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Conditions of limited calcium influx (CLCI) inhibits *IL2* induction and favors expression of anergy-related genes in TCR/CD3 and CD28 costimulated primary human T cells

Sara Monaco[a], Beate Jahraus[b], Yvonne Samstag[b], Hilmar Bading[a,*]

[a] Department of Neurobiology, Interdisciplinary Center for Neurosciences, Heidelberg University, 69120, Heidelberg, Germany
[b] Department of Immunology, Heidelberg University, 69120, Heidelberg, Germany

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ABSTRACT

Calcium is a key regulator of the T cell immune response. Depending on the spatial properties (nucleus versus cytoplasm) of the calcium signals generated after CD3xCD28 stimulation, primary human T cells either mount a productive immune response or develop tolerance. Nuclear calcium acts as a genomic decision maker: during T cell activation, it drives expression of genes associated with a productive immune response while in its absence, stimulated T cells acquire an anergy-like gene profile. Selective inhibition of nuclear calcium signaling in stimulated T cells blocks the productive immune response and directs the cells towards an anergy-like state. Here we show that the two transcriptional programs that include, respectively, the 'activation gene', interleukin 2 (*IL2*) and 'anergy-related genes', *EGR2, EGR3*, and *CREM* have different requirements for transmembrane calcium flux. By either lowering extracellular calcium concentrations with EGTA or using low concentrations of the ORAI blockers, BTP2 or RO2959, we reduced transmembrane calcium flux in human primary T cells stimulated with CD3xCD28. These 'conditions of limited calcium influx' (CLCI) blocked CD3xCD28-induced *IL2* expression but only moderately affected induction of the anergy-related genes *EGR2*, *EGR3*, and *CREM*. We observed no difference in NFAT2 nuclear translocation after CD3xCD28 stimulation between normal conditions and CLCI. These results indicate that CLCI favors expression of anergy-related genes in activated human T cells. CLCI may be used to develop novel means for pro-tolerance immunosuppressive treatments.

1. Introduction

1.1. Calcium signaling in T cell activation

To secure unresponsiveness towards self-antigens, T cell receptor (TCR) stimulation in the absence of co-stimulation (Macian et al., 2004) initiates a state of T cell anergy. This involves the coordinate expression of a number of genes that serve inhibitory roles in the immune response. Calcium signals are central regulators of the immune response and important for specifying T cell fate (Feske, 2007). Calcium signaling is initiated by TCR specific binding to the Major Histocompatibility Complex (MHC) containing an antigenic peptide. This interaction activates phospholipase C (PLC), which in turn leads to production of inositol-1,4,5 trisphosphate (IP3) and a biphasic increase in the intracellular calcium concentration. The initial calcium rise is due to the release of calcium from the endoplasmic reticulum (ER) caused by binding of IP3 to IP3 receptors (IP3R). Depletion of ER calcium stores triggers calcium influx from the extracellular space across plasma membrane channels, which is known as store-operated calcium entry (SOCE). The membrane channels involved in this process are calcium-release-activated calcium (CRAC) channels (Hoth and Penner, 1992), which are formed by the Stromal Interaction Molecules (STIM) and ORAI channels (Niemeyer, 2016). STIMs reside predominantly in the ER membrane and function as ER calcium sensors. ORAI represents the pore-forming unit of the channel (Feske et al., 2006). CRAC channels are essential in T cell activation and abnormal CRAC channel activity

Abbreviations: CLCI, conditions of limited calcium influx; qRT-PCR, quantitative reverse transcriptase PCR; NFAT, nuclear factor of activated T cell; CaM, calmodulin; *IL2*, Interleukin 2; *EGR3*, Early growth response 3; *CREM*, cAMP response element modulator; TCR, T cell receptor; CRAC, calcium-release-activated calcium; SOCE, Store Operated Calcium Entry

* Corresponding author at: Department of Neurobiology, Interdisciplinary Center for Neurosciences, Heidelberg University, Im Neuenheimer Feld 366, 69120, Heidelberg, Germany.
E-mail addresses: monaco@nbio.uni-heidelberg.de (S. Monaco), beate.jahraus@immu.uni-heidelberg.de (B. Jahraus), Yvonne.Samstag@urz.uni-heidelberg.de (Y. Samstag), Bading@nbio.uni-heidelberg.de (H. Bading).

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has been linked to several human diseases, including severe combined immunodeficiency (SCID) disorders, allergy, inflammatory bowel disease and breast cancer (Feske, 2009; Parekh, 2010). Given their importance in the regulation of the immune response, CRAC channels, and in particular ORAIs have been the target of drug discovery initiatives. Several ORAI blockers have been identified. They are reported to have dramatic effects on the T cell immune responses both *in vitro* and *in vivo*, affecting cell proliferation, cytokine production, and effector functions (Jairaman and Prakriya, 2013; Parekh, 2010; Iskikawa, 2003).

