. Studies of PKC θ deficient mice revealed that it negatively regulates calcium flux from both intracellular and extracellular sources (Manicassamy et al., 2006; Pfeiffer et al., 2003). This is of particular importance because initially PKC θ was the only isoform found to translocate to the TCR complex following antigen engagement (Monks et al., 1997). Recently, it has been shown that PKC η is also recruited to the immunological synapse and in similar fashion to PKC θ ,

can regulate $[Ca^{2+}]_i$ in T-cells (Fu et al., 2011). The activity of both of these isoforms is required for proper T-cell activation (Fu et al., 2011; Pfeifhofer et al., 2003; Sun et al., 2000). In support of an antagonistic relationship between PKC and IP_3 signaling, we noted that PMA in combination with ionomycin appeared to be less effective than ionomycin alone in decondensing chromatin in our solubility assays (Figure 1C). It is also possible that these signaling pathways could work independently to decondense chromatin. Clearly, further study on the roles of DAG and IP_3 signaling in the decondensation of chromatin during T-cell activation is warranted.

Acknowledgments

This project was supported by grants from the National Institutes of Health (5 P20 RR016461 and 8 P20 GM103499), the National Science Foundation (EPS-0903795), and the Howard Hughes Medical Institute through an Undergraduate Science Education (USE) award. Further support provided by Furman University's Research and Professional Growth (RPG) and Furman Advantage awards. Special thanks to John F. Wheeler, David M. Hollis, and Samuel R. Wooten.

References

- Aiello FB, Maggiano N, Larocca LM, Piantelli M, Musiani P. Inhibitory effect of cyclosporin A on the OKT3-induced peripheral blood lymphocyte proliferation. *Cell Immunol.* 1986; 97:131–139. [PubMed: 3091267]
- Altman A, Mally MI, Isakov N. Phorbol ester synergizes with Ca^{2+} ionophore in activation of protein kinase C (PKC)alpha and PKC beta isoenzymes in human T cells and in induction of related cellular functions. *Immunology.* 1992; 76:465–471. [PubMed: 1388136]
- Aragon L, Martinez-Perez E, Merkenschlager M. Condensin, cohesin and the control of chromatin states. *Curr Opin Genet Dev.* 2013; 23:204–211. [PubMed: 23312842]
- Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol.* 2000; 1:11–21. [PubMed: 11413485]
- Chi T. A BAF-centred view of the immune system. *Nat Rev Immunol.* 2004; 4:965–977. [PubMed: 15573131]
- Diamantstein T, Ulmer A. The control of immune response in vitro by Ca^{2+} . II. The Ca^{2+} -dependent period during mitogenic stimulation. *Immunology.* 1975; 28:121–125. [PubMed: 1116866]
- Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI. Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. *Nature.* 1997; 386:855–858. [PubMed: 9126747]
- Donnadieu E, Bismuth G, Trautmann A. Calcium fluxes in T lymphocytes. *J Biol Chem.* 1992; 267:25864–25872. [PubMed: 1464601]
- Emmel EA, Verweij CL, Durand DB, Higgins KM, Lacy E, Crabtree GR. Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. *Science.* 1989; 246:1617–1620. [PubMed: 2595372]
- Esser MT, Haverstick DM, Fuller CL, Gullo CA, Braciale VL. Ca^{2+} signaling modulates cytolytic T lymphocyte effector functions. *J Exp Med.* 1998; 187:1057–1067. [PubMed: 9529322]
- Feske S. Calcium signalling in lymphocyte activation and disease. *Nat Rev Immunol.* 2007; 7:690–702. [PubMed: 17703229]
- Flanagan WM, Corthesy B, Bram RJ, Crabtree GR. Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature.* 1991; 352:803–807. [PubMed: 1715516]
- Frauwirth KA, Thompson CB. Regulation of T lymphocyte metabolism. *J Immunol.* 2004; 172:4661–4665. [PubMed: 15067038]
- Fruman DA, Klee CB, Bierer BE, Burakoff SJ. Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A. *Proc Natl Acad Sci U S A.* 1992; 89:3686–3690. [PubMed: 1373887]

