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Dissertation

Calcium signaling dynamics in T cells - role of intra- & extracellular adenine nucleotides

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Declaration about personal contribution to manuscripts/publications in a cumulative thesis (please submit one form per manuscript/publication)

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Writing and editing of manuscript:

I was involved in reviewing and editing of the manuscript.

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Isolation of primary murine CD8+ T cells from WT spleen and lymph nodes and I performed Calcium microdomain imaging using antiCD3/anti-CD28 coated beads, adding apyrase to remove extracellular ATP (Fig. 2). Data analysis using a customised MatLab script, statistical analysis and preparation of figure 2.

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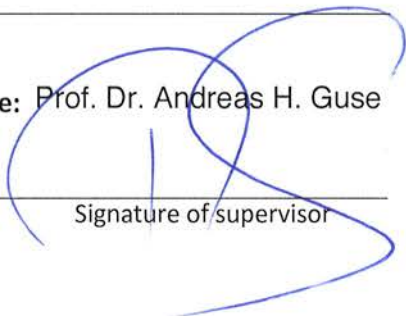
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Method development:

Adapting existing Calcium imaging protocols for global and local Calcium imaging experiments for soluble compound addition. Further, Western Blot protocols were adapted for optimal results.

Data generation and analyses (including contribution to specific figures):

I performed the Calcium microdomain measurements in Jurkat WT and HN1L/JPT2 knockout T cells, primary murine CD4+ T cells and KHYG-1 NK cells using MASTER-NAADP (Fig. 4A-G, S4, S5, S6, S7). Further, I performed global Calcium imaging experiments using Fura2-AM in Jurkat T cells stimulated with MASTER-NAADP, MASTER-NADP and NAADP-AM (Fig. 5, S8). Moreover, I assessed the HN1L/JPT2 expression in the cell lines that were used by Western Blot (Fig. S13). I carried out the final data and statistical analysis of all Calcium imaging experiments and prepared the figures.

Writing and editing of manuscript:

I was involved in the writing process of the first draft of the manuscript, as well as reviewing and editing of the manuscript.

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II Summary

For cells to survive, proliferate, and differentiate, it is essential that both extracellular and intracellular signaling are tightly regulated. In T cells, signaling pathways that depend on Ca^{2+} control their effector functions, resulting in either pro- or anti-inflammatory responses based on how long $[\text{Ca}^{2+}]_i$ remains elevated. Ca^{2+} microdomains that are adhesion-dependent and independent of T cell receptors play a role in the initial sensitizing process, which is marked by local signals with reduced Ca^{2+} amplitudes, such as ATP-dependent Ca^{2+} microdomains through P2X4. The engagement of the TCR/CD3 complex leads to the rapid generation of NAADP, a second messenger that mobilises Ca^{2+} and induces Ca^{2+} microdomains through the action of type 1 ryanodine receptors. These microdomains eventually converge into a global Ca^{2+} response, which is augmented by store-operated Ca^{2+} entry and further intensified by IP_3 and subsequent cADPR signaling. Additionally, the purinergic Ca^{2+} channels P2X4 and P2X7, as well as ATP release by pannexin 1 channels, further influence the initial Ca^{2+} microdomain formation in CD4^+ T cells, fine-tuning the T cell response.

The ability of NAADP to induce Ca^{2+} release from intracellular stores such as the ER and lysosomes has been a topic of debate over the past decade. The recent discovery of NAADP-binding proteins HN1L/JPT2 and Lsm12, along with their binding receptors on the ER (RyR1) and lysosomes (TPCs), underscores the need for a tool to directly investigate NAADP-dependent signaling across various cell types.

One main aim of this thesis was to characterise the recently developed compound MASTER-NAADP, a membrane-permeable and bioreversible NAADP derivative, and its respective control, in their capacity to evoke local Ca^{2+} microdomains and global Ca^{2+} signals in Jurkat T cells by high-resolution live-cell imaging (Publication 2.3). Additionally, using different cell types, e.g. Neuro2A, KHYG-1 and primary murine CD4^+ T cells, working concentrations of MASTER-NAADP in those cell types were established, as well as using knockout models of *Hn1l/jpt2* to validate the involvement of the NAADP binding protein. Furthermore, comparison of MASTER-NAADP to the commercially available NAADP-AM in live-cell-imaging, as well as HPLC studies, contributes to the characterisation of MASTER-NAADP. Finally, the liberated derivative was validated in a permeabilized cell assay and in lipid planar bilayer measurements containing HN1L/JPT2 and RyR1.

Additionally, the involvement of purinergic signaling pathways in initial Ca^{2+} microdomain formation as well as downstream effects was identified in CD8^+ T cells (Publication 2.2). P2X4 and P2X7 signaling were essential for Ca^{2+} microdomain formation, as both knockouts and extracellular ATP removal by apyrase resulted in diminished Ca^{2+} microdomain formation, whereas the global Ca^{2+} response was only mildly affected. Further, NFAT-1 translocation was impaired in *P2rx4^{-/-}* and *P2rx7^{-/-}* CD8^+ T cells compared to the WT, along with reduced Nur77, INF- γ and granzyme B expression. Finally, proliferation was also negatively impacted by *P2rx4^{-/-}* and *P2rx7^{-/-}* in these cells.

As the analysis and quantification of Ca^{2+} microdomains proved to be time-consuming, a modular open-source Python pipeline for (time) efficient analysis was developed (Publication 2.1). DARTS (Deconvolution, Analysis, Registration, Tracking and Shape normalisation) integrates state-of-the-art image postprocessing options, with variable deconvolution algorithms to choose from depending on image quality, deep-learning based cell detection and tracking and bleaching correction of FuraRed. Further, the integrated cell shape normalisation module allows for aggregation of the spatio-temporal information of Ca^{2+} microdomains onto a circular template (dartboard projection) allowing for accessible comparison of Ca^{2+} microdomains across groups.

In summary, the updated method for high-resolution live-cell Ca^{2+} imaging, along with the post-processing and analysis pipeline described here, enables Ca^{2+} microdomain detection across cell types. In CD8^{+} T cells, both P2X4 and P2X7 influence not only Ca^{2+} microdomain formation, but Ca^{2+} microdomains via these channels are necessary for a proper immune response. Further, MASTER-NAADP is established as a *bona fide* NAADP-mimic to unravel NAADP-mediated Ca^{2+} signaling.