Calmodulin (CaM) is the principal target of calcium signaling in T cells, and indeed in most other cell types that use calcium to control adaptive responses (Bading, 2013; Feske, 2007). The calcium/CaM complex binds to and activates several protein kinases and phosphatases. Calcineurin (CN), a calcium/CaM dependent phosphatase, is particularly important in T cells. Activated CN dephosphorylates nuclear factor of activated T cell (NFAT) leading to its translocation from the cytosol to the cell nucleus (Feske, 2007). The gene program induced by NFAT and consequently, the decision on T cell fate depends on whether a co-stimulatory receptor like CD28 is being activated. CD28 induces a signaling cascade leading to activation of PKC and MAPKs, and expression of c-Fos and c-Jun that form the transcription factor AP1. In case of co-stimulation of CD28, NFAT and AP1 form a complex that induces expression of activation genes (e.g., IL2, CD25, IFNɣ), thereby promoting a productive immune reaction. In the absence of CD28 stimulation and AP1 induction, NFAT acts as a homodimer to activate a different gene program that involves anergy genes and culminates in T cell tolerance (Macian et al., 2000).

1.2. Spatial aspects of calcium signaling in T cell fate decision

Our recent work using primary human T cells uncovered that the “T cell calcium signal” is not a single entity but that it harbors two spatially distinct components that serve different functions. Stimulus-evoked calcium increases in the cell nucleus are required for activating a gene program characteristic of a productive immune response (Monaco et al., 2016). In contrast, one of the functions of calcium increases in the cytosol is to activate NFAT translocation to the cell nucleus, which in the absence of nuclear calcium signaling leads to enhanced expression of genes that negatively modulate T cell activation and direct the T cell response towards a hyporesponsive state (Monaco et al., 2016). Thus, calcium signals in different subcellular compartments (nucleus versus cytosol) determine whether T cells mount a productive immune response or develop tolerance. The differential regulation of the T cell response by spatially distinct calcium signals resembles the situation in the nervous system where in particular nuclear calcium has emerged as a key regulator of genomic responses that mediated the consolidation of virtually all neuroadaptations. This includes acquired neuroprotection, memory formation, memory extinction, and chronic pain (Bading, 2013; Mauceri et al., 2011; Simonetti et al., 2013; Weislogel et al., 2013; Zhang et al., 2009).

Calcium regulated processes that are located to different subcellular compartments, serve different functions and are mechanistically distinct, are likely to have different activation thresholds. For example, the amplitude and/or duration of cytoplasmic calcium transients required for induction of NFAT translocation may be different from the calcium levels in the cell nucleus needed to activate CaMKIV/CREB signaling and increase AP1 expression. Therefore, calcium levels may exist that are above threshold for activation of the cytoplasmic but not the nuclear process or vice versa. Given that calcium levels rapidly equilibrate between cytoplasm and nucleus (Eder and Bading, 2007; Monaco et al., 2016), such ‘discriminating calcium rises’ that activate one but not the other mechanism, may be attained under conditions that restrict calcium entry into T cells after stimulation and thus limit the calcium levels reached. To test this hypothesis we established conditions that restrict calcium flux across the plasma membrane in primary human T cells stimulated with a combination of CD3 and CD28 antibodies (CD3xCD28). Our results indicate that ‘conditions of limited calcium influx’ (CLCI) can lead to uncoupling of genes associated with a productive immune response from those characteristic of an anergy-like state.

2. Material and methods

2.1. Human T cell cultures and stimulations

Human peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque (Linaris) density gradient centrifugation of heparinized blood from healthy volunteers. T cells were purified with negative magnetic bead selection using the Pan T cell isolation Kit (Miltenyi Biotech). The isolated T cell population contains predominantly CD4 + and CD8 + T cells and only a small fraction (less than 10%) of regulatory, Natural Killer and unconventional T cells. T cells were grown and stimulated in RPMI media containing 10% FBS, which contains a calcium concentration of 0.7 to 0.8 mM (Feske et al., 2000; unpublished data). For CD3xCD28 stimulation, 24-well plates were pre-coated with 6 µg/ml goat anti-mouse antibodies in PBS (Dianova) overnight. After 30 min blocking with RPMI containing 10% FBS, the wells were incubated with 1 µg/ml antibodies anti-CD3 (eBioscence) and 10 µg/ml anti-CD28 (BD Pharmingen) diluted in RPMI containing 10% FBS for 1 h. The plates were then washed with RPMI and the cells were plated for the indicated time (Wabnitz et al., 2007). The ORAI blockers BTP2 and RO2959 (Aobious) were diluted in DMSO. Both compounds are blockers of STIM/ORAI-mediated store-operated calcium entry (SOCE) (Jairaman et al., 2013).

2.2. Gene expression analysis using quantitative reverse transcriptase PCR (qRT-PCR)

Cells were harvested for total RNA extraction using RNase kit (Qiagen) with an additional on-column DNase I digestion according to the manufacturer’s instructions. cDNAs were synthesized from 1 µg total RNA using oligo(dT) primers and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. QRT-PCR was done using TaqMan Universal PCR Master Mix (Applied Biosystems) with Assays-on Demand Gene Expression Products. The human probes for *IL2*, *CREM*, *EGR2*, and *EGR3* were Assays-on-Demand Gene Expression Products with TaqMan MGB probes, FAM dye labelled. The thermal cycling conditions were 10 min at 95 °C, 55 cycles of 15 s for denaturation at 95 °C, and 60 s for annealing and extension at 60 °C. The expression levels of the target mRNA was normalized to the relative ratio of the expression of B2M mRNA using the ΔΔCt cycle threshold method. Each qRT-PCR assay was performed at least three times, and the results are expressed as the means ± SEM (Zhang et al., 2011a).