- Fu G, Hu J, Niederberger-Magenat N, Rybak V, Casas J, Yachi PP, Feldstein S, Ma B, Hoerter JA, Ampudia J, Rigaud S, Lambalez F, Gavin AL, Sauer K, Cheroute H, Gascoigne NR. Protein kinase C η is required for T cell activation and homeostatic proliferation. *Sci Signal*. 2011; 4:ra84. [PubMed: 22155788]
- Gatzka M, Piekorz R, Moriggl R, Rawlings J, Ihle JN. A role for STAT5A/B in protection of peripheral T-lymphocytes from postactivation apoptosis: insights from gene expression profiling. *Cytokine*. 2006; 34:143–154. [PubMed: 16757175]
- Gelfand EW, Cheung RK, Mills GB, Grinstein S. Uptake of extracellular Ca^{2+} and not recruitment from internal stores is essential for T lymphocyte proliferation. *Eur J Immunol*. 1988; 18:917–922. [PubMed: 3133231]
- Gosling KM, Makaroff LE, Theodoratos A, Kim YH, Whittle B, Rui L, Wu H, Hong NA, Kennedy GC, Fritz JA, Yates AL, Goodnow CC, Fahrner AM. A mutation in a chromosome condensin II subunit, kleisin beta, specifically disrupts T cell development. *Proc Natl Acad Sci U S A*. 2007; 104:12445–12450. [PubMed: 17640884]
- Gouy H, Cefai D, Christensen SB, Debre P, Bismuth G. Ca^{2+} influx in human T lymphocytes is induced independently of inositol phosphate production by mobilization of intracellular Ca^{2+} stores. A study with the Ca^{2+} endoplasmic reticulum-ATPase inhibitor thapsigargin. *Eur J Immunol*. 1990; 20:2269–2275. [PubMed: 1700752]
- Greene WC, Parker CM, Parker CW. Calcium and lymphocyte activation. *Cell Immunol*. 1976; 25:74–89. [PubMed: 183895]
- Grumont R, Lock P, Mollinari M, Shannon FM, Moore A, Gerondakis S. The mitogen-induced increase in T cell size involves PKC and NFAT activation of Rel/NF- κ B-dependent c-myc expression. *Immunity*. 2004; 21:19–30. [PubMed: 15345217]
- Gwack Y, Feske S, Srikanth S, Hogan PG, Rao A. Signalling to transcription: store-operated Ca^{2+} entry and NFAT activation in lymphocytes. *Cell Calcium*. 2007; 42:145–156. [PubMed: 17572487]
- Harris DT, Kozumbo WJ, Testa JE, Cerutti PA, Cerottini JC. Molecular mechanisms involved in T cell activation. III. The role of extracellular calcium in antigen-induced lymphokine production and interleukin 2-induced proliferation of cloned cytotoxic T lymphocytes. *J Immunol*. 1988; 140:921–927. [PubMed: 3276784]
- Hirano T. Condensins: universal organizers of chromosomes with diverse functions. *Genes Dev*. 2012; 26:1659–1678. [PubMed: 22855829]
- Hogan PG, Chen L, Nardone J, Rao A. Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev*. 2003; 17:2205–2232. [PubMed: 12975316]
- Hogan PG, Lewis RS, Rao A. Molecular basis of calcium signaling in lymphocytes: STIM and ORAI. *Annu Rev Immunol*. 2010; 28:491–533. [PubMed: 20307213]
- Ihle JN, Witthuhn BA, Quelle FW, Yamamoto K, Silvennoinen O. Signaling through the hematopoietic cytokine receptors. *Annu Rev Immunol*. 1995; 13:369–398. [PubMed: 7612228]
- Isakov N, Altman A. Protein kinase C(θ) in T cell activation. *Annu Rev Immunol*. 2002; 20:761–794. [PubMed: 11861617]
- Jaehning JA, Stewart CC, Roeder RG. DNA-dependent RNA polymerase levels during the response of human peripheral lymphocytes to phytohemagglutinin. *Cell*. 1975; 4:51–57. [PubMed: 1116173]
- Janmey PA. Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. *Annu Rev Physiol*. 1994; 56:169–191. [PubMed: 8010739]
- June CH, Ledbetter JA, Rabinovitch PS, Martin PJ, Beatty PG, Hansen JA. Distinct patterns of transmembrane calcium flux and intracellular calcium mobilization after differentiation antigen cluster 2 (E rosette receptor) or 3 (T3) stimulation of human lymphocytes. *J Clin Invest*. 1986; 77:1224–1232. [PubMed: 2420827]
- Kankaanranta H, Luomala M, Kosonen O, Moilanen E. Inhibition by fenamates of calcium influx and proliferation of human lymphocytes. *Br J Pharmacol*. 1996; 119:487–494. [PubMed: 8894168]
- Lin J, Weiss A. T cell receptor signalling. *J Cell Sci*. 2001; 114:243–244. [PubMed: 11148124]
- Lin JX, Leonard WJ. Signaling from the IL-2 receptor to the nucleus. *Cytokine Growth Factor Rev*. 1997; 8:313–332. [PubMed: 9620644]