III Zusammenfassung

Damit Zellen überleben, proliferieren und differenzieren können ist es essentiell, dass sowohl die extrazelluläre, als auch die intrazelluläre Signalweiterleitung streng reguliert werden. In T Zellen sind Signalwege, die Ca^{2+} -abhängig sind, dafür verantwortlich, dass die Effektorfunktionen, die sowohl pro- als auch anti-inflammatorische Antworten beinhalten über die Stärke und Dauer der Erhöhung der $[\text{Ca}^{2+}]_i$ kontrolliert werden. T-Zell Rezeptor unabhängige, aber Adhäsions-abhängige Ca^{2+} Mikrodomänen spielen eine entscheidende Rolle in der initialen Sensibilisierung der T-Zellen. Dieser Prozess ist gekennzeichnet durch lokale Signale mit reduzierten Ca^{2+} Amplituden, welche ebenfalls in ATP-abhängigen Ca^{2+} Mikrodomänen durch P2X4 zu beobachten sind. Die Aktivierung des T-Zell Rezeptor/CD3 Komplex führt zur schnellen Produktion von NAADP, einem Ca^{2+} mobilisierenden sekundären Botenstoff, der die Bildung von Ca^{2+} Mikrodomänen über den Ryanodin Rezeptor Typ I (RyR1) herbeiführt. Letztendlich gehen die Ca^{2+} Mikrodomänen in ein globales Ca^{2+} Signal über, welches über speichergesteuerten Ca^{2+} Einstrom und weitere sekundäre Botenstoffe wie IP_3 und cADPR amplifiziert wird. Zusätzlich beeinflussen die purinergen Ca^{2+} Kanäle P2X4 und P2X7, sowie die ATP-Freisetzung durch pannexin 1 Kanäle, die Bildung von initialen Ca^{2+} Mikrodomänen in CD4^+ T Zellen und regulieren somit zusätzlich die T-Zell Antwort.

Wie NAADP Ca^{2+} freisetzt und aus welchen intrazellulären Speichern Ca^{2+} freigesetzt wird, ist bis heute unklar. Die vor kurzen identifizierten NAADP-Bindeproteine, HN1L/JPT2 und LSM12, und deren Bindung an die möglichen Rezeptoren am ER (RyR1) und Lysosomen (TPCs), unterstreicht die Notwendigkeit einer Methode um NAADP-abhängige Signalwege direkt und in verschiedenen Zelltypen untersuchen zu können.

Ein Ziel dieser Arbeit war die Charakterisierung der kürzlich entwickelten Verbindung MASTER-NAADP, ein membran-gängiges und bioreversibles NAADP-Derivat und dessen Kontrolle, hinsichtlich ihrer Möglichkeit der Freisetzung von lokalen oder globalen Ca^{2+} Signalen, mit Hilfe von hoch-auflösender Lebendzell-Mikroskopie (Publikation 2.3: Krukenberg et al. (2024)). Zusätzlich wurden in verschiedenen Zelltypen, wie Neuro2a, KHYG-1 und primären murinen CD4^+ T Zellen, sowohl die in der jeweiligen Zelllinie zu verwendenden Konzentrationen etabliert, als auch ein Knockout-Modell des NAADP-Bindeproteins (HN1L/JPT2) verwendet, um dessen Rolle in NAADP-abhängigen Ca^{2+} Signalen zu verifizieren. Des weiteren wurde MASTER-NAADP mit dem kommerziell erhältlichen NAADP-AM hinsichtlich der Ca^{2+} Freisetzung und in HPLC Studien verglichen. Anschließend wurde die durch Esterase freigesetzte Verbindung in permeabilisierten Zell-Tests, als auch in *lipid-planar bilayer* Experimenten validiert.

Des weiteren wurde die Rolle von purinergen Kanälen in der Bildung initialer Ca^{2+} Mikrodomänen und deren Auswirkung auf nachfolgende intrazelluläre Signalwege in CD8^+ T Zellen untersucht (Publikation 2.2: Brock et al. (2024)). Sowohl P2X4, als auch P2X7 waren essentiell für die Bildung von Ca^{2+} Mikrodomänen in CD8^+ T Zellen, da nicht nur die genetische Deletierung der Kanäle, sondern auch die Eliminierung von extrazellulären ATP durch Zugabe von Apyrase,

zu einer signifikant reduzierten Bildung von Ca^{2+} Mikrodomänen führte, wobei die globale Ca^{2+} Antwort nur zu einem geringen Anteil beeinflusst wurde. Außerdem war die Translokation des Transkriptionsfaktors NFAT-1 in $P2rx4^{-/-}$ und $P2rx7^{-/-}$ CD8^{+} T Zellen im Vergleich zum WT signifikant verringert, zusammen mit reduzierten Nur77, INF- γ und Granzym B Expression. Zusätzlich war die Proliferation ebenfalls negativ beeinflusst durch $P2rx4^{-/-}$ und $P2rx7^{-/-}$.

Da die Analyse und Quantifizierung von Ca^{2+} Mikrodomänen sich als (Zeit)-intensiv heraus stellte, wurde ein modulares, frei verfügbares Python Skript für eine (Zeit)-effiziente Analyse entwickelt (Publikation 2.1: Woelk et al. (2024)). DARTS integriert den neuesten Stand von Post-Prozessierungsoptionen, mit variabel wählbaren Dekonvolutionsalgorithmen je nach Bildqualität, Deep Learning basierten Zell-Detektions und Verfolgungsmethoden und einer Bleichkorrektur für FuraRed. Des weiteren erlaubt das integrierte Modul für Zellformnormalisierung die Aufsummierung der Raum-zeitlichen Information von Ca^{2+} Mikrodomänen und ermöglicht damit einen direkten Vergleich zwischen verschiedenen Gruppen.