2.3. Immunoblot analysis

T cells were stimulated with CD3xCD28 antibodies for 6 h after a pre-treatment of 1 h with the indicated concentrations of BTP2 and RO2959. During the last 4 h of stimulation, 10 µg/ml Brefeldin A (Invitrogen) was added to obtain intracellular accumulation of IL2. Cells were lysed and subjected to standard immunoblot analysis. Antibodies against EGR2 (Novus biologicals), EGR3 (Santacruz Biotechnologies), IL2 (AB clonal) and beta actin (Santacruz biotechnologies) were used.

2.4. Calcium measurements

To measure intracellular calcium levels, 1.5 million cells were loaded with Indo-1 (0.2 µg/ml) for 45 min, washed and resuspended in 500 µl RPMI containing 10% FBS. Calcium fluxes were analyzed by flow cytometry (FACSAria cytometer, Becton Dickinson) by monitoring changes in Indo-1 fluorescence over time. CD3 antibodies (1 µg/ml)

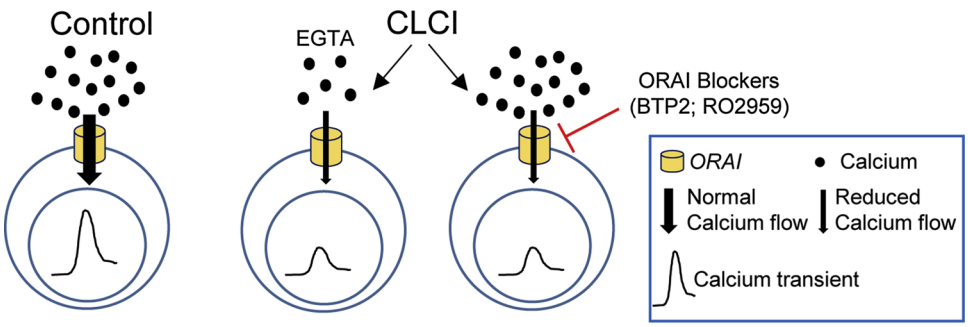


Fig. 1. Schematic illustration of the generation of conditions of limited calcium influx (CLCI) in human T cells during CD3xCD28 stimulation. Compared to physiologic conditions (Control, left part), CLCI generated either by reducing extracellular calcium concentration using EGTA (middle part) or by treatment with low concentrations of the ORAI blockers, BTP2 or RO2959 (right part) leads to limited calcium influx from the extracellular space in CD3xCD28-stimulated human T cells and to smaller intracellular calcium transients.

were added after 1 min of measurement providing the baseline fluorescence signal; after additional 2 min 0.6 µg/ml goat anti-mouse antibody was added to induce cross-linking. The signal was measured for 9 min. Indo-1 was excited at a wavelength of 350 nm and the ratios of the emission fluorescence signal intensities at a wavelength of 390 nm versus 496 nm (calcium bound Indo-1/free Indo-1) were determined. The kinetics of calcium transients were analyzed using Flow-Jo.

2.5. NFAT2 immunocytochemistry

Analysis of the subcellular localization of NFAT2 was done as described (Monaco et al., 2016). Briefly, cells were stimulated with antibodies against CD3xCD28 for 6 h in the presence or absence of different concentrations of BTP2 or RO2959 (preincubated for 1 h) or were left unstimulated. The cells were centrifuged in a cytospin for 5 min at 500 rpm onto poly-L-lysine coated coverslips (Sigma Aldrich), fixed in 4% PFA for 20 min at RT, permeabilized by washing 3 times in wash buffer (PBS containing 0.5% NP-40 and 0.01% NaN3), and nonspecific binding was blocked by incubation with wash buffer containing 10% FBS for 30 min at RT. The antibody incubation was done for 1 h at RT using anti-NFAT2 antibodies (7A6) (Enzo Lifescience), followed by incubation with Alexa 488 conjugated goat anti-rabbit antibodies (Invitrogen) for 1 h at RT. Counterstain of nuclei was done with Hoechst 33258 (2 µg/ml; Serva, Germany). Samples were mounted in Mowiol. Stained samples were analyzed using a CCD camera (Spot insight2 Visitron Systems). For quantitative analysis of nuclear versus cytoplasmic localization of NFAT2, Hoechst 33258 staining was used to identify the nucleus and the cytoplasm (see Fig. 5B). The fluorescence intensities of NFAT2 immunoreactivity in the cytoplasm and in the nucleus was measured using ImageJ in 30 cells for each condition and the ratios of the average fluorescence intensities of both areas was calculated.

2.6. Statistics

Data represent means +/- SEM. ANOVA with Tukey´s post hoc test was used for statistical analysis.

2.7. Study approval

The blood samples were taken from healthy donors. Written informed consent was received from the donors prior to inclusion in the study.