- Loh C, Shaw KT, Carew J, Viola JP, Luo C, Perrino BA, Rao A. Calcineurin binds the transcription factor NFAT1 and reversibly regulates its activity. *J Biol Chem.* 1996; 271:10884–10891. [PubMed: 8631904]
- Lytton J, Westlin M, Hanley MR. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. *J Biol Chem.* 1991; 266:17067–17071. [PubMed: 1832668]
- Macian F. NFAT proteins: key regulators of T-cell development and function. *Nat Rev Immunol.* 2005; 5:472–484. [PubMed: 15928679]
- Manicassamy S, Sadim M, Ye RD, Sun Z. Differential roles of PKC-theta in the regulation of intracellular calcium concentration in primary T cells. *J Mol Biol.* 2006; 355:347–359. [PubMed: 16309697]
- Monks CR, Kupfer H, Tamir I, Barlow A, Kupfer A. Selective modulation of protein kinase C-theta during T-cell activation. *Nature.* 1997; 385:83–86. [PubMed: 8985252]
- Morgan AJ, Jacob R. Ionomycin enhances Ca²⁺ influx by stimulating store-regulated cation entry and not by a direct action at the plasma membrane. *Biochem J.* 1994; 300(Pt 3):665–672. [PubMed: 8010948]
- Moriggl R, Topham DJ, Teglund S, Sexl V, McKay C, Wang D, Hoffmeyer A, van Deursen J, Sangster MY, Bunting KD, Grosveld GC, Ihle JN. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. *Immunity.* 1999; 10:249–259. [PubMed: 10072077]
- Morley SJ, Rau M, Kay JE, Pain VM. Increased phosphorylation of eukaryotic initiation factor 4 alpha during early activation of T lymphocytes correlates with increased initiation factor 4F complex formation. *Eur J Biochem.* 1993; 218:39–48. [PubMed: 8243475]
- Nasmyth K, Haering CH. Cohesin: its roles and mechanisms. *Annu Rev Genet.* 2009; 43:525–558. [PubMed: 19886810]
- Nobrega AF, Maldonado MS, Dos Reis GA. Analysis of isolated and combined effects of calcium ionophore and phorbol ester on T lymphocyte activation. *Clin Exp Immunol.* 1986; 65:559–569. [PubMed: 3096619]
- Panigrahi AK, Zhang N, Mao Q, Pati D. Calpain-1 cleaves Rad21 to promote sister chromatid separation. *Mol Cell Biol.* 2011; 31:4335–4347. [PubMed: 21876002]
- Paul, WE. *Fundamental immunology*. 7. Wolters Kluwer Health/Lippincott Williams & Wilkins; Philadelphia: 2013.
- Pfeifhofer C, Kofler K, Gruber T, Tabrizi NG, Lutz C, Maly K, Leitges M, Baier G. Protein kinase C theta affects Ca²⁺ mobilization and NFAT cell activation in primary mouse T cells. *J Exp Med.* 2003; 197:1525–1535. [PubMed: 12782715]
- Rawlings JS, Gatzka M, Thomas PG, Ihle JN. Chromatin condensation via the condensin II complex is required for peripheral T-cell quiescence. *EMBO J.* 2011; 30:263–276. [PubMed: 21169989]
- Rawlings JS, Rosler KM, Harrison DA. The JAK/STAT signaling pathway. *J Cell Sci.* 2004; 117:1281–1283. [PubMed: 15020666]
- Sarkadi B, Tordai A, Homolya L, Scharff O, Gardos G. Calcium influx and intracellular calcium release in anti-CD3 antibody-stimulated and thapsigargin-treated human T lymphoblasts. *J Membr Biol.* 1991; 123:9–21. [PubMed: 1723105]
- Sawada S, Suzuki G, Kawase Y, Takaku F. Novel immunosuppressive agent, FK506. In vitro effects on the cloned T cell activation. *J Immunol.* 1987; 139:1797–1803. [PubMed: 2442255]
- Saxena HM, Dikshit M. Abrogation of DTH response and mitogenic lectin- and alloantigen-induced activation of lymphocytes by calcium inhibitors TMB-8 and BAPTA-AM. *Immunol Lett.* 2005; 101:60–64. [PubMed: 15893385]
- Shapiro DN, Adams BS, Niederhuber JE. Antigen-specific T cell activation results in an increase in cytoplasmic free calcium. *J Immunol.* 1985; 135:2256–2261. [PubMed: 2411788]
- Sun Z, Arendt CW, Ellmeier W, Schaeffer EM, Sunshine MJ, Gandhi L, Annes J, Petrzilka D, Kupfer A, Schwartzberg PL, Littman DR. PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes. *Nature.* 2000; 404:402–407. [PubMed: 10746729]
- Tan SL, Parker PJ. Emerging and diverse roles of protein kinase C in immune cell signalling. *Biochem J.* 2003; 376:545–552. [PubMed: 14570590]