Zusammenfassend kann die hier beschriebene Methode für hochauflösende Lebendzell-Mikroskopie und deren Postprozessierung und Analyse zur Detektierung von Ca^{2+} Mikrodomänen in diesen Zelltypen verwendet werden. In CD8^{+} T Zellen beeinflussen sowohl P2X4 als auch P2X7 nicht nur die Bildung von Ca^{2+} Mikrodomänen, sondern sind auch notwendig für die darauffolgende korrekte Immunantwort. Des weiteren wurde MASTER-NAADP als *bona fide* NAADP-nachahmende Verbindung verifiziert um NAADP-induzierte Ca^{2+} Signalwege zu untersuchen.

III Index of abbreviations

[Ca ²⁺] _i	free cytosolic Ca ²⁺ concentration
AB	acyloxybenzyl
ACB	alkoxycarbonyloxybenzyl
ADCM	adhesion-dependent Ca ²⁺ microdomains
ADPR	adenosine diphosphate ribose
AM	acetoxymethyl
AP	activator protein
APC	antigen presenting cell
ASPDH	aspartate dehydrogenase domain-containing protein
ATP	adenosine triphosphate
cADPR	cyclic adenosine diphosphate ribose
CaM	Calmodulin
cAMP	cyclic adenosine monophosphate
CD38	cyclic ADP ribose hydrolase / cluster of differentiation 38
CRAC	Calcium release-activated channels
CTL	cytotoxic T lymphocyte
DAG	diacylglycerol
DC	dendritic cell
deMASTER-NAADP	deprotected Membrane permeAble, STabilized, bio-rEversibly protected precursor of NAADP
DL	deep learning
DUOX	dual oxidase
EAE	experimental autoimmune encephalomyelitis
fps	frames per second
GECI	genetically encoded fluorescent Ca ²⁺ indicators
HN1L	hematological and neurological expressed 1
kDa	kilo Dalton
HPLC	high-performance liquid chromatography
IL	interleukin
IP ₃	D- <i>myo</i> -inositol-1,4,5-trisphosphate
IP ₃ -R	D- <i>myo</i> -inositol-1,4,5-trisphosphate receptor
ITAM	immunoreceptor tyrosine-based activation motif
ITC	isothermal titration calorimetry
JPT2	jupiter microtubule associated homolog 2
LR	Lucy-Richardson
LSM12	Sm-like (LSM) protein 12

MASTER-NAADP	Membrane permeable, Stabilized, bio-reversibly protected precursor of NAADP
MHC	major histocompatibility complex
ML	machine learning
MS	multiple sclerosis
NAADP	nicotinic acid adenine dinucleotide phosphate
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	nuclear factor of activated T cells
NK cell	natural killer cell
NOX	NADPH oxidase
PAL	photo-affinity labelling
PAMPs	pathogen-associated molecular patterns
PANX1	pannexin-1
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PLC	phospholipase C
PM	plasma membrane
PMCA	plasma membrane Ca^{2+} ATPase
PSF	point spread function
PTK	protein tyrosine kinase
ROI	region of interest
ROS	reactive oxygen species
RyR	ryanodine receptor
SARM1	sterile alpha toll/interleukin receptor motif containing-1
SERCA	sarcoplasmic / endoplasmatic reticulum Ca^{2+} ATP-ase
SNR	signal-to-noise ratio
SOCE	store-operated Ca^{2+} entry
SoRa	confocal spinning disk super resolution
SR	super resolution
STIM	stromal interaction molecules
TCR	T cell receptor
TD ER	time-dependent entropy-like deconvolution
TDCM	T cell receptor dependent Ca^{2+} microdomains
T _h	T helper
TIRF	Total Internal Reflection Fluorescence

TM	transmembrane
TPC	two pore channel
TPC2-A1-N	1, 2-cyano-3-(3,5-dichlorophenyl)-3-oxo- <i>N</i> -(4-(trifluoromethyl)phenyl)propanamide
TPC2-A1-P	2, 5-(5-bromo-2-(trifluoromethoxy)phenyl)-1-(cyclohexylmethyl)-2-methyl-1 <i>H</i> -pyrrole-3-carboxylic acid

1 INTRODUCTION

1.1 The immune system

1.1.1 Innate vs. adaptive immune response

The immune system is an essential part of every living organism's line of defence to protect the body from pathogens such as fungi, bacteria, viruses and parasites. Moreover, infected or otherwise compromised cells (e.g. tumor cells) get eliminated by a carefully orchestrated immune response. This response is traditionally classified into two major pathways: i) the innate and ii) the adaptive immune response (Murphy et al. 2017).

The innate immune system comprises the first line of defence, hence being active minutes to hours after infection, activating antimicrobial effectors and the complement system leading to opsonisation via plasma proteins. Moreover, pathogen-associated molecular patterns (PAMPs), which are only present in pathogenic microbes and some viruses, are presented on the cell surface of infected cells, which in return are recognised via pathogen-recognition receptors on phagocytes, like macrophages, natural killer (NK) or dendritic cells (DCs) and lead to phagocytosis (Ma et al. 2024).

To activate the adaptive immune response, DCs transport the antigen from the site of infection to the lymphoid organs. There, antigenic peptides are presented by DCs via specific major histocompatibility complex (MHC) molecules and specifically recognized by naive B and T cells. Antigen recognition by naive B cells depends on T helper (T_h) cell interaction, leading to proliferation and differentiation to antibody-secreting plasma cells (Murphy et al. 2017). Both, secreted antibodies and activation of the complement system are part of the humoral immunity, either belonging to the adaptive (antibodies) or the innate (complement system) immune response.

Naive T cells proliferate and differentiate to effector T cells after antigen recognition. The matured T cells then infiltrate the site of infection to kill (cytotoxic T cells, $CD8^+$) the infected cells or attract other immune cells, such as T helper (T_h , $CD4^+$) cells by cytokine secretion. Differentiation of $CD4^+$ T cells into specific effector subtypes, e.g. $Th1$, $Th2$, $Th17$ and T_{reg} further ameliorates the immune response towards different pathogens (Hwang et al. 2020; Chopp et al. 2023). This process must be tightly regulated to circumvent autoimmunity or exhaustion. After clearance of an infection most B and T cells die, only a handful of subsets like T_{regs} and memory cells survive and patrol the body, enabling a more rapid re-activation of the adaptive immune response if the infection reoccurs (Murphy et al. 2017).