3. Results and discussion

3.1. Selection of representative CD3xCD28-driven genes for transcriptional analysis

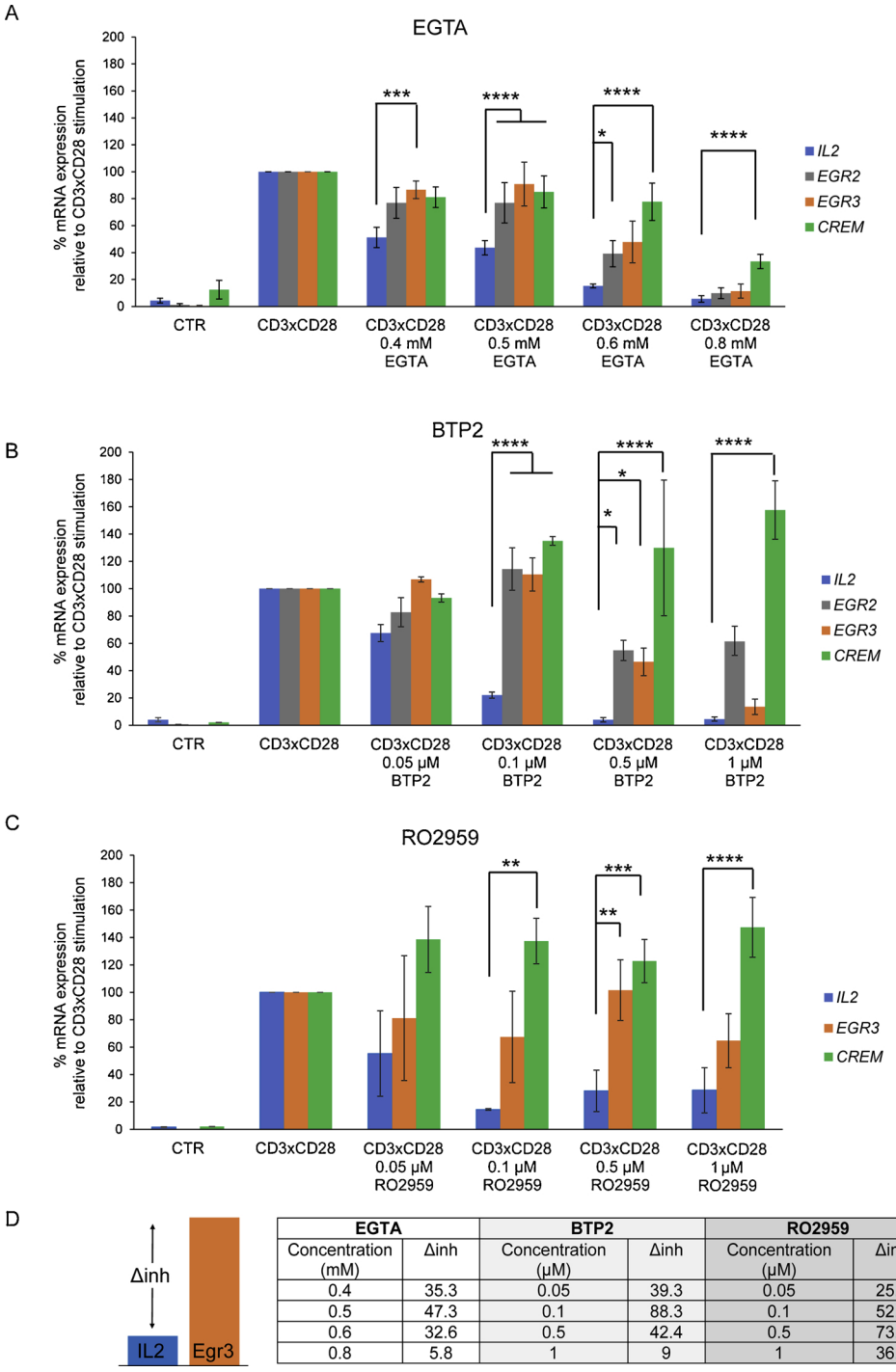
For clarity and simplicity, we refer to those genes that are required for a productive immune response as ‘activation genes’ and those genes that mediate the induction of the anergy-like state as ‘anergy-related genes’. Activation genes that include *IL2*, *INFY*, *CD25*, *CD69* are controlled by nuclear calcium while anergy-related genes, which include *EGR2*, *EGR3*, *CREM*, *DGKα*, *CTLA-4* are transcriptionally induced by cytosolic calcium under conditions of nuclear calcium signaling blockade (Monaco et al., 2016). For this study, we have chosen *IL2*, a key regulator of T cell immune response (Feske et al., 2001), as a representative of the activation genes and *EGR2*, *EGR3*, and *CREM*, as representatives of the anergy-related genes (Safford et al., 2005; Wells, 2009).