- Tsien RY. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry*. 1980; 19:2396–2404. [PubMed: 6770893]
- Villunger A, Ghaffari-Tabrizi N, Tinhofer I, Krumbock N, Bauer B, Schneider T, Kasibhatla S, Greil R, Baier-Bitterlich G, Uberall F, Green DR, Baier G. Synergistic action of protein kinase C theta and calcineurin is sufficient for Fas ligand expression and induction of a crmA-sensitive apoptosis pathway in Jurkat T cells. *Eur J Immunol*. 1999; 29:3549–3561. [PubMed: 10556809]
- Weiss MJ, Daley JF, Hodgdon JC, Reinherz EL. Calcium dependency of antigen-specific (T3-Ti) and alternative (T11) pathways of human T-cell activation. *Proc Natl Acad Sci U S A*. 1984; 81:6836–6840. [PubMed: 6093124]
- Willerford DM, Chen J, Ferry JA, Davidson L, Ma A, Alt FW. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. *Immunity*. 1995; 3:521–530. [PubMed: 7584142]
- Wood AJ, Severson AF, Meyer BJ. Condensin and cohesin complexity: the expanding repertoire of functions. *Nat Rev Genet*. 2010; 11:391–404. [PubMed: 20442714]
- Zhao K, Wang W, Rando OJ, Xue Y, Swiderek K, Kuo A, Crabtree GR. Rapid and phosphoinositol-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling. *Cell*. 1998; 95:625–636. [PubMed: 9845365]
- Zweifach A, Lewis RS. Mitogen-regulated Ca^{2+} current of T lymphocytes is activated by depletion of intracellular Ca^{2+} stores. *Proc Natl Acad Sci U S A*. 1993; 90:6295–6299. [PubMed: 8392195]

HIGHLIGHTS

- Chromatin rapidly decondenses during T-cell activation
- Mobilization of intracellular calcium is required for proper chromatin decondensation
- Activation-induced decondensation occurs independently of NFAT activation
- Extracellular calcium is dispensable for the induction of chromatin decondensation
- Mobilization of intracellular calcium is sufficient to induce chromatin decondensation

FIGURE 1. Activation of either the DAG or the IP₃ pathway decondenses chromatin
(A) Splenocytes were left untreated (tinted histogram) or stimulated with 1 μ g/mL anti-CD3 antibodies, 10 μ g/mL PMA, 250ng/mL ionomycin, or PMA in combination with ionomycin for 1 hour (dotted line), 3 hours (dashed line) or 6 hours (solid line). Cells were then analyzed by flow cytometry to determine chromatin accessibility in CD4⁺ cells (top row) and CD8⁺ cells (bottom row) by intracellular staining for H3K4me1. Data are representative of at least three independent experiments. **(B)** Biological triplicates were treated as in A. Data are the means $\pm$ SEM and are presented as the mean fluorescence intensity of H3K4me1 staining, calibrated to the 0 hour vehicle control. * p <0.05, ** p <0.005 (Student's *t*-test) compared with the 0 hour time point. **(C)** Purified naïve T-cells were left untreated or stimulated with anti-CD3 antibodies, PMA, ionomycin, or PMA in combination with Ionomycin for the times indicated. Proteins were resolved via SDS-PAGE and histone solubility was measured via detection of histone H3 as assayed by western blot. Detection of actin serves as a loading control. Data are representative of at least three independent experiments.