1.1.2 Activation of T cells by T cell receptor stimulation

Activation of naive T cells in the lymph nodes requires the formation of the immune synapse. Here, T cells encounter antigen presenting cells (APCs) by sensing APC produced cytokines, recognising and binding antigenic peptide MHC molecules, via the T cell receptor (TCR) complex and their respective CD4 (MHC class II) or CD8 (MHC class I) co-receptors (Brownlie and Zamoyska 2013). For complete naive T cell activation co-stimulatory-ligands need to be present, e.g. CD28 on T cells and B7 family members (CD80/CD86) on APCs (Azuma 2019).

The TCR is a heterodimer, formed by random rearrangement and pairing of conventional TCR α and TCR β chains, or non-conventional $\delta\gamma$ TCR chains, leading to 5×10^6 expressed TCR $\alpha\beta$ combinations in humans and thus a high variability (Godfrey et al. 2015). A functional TCR complex requires association of a CD3 $\epsilon\gamma$ dimer and a CD3 $\epsilon\delta$ dimer, forming the CD3 complex and the ζ chain which is present as a homodimer. Both CD3 dimers possess an extracellular immunoglobulin-like domain, while the ζ chain only has a short extracellular domain. Initial signaling via the TCR complex is transduced by tyrosine phosphorylation of cytoplasmic regions on the CD3 chains by protein tyrosine kinases (PTKs) (Brownlie and Zamoyska 2013). Each CD3 $\epsilon\delta\gamma$ chain possesses one immunoreceptor tyrosine-based activation motif (ITAM), characterised by a tandem duplication of a tyrosine containing sequence (YXXL/I), whereas ζ contains 3 ITAMs. Initially, Lck and to a lower extent Fyn, both Src family kinase members, that are associated with the cytoplasmic domains of CD4 and CD8 co-receptors, are recruited and phosphorylate ITAMS in CD ϵ , γ , δ and ζ chains (Brownlie and Zamoyska 2013). The phosphorylated ITAMs further recruit and activate the tyrosine kinase ZAP-70 (ζ chain of TCR associated protein 70), a Syk PTK family member, to the proximity of the plasma membrane (PM). The phosphorylated ζ subunit provides the binding site for ZAP-70 via Src homology protein 2 (SH2) domains. Upon binding, ZAP-70 is further phosphorylated and subsequently activated by Lck. This further leads to the phosphorylation of scaffold protein LAT (linker for activated T cells) and adaptor protein SLP-76 (SRC-homology-2-domain-containing leukocyte protein of 76 kDa) which are linked by the adaptor protein Gads (Grb2-related adaptor downstream of Shc) (Murphy et al. 2017). Moreover, phosphoinositide 3-kinase is recruited and activated, resulting in formation of PM tethered phosphatidylinositol-(3, 4, 5)-triphosphate (PIP₃) by phosphorylation of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂). Afterwards, the TCR signaling cascade branches into different signaling modules affecting e.g. transcription regulation, metabolism, cell adhesion and actin polymerisation (Murphy et al. 2017).

Here, the focus will be on phospholipase C- γ (PLC- γ) activation, which is recruited to the PM and is further phosphorylated by interleukin-2-inducible T cell kinase (Itk) to be fully activated. Activated PLC- γ cleaves membrane-tethered PI(3,5)P₂ into the membrane lipid diacylglycerol (DAG) and the soluble second messenger D-myo-inositol-1,4,5-trisphosphate (IP₃). Both, IP₃ and DAG induce several intracellular signaling pathways, like calcium (Ca⁺²) signaling, which will be further discussed in the following section, and the protein kinase C (PKC) pathway (Courtney

et al. 2018). Subsequently activating and translocating transcription factors like nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), activator protein (AP-1) and nuclear factor of activated T cells (NFAT), followed by gene expression responsible for T cell activation, proliferation and cytokine production (Trebak and Kinet 2019) (Overview: Fig. 1).

1.2 Calcium as a universal signaling ion

Ca^{2+} is a universal molecule in all eukaryotes, serving both nutritional and signaling purposes (Luan and Wang 2021). In immune cells, like T cells, B cells, NK cells, mast cells, DCs, monocytes and macrophages, changes in Ca^{2+} concentration serve as an important signaling prompt for their activation, but also induce effector functions, gene expression and differentiation (Feske 2007). However, unregulated cellular Ca^{2+} overload can result in cytotoxicity by inducing apoptotic or necrotic cell death (Orrenius et al. 2003). Thus, Ca^{2+} homeostasis inside the cell needs to be tightly regulated.

In T cells the resting cytosolic Ca^{2+} concentration is maintained in the low nanomolar (30 - 100 nM) range, whereas the extracellular Ca^{2+} concentration lies around 1 mM (Berridge et al. 2000; Feske 2007). Moreover, in the intracellular Ca^{2+} stores, the luminal Ca^{2+} concentration varies between 0.1 μM in mitochondria, 400 - 600 μM in lysosomes and up to 1 mM in the endoplasmic reticulum (ER) (Feske 2007). Due to the PM resting potential between -60/-50 mV, Ca^{2+} influx to the cytosol by voltage-gated Ca^{2+} channels is favoured. Upon stimulation, a rapid rise in the cytosolic Ca^{2+} concentration (up to 1000 nM) is triggered by either Ca^{2+} entry from the extracellular space or through Ca^{2+} release from intracellular stores. To maintain Ca^{2+} homeostasis, the Ca^{2+} stores are replenished by pumping Ca^{2+} from the cytosol back to the ER by sarco/endoplasmic reticulum Ca^{2+} -ATPases (SERCA) pumps sitting on the intracellular membranes and by pumping Ca^{2+} into the extracellular space via ATPases in the plasma membrane (PMCA). In addition, multiple Ca^{2+} binding proteins, like Calreticulin or Calmodulin (CaM), with nanomolar to micromolar high affinities for Ca^{2+} , buffer Ca^{2+} inside the cell (Luan and Wang 2021).