3.2. Establishing conditions of limited calcium influx (CLCI) during T cell activation

Previous work uncovered different spatial requirements for calcium signals in the transcriptional induction of activation genes versus anergy-related genes (Monaco et a., 2016). To investigate whether in addition, the two gene programs have also different requirements for transmembrane calcium entry, we used two strategies to establish conditions of limited calcium influx (CLCI) during CD3xCD28 stimulation of primary human T cells. First, we lowered the extracellular free calcium concentration by adding increasing amounts of the calcium chelator, EGTA to the media (Fig. 1, middle part). In this study, human T-cells were grown and stimulated in RPMI media containing 10% fetal bovine serum (FBS) which contains a calcium concentration of 0.7 to 0.8 mM (Feske et al., 2000; unpublished data). The addition of EGTA in the range from 0.4 mM and 0.8 mM lowers the free calcium concentration to the range of 0.3-0.4 mM to less than 0.02 mM, which affected activation and anergy-related genes differently (see below). As a second means of decreasing the driving force of calcium influx into CD3xCD28-stimulated human T cells we used BTP2 (also known as YM-58483) and RO2959. Both compounds are blockers of STIM/ORAI-mediated, CRAC channel-regulated store-operated calcium entry (SOCE) (Jairaman et al., 2013) (Fig. 1, right part). While their use at a concentration of 1 µM is known to block cytokine production and T cell proliferation (Chen et al., 2013; Ishikawa et al., 2003), we found that treatment with low concentrations (0.05–0.5 µM) of BTP2 and RO2959 led to CLCI and differential induction of gene expression.

3.3. Uncoupling of activation and anergy-related gene expression in CLCI

Our gene expression analysis revealed that in human primary T cells stimulated for 6 h with CD3xCD28, the induction of *IL2* expression was more sensitive to CLCI compared to that of the anergy-related genes *EGR2*, *EGR3*, and *CREM* (Fig. 2). We identified EGTA concentration between 0.4 mM and 0.6 mM as the critical range for differential regulation. In this concentration range, the reduction of *IL2* expression was significantly stronger than the reduction of *EGR2*, *EGR3*, and *CREM* expression (Fig. 2A). At 0.8 mM EGTA, which gives rise to a free calcium in the media of less than 0.02 mM, the induction of all genes tested was blocked except for *CREM* (Fig. 2A) that appears to require very little transmembrane calcium flux for its induction after CD3xCD28-stimulation. Similar results were obtained using the



EGTA		BTP2		RO2959	
Concentration (mM)	Δinh	Concentration (µM)	Δinh	Concentration (µM)	Δinh
0.4	35.3	0.05	39.3	0.05	25.8
0.5	47.3	0.1	88.3	0.1	52.9
0.6	32.6	0.5	42.4	0.5	73.4
0.8	5.8	1	9	1	36.2

Fig. 2. Effects of CLCI on transcriptional induction of activation and anergy-related genes in human T cells upon CD3xCD28 stimulation. CLCI was generated using increasing concentrations of EGTA in the media (**A**), or increasing concentrations of the ORAI blockers, BTP2 (**B**) or RO2959 (**C**). Primary human T cells were stimulated for 6 h with a combination of CD3 and CD28 antibodies (CD3xCD28) and expression of *IL2, EGR2*, *EGR3*, and *CREM* was analyzed using qRT-PCR. Cells were pre-treated with BTP2 and RO2959 for 1 h. Results are given as percentage of mRNA expression relative to mRNA expression after CD3xCD28 stimulation in media without EGTA (**A**) or without blockers (**B**), which was set to 100%. Asterisks indicate statistically significant differences in % inhibition by EGTA (**A**) BTP2 (**B**) and RO2959 (**C**) of the indicated genes relative to respective inhibition of *IL2*. (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0005). (**D**) Quantitative analysis of uncoupling of CD3xCD28 induced mRNA expression of *EGR3* and *IL2* after the indicated treatments. As shown schematically (left panel), Δinh is calculated as follows for a given condition: mean of the percentage of induction of mRNA expression of *EGR3* – mean of the percentage of induction of mRNA expression of *IL2* as shown in A–C. The values obtained for each treatment and condition are given in the table.

inhibitors of SOCE (Fig. 2B, C). The use of BTP2 and RO2959 was even more effective than EGTA in uncoupling the inhibition of *IL2* expression from that of anergy-related genes in CD3xCD28 stimulated primary human T cells. The pre-treatment with BTP2 or RO2959 was done for 1 h followed by CD3xCD28 stimulation for 6 h and subsequent gene expression analysis. Most effective uncoupling was observed with BTP2 at 0.1 µM (Fig. 2B) and with RO2959 at 0.1 µM and 0.5 µM (Fig. 2C). At these concentrations, the induction of *IL2* expression was inhibited by about 80% while induction of *EGR3* and *CREM*, as well as that of *EGR2* (data not shown) was very little affected (Fig. 2C). Similar to the result obtained with EGTA, the induction of *CREM* expression was least sensitive to blockade of SOCE. Even at 1 µM of either BTP2 or RO2959, expression of *CREM* was still induced after CD3xCD28 stimulation. To quantitatively determine the uncoupling of CD3xCD28 induced expression of the two classes of genes (i.e., activation genes and anergy-related genes) in CLCI, we defined the inhibition index 'Δinh' (Fig. 2D). Δinh is calculated based on the differences in expression (in control conditions versus CLCI) of *IL2* and of *EGR3*, which were selected as the representative examples of the activation and anergy-related groups of genes, respectively. Δinh was calculated as follows for a given condition: mean of the percentage of induction of mRNA expression of *EGR3* – mean of the percentage of induction of mRNA expression of *IL2* (see schematic illustration in Fig. 2D, left). These results indicate that during T cell stimulation, induction of activation genes and anergy-related genes have different requirements for transmembrane calcium entry. At CLCI, generated either with EGTA in the media or through