FIGURE 2. Mobilization of calcium is required to initiate chromatin decondensation

Splenocytes were pretreated for 30 minutes with vehicle (DMSO) or 10 μM BAPTA-AM and then stimulated with anti-CD3 antibodies for the times indicated. Chromatin accessibility was determined by flow cytometry for CD4+ (A,B) and CD8+ (C,D) T-cells by staining for H3K4me1. Data are the means ± SEM of triplicates and are presented as the mean fluorescence intensity of H3K4me1 staining, calibrated to the 0 hour vehicle control (A,C) or calibrated to the 0 hour time point of each treatment group showing the fold change in chromatin decondensation in response to TCR stimulation (B,D). Similar results were obtained with three independent experiments. *p<0.05, **p<0.005 (Student's t-test) compared with vehicle treatment at each time point.

FIGURE 3. Inhibition of the NFAT activation has a transient effect on initial chromatin decondensation

Splenocytes were pretreated for 30 minutes with vehicle (DMSO), 100nM FK506, or 1 μ g/mL Cyclosporin A (CsA) and then stimulated with anti-CD3 antibodies for the times indicated. Chromatin accessibility was determined via flow cytometry for CD4+ (A,B) and CD8+ (C,D) cells by staining for H3K4me1, calibrated to the 0 hour vehicle control (A,C) or calibrated to the 0 hour time point of each treatment group showing the fold change in chromatin decondensation in response to TCR stimulation (B,D). Similar results were obtained with three independent experiments. * $p < 0.05$, ** $p < 0.005$ (Student's t-test) compared with vehicle treatment at each time point.

FIGURE 4. Extracellular calcium is not required for initial chromatin decondensation
(A) Splenocytes were left untreated (tinted histogram) or stimulated with either 1 $\mu\text{g}/\text{mL}$ anti-CD3 antibodies or 250 ng/mL ionomycin in T-cell media supplemented with 5 mM EGTA for 1 hour (dotted line), 3 hours (dashed line) or 6 hours (solid line). Chromatin accessibility was determined via flow cytometry for CD4⁺ and CD8⁺ T-cells by staining for H3K4me1. Data are representative of three independent experiments. **(B)** Biological triplicates were treated as in A. Data are the means $\pm$ SEM and are presented as the mean fluorescence intensity of H3K4me1 staining, calibrated to the 0 hour vehicle control. ** $p < 0.005$ (Student's t-test) compared with 0 hour time point. **(C)** Purified naïve T-cells were treated and chromatin solubility was determined via western blot analysis of histone H3. Detection of actin serves as a loading control. Data are representative of at least three independent experiments.

FIGURE 5. Mobilization of calcium from intracellular stores is sufficient to induce chromatin decondensation

Splenocytes were left untreated (tinted histogram) or stimulated with 1 μ M thapsigargin in T-cell media (**A**) or T-cell media supplemented with 5mM EGTA (**B**) for 1 hour (dotted line), 3 hours (dashed line) or 6 hours (solid line). Chromatin accessibility was determined via flow cytometry for CD4⁺ and CD8⁺ T-cells by staining for H3K4me1. Data are representative of three independent experiments. (**C**) Biological triplicates were treated as in B. Data are the means $\pm$ SEM and are presented as the mean fluorescence intensity of H3K4me1 staining, calibrated to the 0 hour vehicle control. ** $p < 0.005$ (Student's *t*-test) compared with 0 hour time point. (**D**) Purified naïve T-cells were treated as above and chromatin solubility was determined via western blot analysis of histone H3. Detection of actin serves as a loading control. Data are representative of at least three independent experiments.