1.2.1 Ca^{2+} signaling in T cells

As described in **1.1.2**, TCR complex engagement with the antigen-peptide presented on APCs initiates multiple phosphorylation cascades, consequently activating the T cell. To transduce the initial activational signal, Ca^{2+} mobilizing second messengers, namely IP_3 , cyclic adenosine diphosphate ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) are recruited. NAADP is rapidly formed within the first seconds after stimulation and releases Ca^{2+} from intracellular Ca^{2+} stores, which is orchestrated by NAADP binding proteins like Sm-like protein (LSM12) or hematological and neurological expressed 1/ jupiter microtubule associated homolog 2 (HN1L/JPT2) and subsequent binding to their target channels type I ryanodine receptor (RyR1) on the ER or two pore channels (TPC1 & 2) localised on (endo-)lysosomes,

presumably cell type dependent (Fig. 1). A detailed overview of the formation of NAADP, the proposed binding proteins and subsequent targets will be given in a later section.

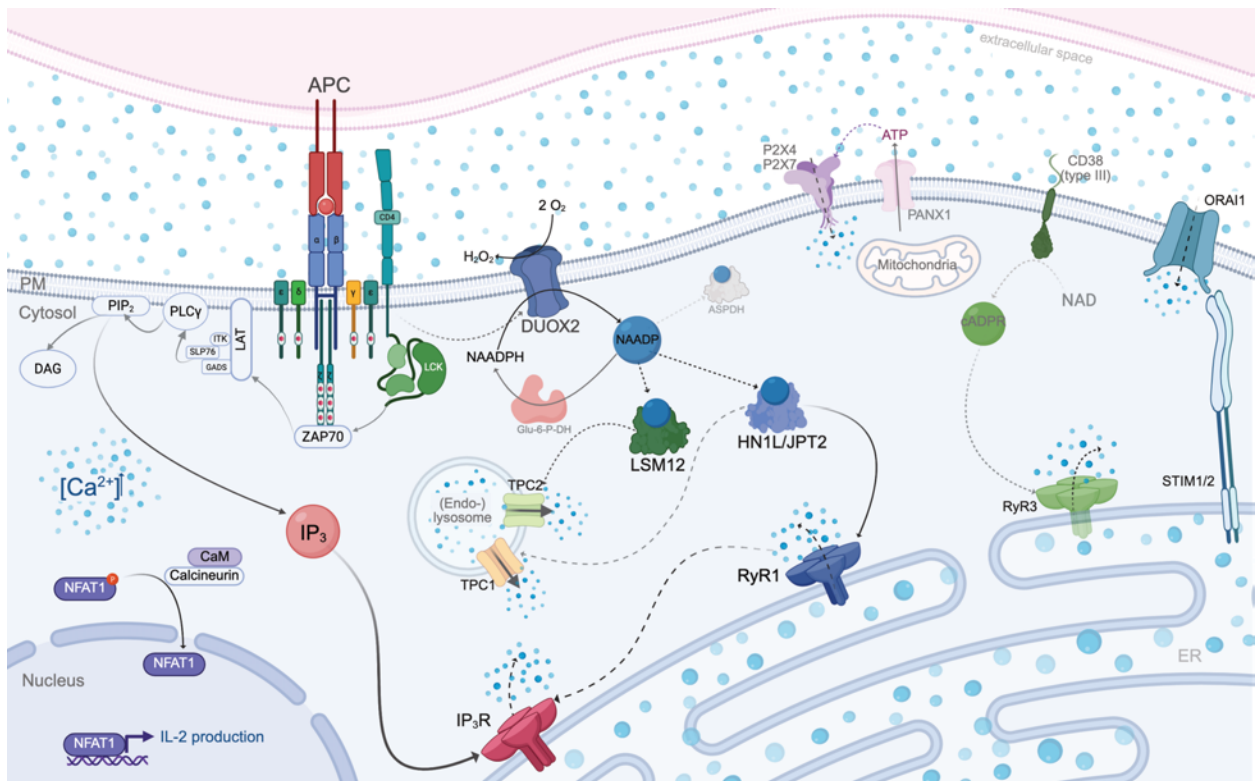


Figure 1: Ca²⁺ signaling in T cells. After TCR/CD3 stimulation by an APC, Ca²⁺ releasing second messengers like NAADP and IP₃ are formed and induce Ca²⁺ release from internal stores. Ca²⁺ depletion in the ER is sensed by STIM1/2, leading to a conformational change and activating store-operated Ca²⁺ entry channels (ORAI1), further elevating [Ca²⁺]_i. Increasing [Ca²⁺]_i is sensed by Calmodulin (CaM), activating Calcineurin to dephosphorylate NFAT1, translocating into the nucleus to initiate NFAT1-dependent gene transcription and subsequent interleukin-2 (IL-2) production. Figure created with BioRender.

The most intensively studied second messenger in Ca^{2+} signaling is IP_3 . The formation of IP_3 by PLC- γ reaches its peak concentration around 2 to 5 minutes after stimulation and triggers a moderate and transient Ca^{2+} release from the ER through binding to the three IP_3 receptor (IP_3R) isoforms (type I, II, III) (Kummerow et al. 2009). The IP_3R isoforms consist of homo- or heteromers, with varying sensitivity to IP_3 and an allosteric regulation by Ca^{2+} (Trebak and Kinet 2019). In T cells, type I IP_3R is the main isoform responsible for IP_3 induced Ca^{2+} release from the ER. In a recent publication by Weiß et al. (2023), IP_3 production by PLC γ activation through integrins and subsequent Ca^{2+} signaling via IP_3R have been identified to play an important role in adhesion-dependent Ca^{2+} microdomains (ADCM). These ADCMs sensitise the T cell for further activation through the TCR (Weiß et al. 2023; Diercks 2024). In addition, this low-level sensitisation is dependent on STIM1/ORAI1 pre-formed clusters (Diercks et al. 2018), as well as autocrine activation of P2X4 by basal ATP release via PANX1 (Brock et al. 2022).

In 1987 Clapper and colleagues discovered that IP_3 induces Ca^{2+} release from internal stores in sea urchin egg homogenates, whereas a metabolite of nicotinamide adenine dinucleotide (NAD), E-NAD, induced Ca^{2+} release with a lag period; this E-NAD was later identified as cADPR (Clapper et al. 1987; Lee et al. 1989). In T cells, levels in cADPR rise about ten minutes after T cell activation and evoke Ca^{2+} release from the ER via RyR3, therefore, both IP_3 and cADPR sustain the elevated $[Ca^{2+}]_i$ levels (Schwarzmann et al. 2002; Ernst et al. 2013).