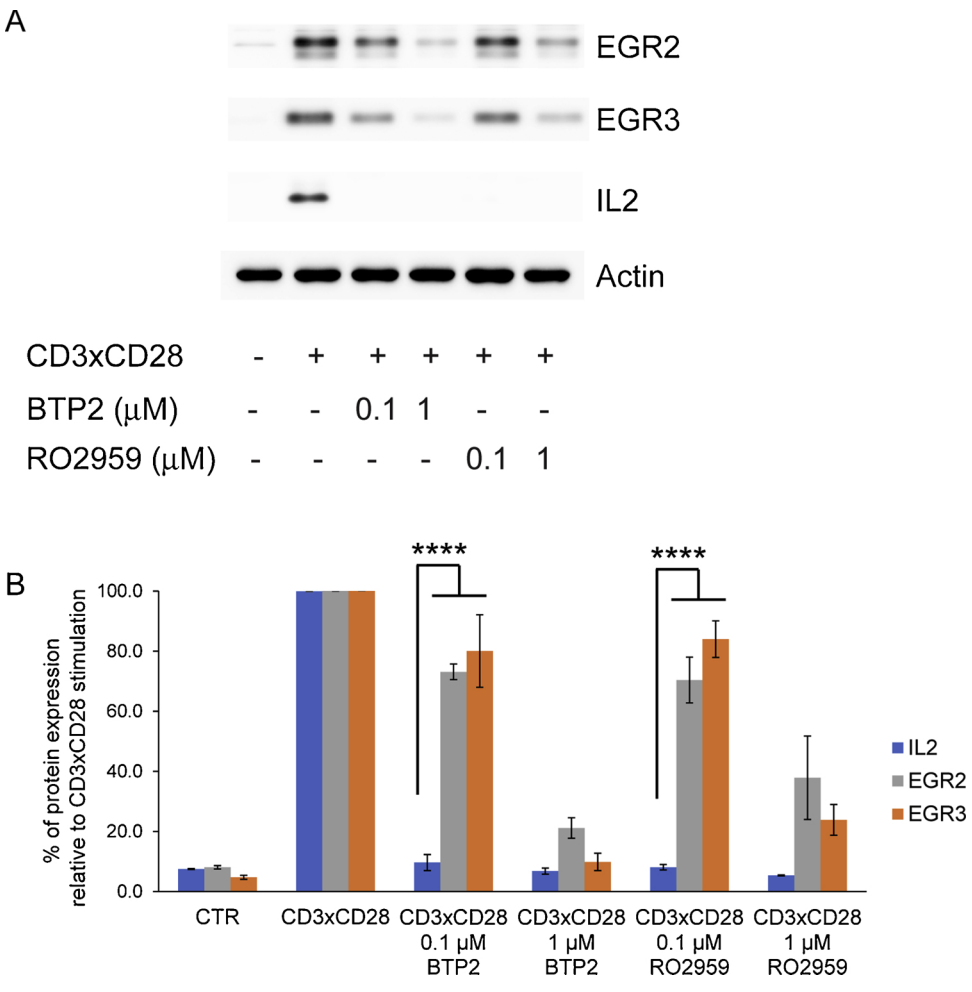


Fig. 3. Effects of CLCI on induction of activation and anergy-related protein upon CD3xCD28 stimulation. (**A**) Representative immunoblot analysis of EGR2, EGR3, IL2 and beta actin (Actin) in T cells stimulated with a combination of CD3 and CD28 antibodies (CD3xCD28) with or without pre-treatment with the indicated concentrations of BTP2 or RO2959. (**B**) Quantitative analysis of three different experiments. EGR2, EGR3 and IL2 protein levels were normalized to the expression levels of Actin. Results are given as percentage of protein expression relative to protein expression after CD3xCD28 stimulation without BTP2 and RO2959, which was set to 100%. Bars represent mean ± SEM. Asterisks indicate statistically significant differences in % inhibition by BTP2 and RO2959 of the expression of the proteins indicated relative to inhibition of IL2 under the same conditions (****P < 0.0005).