The decrease in ER $[Ca^{2+}]$ is sensed by stromal interaction molecules (STIM) 1 and 2, leading to a conformational change, translocation to the plasma membrane and formation of STIM/ORAI clusters. Followed by activation of Calcium release-activated channels (CRAC), namely ORAI1/2 channels sitting in the PM, hence resulting in Ca^{2+} influx into the cytosol. This mechanism is termed store-operated Ca^{2+} entry (SOCE), further elevating $[Ca^{2+}]_i$ for minutes to hours. Prolonged Ca^{2+} microdomains by SOCE result in the translocation of NFAT to the nucleus, leading to the secretion of IL-17 and IFN- γ (Lee et al. 2018; Gu et al. 2021; Weiß et al. 2023).

1.2.2 Purinergic signaling in T cells

Purines, such as adenosine triphosphate (ATP) and adenosine, are key regulators of intracellular energy homeostasis and nucleotide production, e.g., NAADP, ADPR and cAMP (Huang et al. 2021). Extracellularly, these purines induce a variety of pro- or anti-inflammatory signaling pathways in immune cells by engaging purinergic P1 and P2 receptors, as well as a number of enzymes and transporters regulating the extracellular nucleotide concentration (Junger 2011; Cekic and Linden 2016). This concept is referred to as purinergic signaling. Fig. 2 shows the basic mechanisms of purinergic signaling, i) production and release of ATP into the extracellular space, ii) purinergic receptors (P2X, P2Y) activated by ATP or its metabolites and iii) mechanisms terminating purinergic signaling by breaking down extracellular ATP (eATP), ATP uptake or diffusion (Ledderose and Junger 2020). Under physiological conditions, eATP concentrations are considered to be in the low nanomolar range (1 - 10 nM), while cytoplasmic ATP concentrations are high (5 - 10 mM) (Trabanelli et al. 2012). Controlled release of intracellular ATP by exocytosis or by osmo- or mechano-sensitive nonjunctional hemichannels, e.g. pannexin1 (PANX1) and connexin (Schenk et al. 2008), or by cell death (e.g. apoptosis, necrosis) induce physiological processes including chemotaxis or T cell activation (Junger 2011; Trabanelli et al. 2012). Extracellular ATP is metabolised by the ectonucleotidases CD39 and CD73, hydrolysing ATP to AMP and subsequently to adenosine, activating adenosine receptors (Cekic and Linden 2016).

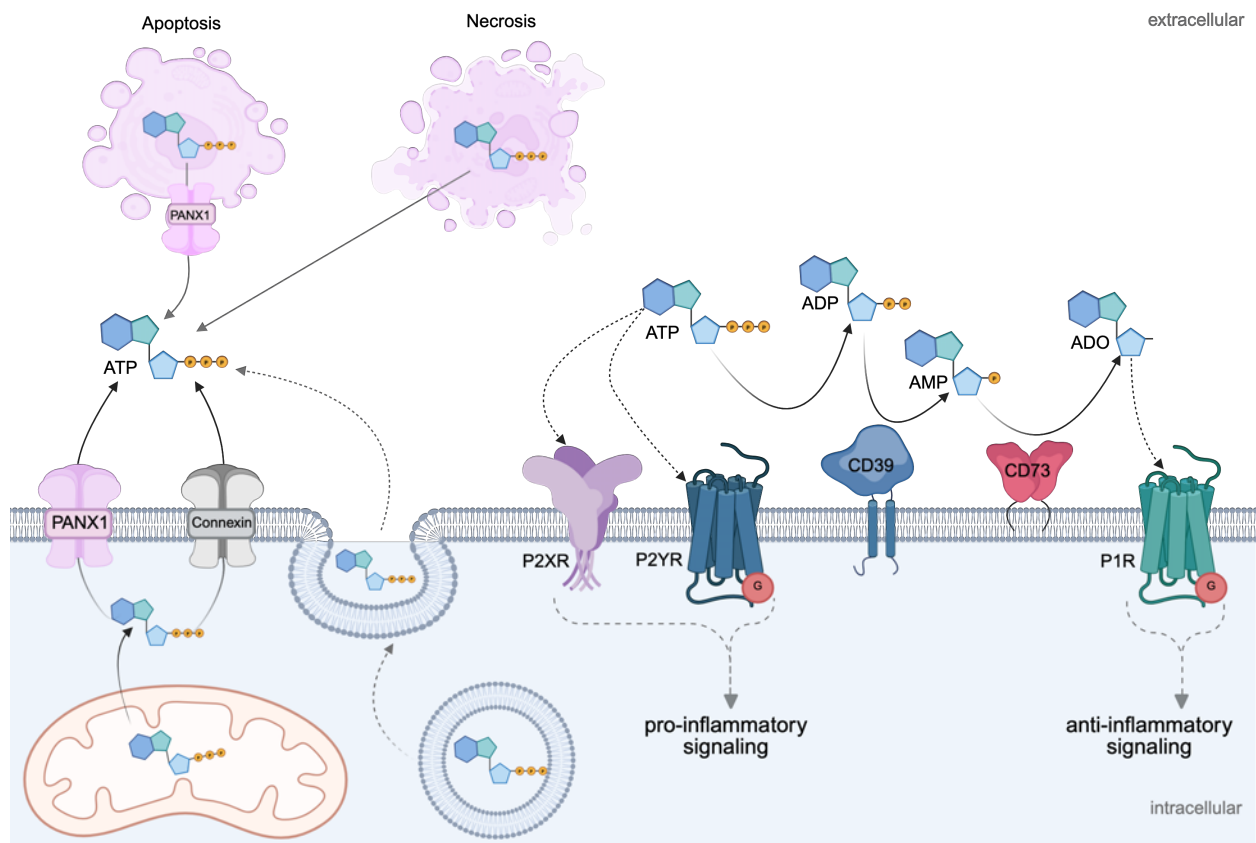


Figure 2: Purinergic signaling pathway. ATP is released into the extracellular space by hemichannels, e.g. connexin and PANX1, by exocytosis or by cell death. Extracellular ATP activates P2X or P2Y receptors leading to pro-inflammatory responses. Further, eATP is degraded by ectonucleotidases CD39 to AMP and CD73 to adenosine. Adenosine induces P1 receptor signaling mediating anti-inflammatory responses. Figure created with BioRender.

Role of P2X receptors in T cells

P2 receptors are further divided into two classes, the purinergic heterotrimeric G-protein coupled P2Y receptors and ligand-gated cation channels (P2X) (Schenk et al. 2011). P2X receptors are ATP-gated ion channels forming homo- and hetero-trimers and are permeable for Na^+ , K^+ and Ca^{2+} (Wang and Yu 2016). So far, 7 subtypes of P2X receptors (P2X1 - P2X7) have been identified and are abundantly expressed in excitatory and non-excitatory cells (Burnstock 2016a, b). In immune cells, eATP activates purinergic P2X receptors, e.g. P2X1, P2X4 and P2X7, triggering an amplification of global Ca^{2+} signaling affecting activation and inflammation (Junger 2011; Burnstock and Boeynaems 2014). In a resting T cell, P2X1, P2X4, P2X7 and PANX1 are uniformly distributed. Upon T cell activation, P2X1 and P2X4, as well as PANX1 translocate to the immune synapse, co-localising with ORAI1 and STIM1 molecules, whereas P2X7 remains uniformly distributed (Woehrle et al. 2010; Junger 2011). Here, the increasing $[\text{Ca}^{2+}]_i$ by SOCE through ORAI1 promotes mitochondrial ATP production (Ledderose et al. 2014), followed by localised ATP release through PANX1 at the IS, resulting in an autocrine activation of P2X4 and P2X7, further contributing to Ca^{2+} influx necessary for full T cell activation (Woehrle

et al. 2010; Ledderose et al. 2016; Brock et al. 2022). Silencing of P2X1 or P2X4 reduced Ca^{2+} entry upon T cell activation, leading to lower NFAT translocation and decreased IL-2 production (Woehrle et al. 2010), highlighting the importance of purinergic signaling for T cell activation and function. In addition to amplifying the global Ca^{2+} response upon TCR stimulation, P2X4, P2X7 as well as ATP release by PANX1 are essential in initial Ca^{2+} microdomain formation in CD4^+ T cells (Brock et al. 2022).

1.3 Nicotinic acid adenine dinucleotide phosphate (NAADP)

NAADP (Fig. 3) has been identified from nicotinamide adenine dinucleotide phosphate (NADP) impurities in the late 1980s and was shown to be responsible for the evoked Ca^{2+} release from sea urchin egg homogenates by NADP (Clapper et al. 1987; Lee and Aarhus 1995; Chini et al. 1995). In the following years, NAADP was established as the most potent endogenous Ca^{2+} mobilizing second messenger, since as low as 10 - 50 nM concentrations are sufficient to release Ca^{2+} from intracellular stores (Lee and Aarhus 1995; Albrieux et al. 1998; Cancela et al. 1999). In Jurkat T cells Berg et al. (2000) discovered a dose-dependency, where NAADP acts in a bell-shaped curve, hence releasing Ca^{2+} by microinjection of low concentrations of NAADP, but shows an inhibiting effect at higher concentrations (Berg et al. 2000; Gasser et al. 2006b, a). Moreover, both, IP_3 and cADPR signaling, are dependent on NAADP induced Ca^{2+} release; as co-injection of self-inactivating NAADP concentrations inhibited both IP_3R and cADPR induced signaling and resulted in diminished Ca^{2+} signals upon subsequent TCR stimulation (Lee and Aarhus 1995; Chini et al. 1995; Berg et al. 2000). Further, it has been recently discovered by our group that NAADP is crucial for Ca^{2+} microdomain formation in T cells (Wolf et al. 2015; Diercks et al. 2018; Gu et al. 2021; Roggenkamp et al. 2021).

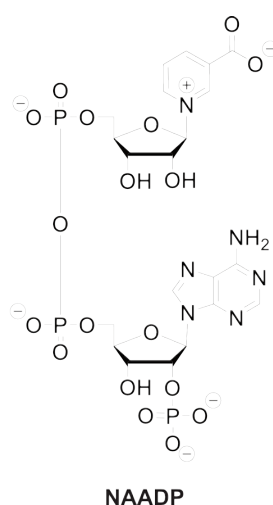


Figure 3: Structure of Nicotinic acid adenine dinucleotide phosphate (NAADP). Created with Chemdraw 23.1.2.

Since its discovery, many studies tried to resolve the question of i) how NAADP is formed, ii) what are the target receptors of NAADP or iii) if NAADP acts via binding proteins. This will be discussed in detail in the following sections.

1.3.1 NAADP formation

cyclic ADP ribose hydrolase/ cluster of differentiation 38 (CD38)

NAD-glycohydrolases, like the membrane-bound leukocyte antigen CD38 and sterile alpha toll/interleukin receptor motif containing-1 (SARM1) have been identified to catalyze reactions to produce NAD(P)-derived second messengers, like cADPR, ADPR, 2'-deoxy-ADPR and NAADP (Kim et al. 1993; Howard et al. 1993; Zocchi et al. 1993; Essuman et al. 2017; reviewed in Guse 2023a).

CD38 is a type II membrane protein, with its active catalytic site in the extracellular space, opening up the question how the second messengers produced are transported to their site of action. Addressing this question, two groups found that a small portion of CD38 is present in type III orientation with the catalytic domain facing the cytoplasm in cell lines (Zhao et al. (2012): HL-60, U937; Fliegert et al. (2017): Jurkat) and human primary monocytes (Zhao et al. 2012), hence resolving this problem.

In vitro, CD38 can catalyze the base-exchange reaction using NADP as a substrate, requiring an acidic pH (4-5) and high excess of nicotinic acid to form NAADP (Aarhus et al. 1995). *In vivo*, these conditions are not present in the cytosol and might only be met in endo-lysosomes, but this also requires the transport of NA and NADP into lysosomal lumen and export of NAADP into the cytosol (Nam et al. 2020; Guse 2023b). Targeting CD38 to endo-lysosomes increased endogenous NAADP levels (Fang et al. 2018). Moreover, various *Cd38* knockout models in e.g. lymphokine-activated killer cells (Rah et al. 2005; Nam et al. 2020) and pancreatic acinar cells (Cosker et al. 2010) propose a physiological role for CD38 in producing NAADP.