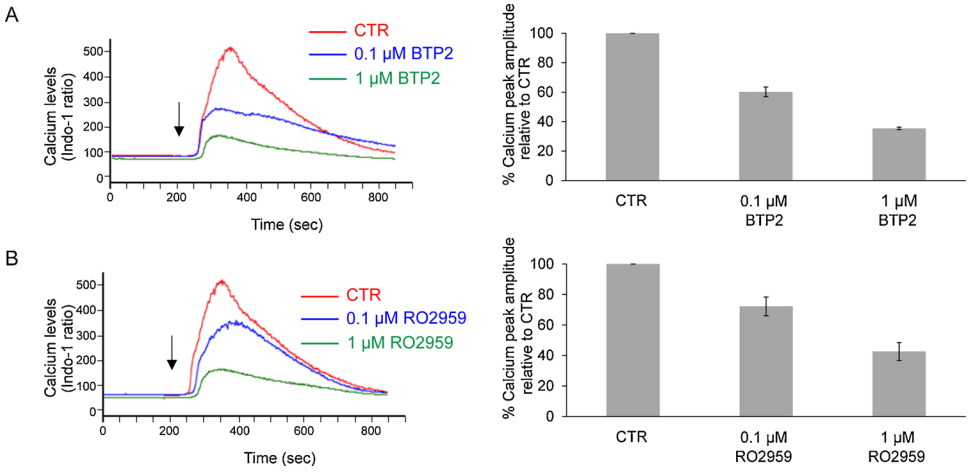


Fig. 4. Stimulus-induced calcium transients in CLCI. Calcium transients were measured by flow cytometry using the ratiometric indicator, Indo-1 in T cells pre-treated with vehicle (CTR) or with different concentrations of BTP2 (**A**) or RO2959 (**B**) and stimulated by exposure to CD3 antibodies followed by crosslinking with anti-mouse IgG antibodies as indicated with arrows. Representative example traces are shown on the left; quantitative analyses of calcium peak amplitudes are shown on the right. Results are given as percentage of peak amplitude in control conditions (CTR), which was set to 100%. Bars show mean ± SEM (n = 3).

pharmacological inhibition of SOCE, expression of anergy-related genes is favored in CD3xCD28 stimulated primary human T cells. An uncoupling of the transcriptional responses with cyclosporin A (CsA) was not possible. CsA blocks T cell activation through inhibiting calcineurin, thereby preventing the dephosphorylation and nuclear translocation of NFAT activation (Macian et al., 2002). NFAT dephosphorylation and nuclear translocation is required for induction of both activation and anergy-related genes (Macian et al., 2001). Therefore, as expected, we observed with any of the CsA concentration used, including as little as 50 nM, a concomitant reduction of induction of *IL2* and anergy-related genes (data not shown).

We next investigated whether the uncoupling of activation and anergy-related gene induction in CLCI translates into differential expression of the respective proteins. Indeed, we observed striking differences in the extent to which protein expression of activation and anergy-related genes was affected in CLCI. In CLCI generated with 0.1 μM BTP2 and 0.1 μM RO2959, which yields high level uncoupling of mRNA expression of activation and anergy-related genes (see Fig. 2), CD3xCD28 induced expression of EGR2 protein and EGR3 protein were only slightly reduced compared to CD3xCD28 stimulated control, while induction of expression of IL2 protein was virtually eliminated (Fig. 3). At 1 μM of either BTP2 or RO2959, CD3xCD28 induced protein

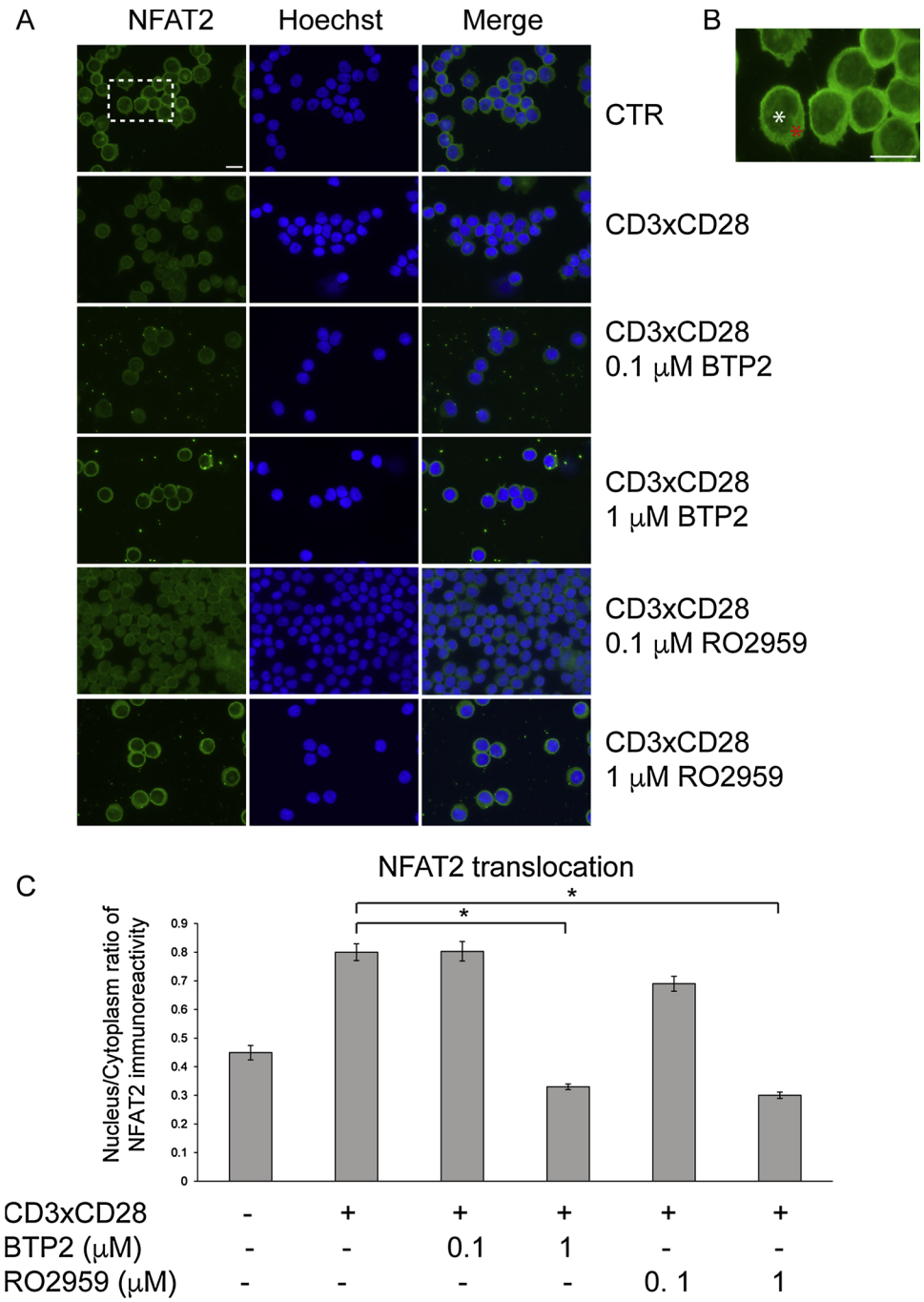


Fig. 5. Effect of CLCI on NFAT2 nuclear translocation in human T cells upon CD3xCD28 stimulation. (**A**) Immunocytochemical analysis of the intracellular distribution of NFAT2 in unstimulated (CTR) primary human T cells and in T cells stimulated with CD3xCD28 with or without pretreatment for 1 h with BTP2 or RO2959 using the indicated concentrations. Nuclei were counterstained with Hoechst 33,258. Scale bar is 10 μm. (**B**) Higher magnification of the region indicated with a dashed rectangle in (**A**). The white and the red asterisks indicate the regions used to quantify the nuclear and cytoplasmic NFAT2 immunoreactivity, respectively. (**C**) Quantitative analysis of NFAT2 subcellular localization. Results are given as the ratio of nuclear and cytoplasmic (Nucleus/Cytoplasm) NFAT2 immunoreactivity. Bars show mean ± SEM (n = 3). Statistically significant differences are indicated with asterisks (*P < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

expression of both the anergy-related genes *EGR2* and *EGR3*, and protein expression of the activation gene, *IL2* were robustly inhibited (Fig. 3). Thus, uncoupling of CD3xCD28 induced expression of the two classes of genes (i.e., activation genes and anergy-related genes) in CLCI takes place at the level of mRNA and protein expression.

3.4. Calcium transients in CLCI

We next determined the impact of CLCI on stimulus-induced calcium transients. Amplitude and duration of calcium signals in immune cells are critical determinants of the activation of transcriptional regulators and thus specify the type of transcriptional response (Dolmetsch et al., 1997). We measured calcium profiles by flow cytometry using the ratiometric indicator, Indo-1 in primary human T cells after stimulation by crosslinking of cell-bound CD3 antibodies (Fig. 4). We found that in control conditions, CD3 crosslinking induced the typical calcium profile of stimulated T cells. In CLCI induced by treatment with 0.1 μM BTP2 and 0.1 μM RO2959, the amplitudes of the stimulus-evoked calcium transients were reduced by 40% and 30%, respectively (Fig. 4A, B). As expected, treatment with high concentrations (1 μM) of either ORAI blocker almost completely eliminated the calcium transients.

3.5. NFAT2 translocation in CLCI

We finally investigated the nuclear translocation of NFAT2 in CLCI. Because low-amplitude sustained calcium signals can cause NFAT nuclear translocation (Dolmetsch et al., 1997), we predicted that in CLCI, NFAT2 will undergo this translocation. Indeed, we found that both, in control conditions and in the presence of 0.1 μM of either BTP2 or RO2959, NFAT2, one of the most abundant NFAT isoforms in T cells

activated during anergy (Srinivasan and Frauwirth, 2007), translocated to the cell nucleus of human T cells upon stimulation with CD3xCD28 (Fig. 5A, C). In contrast, and as expected, at 1 μM of BTP2 or 1 μM of RO2959, NFAT2 translocation was blocked (Fig. 5A, C). Thus, CLCI induced by low concentrations of BTP2 and RO2959 allows for NFAT2 translocation in CD3xCD28-stimulated human T cells and consequently for NFAT2-mediated expression of anergy-related genes.

4. Conclusion

Using simple means of restricting calcium entry into primary human T cells during CD3xCD28 stimulation, we disentanglement the transcriptional events that guide T cells either towards a productive response or an anergy-like state. CLCI can effectively inhibit *IL2* expression yet allowing for expression of genes that direct T cells towards tolerance. Exploiting the different requirements for transmembrane calcium entry for gene activation may help establishing new strategies for pro-tolerance immunosuppressive therapies.

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Author contributions

HB conceived the project. HB and SM wrote the manuscript. YS contributed to experimental methodology and the editing of the manuscript. SM performed experiments and analyzed data. BJ purified human T cells and performed experiments.

Declaration of Competing Interest

None.

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