Contradicting to these findings, knockout of *Cd38* in CD4⁺ T cells neither affected Ca²⁺ microdomain formation nor global Ca²⁺ signaling (Gu et al. 2021). Moreover, in spleens and thymus from *Cd38* deficient mice endogenous NAADP levels were elevated (Schmid et al. 2011), shifting the perspective from a NAADP producing to a NAADP degrading role for CD38, at least in T cells.

Sterile alpha toll/interleukin receptor motif containing-1 (SARM1)

SARM1, a protein that is sequentially and structurally completely different from CD38 and is linked to axonal degradation in neurons, possesses similar NAD(P) metabolizing activity to ADPR, cADPR and NAADP as CD38 (Summers et al. 2016; Zhao et al. 2019; Guse 2023a). Interestingly, SARM1 produces higher cADPR but lower ADPR levels in comparison to CD38. Just recently, Angeletti and coworkers found that the base-exchange activity of SARM1 can take

place at neutral pH (Angeletti et al. 2022), highlighting SARM1 as an interesting candidate for endogenous cytosolic NAADP producing enzyme, but further research is needed.

NADPH (dual) oxidases

Since the evidence for CD38 as the NAADP producing enzyme is rather contradictory, another synthesis route ought to be explored. Members of the (dual) NADPH oxidase family (NOX/DUOX) produce reactive oxygen species (ROS), which is associated with inflammatory processes (Yarosz and Chang 2018) while oxidising NADPH to NADP (Leto et al. 2009; Taylor and Tse 2021). Consequently, the question arose whether they could also oxidise NAADPH to NAADP.

The NOX/DUOX enzyme family comprises seven members (NOX 1-5; DUOX1/DUOX2) with a conserved C-terminal domain containing FAD and NADPH binding sites and six conserved transmembrane domains for NOX1-5 and an additional N-terminal transmembrane domain for DUOX1/2 containing a peroxidase-homology domain (reviewed in: Bedard and Krause 2007; Taylor and Tse 2021). Moreover, NOX5, DUOX1 and DUOX2 contain EF hand Ca^{2+} binding domains. The maturation factors DUOXA1 and DUOXA2 are required for functional DUOX1 or DUOX2 activity, as they direct the transport of the functional protein from the ER to the PM (Grasberger and Refetoff 2006; Taylor and Tse 2021).

Gu and coworkers demonstrated, in NOX5, DUOX1 and DUOX2 overexpressing HEK293 membranes, the formation of NAADP from NAADPH under physiological conditions (Gu et al. 2021). To decipher the physiological relevance, knockout models of *Nox1*, *Nox2*, *Duox1*, *Duox2* or a functional knockout of *Duoxa1/Duoxa2* were used, as these are the isoforms mainly expressed in T cells. Interestingly, neither in *Nox1* nor *Nox2*-deficient mice an effect in local and global Ca^{2+} signaling upon TCR/CD3 stimulation, could be observed. The functional double knockout of DUOX1/2 by gene deletion of the maturation factors DUOXA1 and DUOXA2 (Grasberger and Refetoff 2006; Gunaratne et al. 2021) revealed that Ca^{2+} microdomain formation as well as global Ca^{2+} signaling relies on functional DUOX1/2 (Gu et al. 2021). Further, single knockout of *Duox2*, but not *Duox1* affected Ca^{2+} microdomain formation in the first 15 seconds after TCR stimulation, but both knockouts resulted in decreased global Ca^{2+} responses, therefore rendering DUOX2 as the main NAADP producing enzyme in T cells (Gu et al. 2021).

ROS homeostasis plays an important role in the regulation of inflammation in general. In T cells low-level ROS influences TCR signaling and NFAT translocation leading to the regulation of T cell differentiation (reviewed in Basso et al. 2021; Liu et al. 2023). Therefore, ROS production by NOX/DUOX enzymes, like production of H_2O_2 by oxidising NAADPH to NAADP, could further influence Ca^{2+} signaling, by activation of transient receptor potential melastatin 2 (TRPM2) channel or indirect inhibition of SERCA pumps (Gu et al. 2021). However, no evidence was found for involvement of H_2O_2 in initial Ca^{2+} microdomain formation, probably due to the calculated low nanomolar concentrations of H_2O_2 (Gu et al. 2021).

The redox cycle requires not only oxidation of NAADPH to NAADP by NOX/DUOX but also the

rapid reduction of NAADP to NAADPH, catalysed by glucose 6-phosphate dehydrogenase, but not by other NADP-dependent dehydrogenases (Billington et al. 2004; Gu et al. 2021). To summarize, DUOX2 and to a lower extent DUOX1 can be described as the NAADP forming enzymes upon TCR/CD3 stimulation.

1.3.2 The *unifying hypothesis*: NAADP binding proteins and target receptors

As mentioned above, NAADP target receptor(s) and subsequent targeted organelle(s) are controversially discussed in the literature. Up to date, members of voltage-gated ion channel superfamily, e.g. TPCs on endo-lysosomes and Ca^{2+} ion channels, e.g. RyR on ER (or sarcoplasmic reticulum) membranes have been proposed as target channels of NAADP. Although several groups could show that either TPCs or RyRs are regulated by NAADP; on neither TPC nor RyR, a direct binding site for NAADP could be identified (reviewed in Walseth and Guse (2021)). Usage of photo-affinity labelling (PAL) of NAADP binding proteins, by radioactively-labeled 5-azido-NAADP ([^{32}P]-5- N_3 -NAADP), resulted in labelling of small soluble proteins at 22/23 kDa in mammalian cell lines and tissues, as well as in sea urchin eggs and can be competed by free NAADP (Lin-Moshier et al. 2012; Walseth et al. 2012). These findings are in line with the idea by Gerasimenko et al. (2003), proposing binding proteins for NAADP and cADPR to activate RyR, hence a *unifying hypothesis* was introduced by Guse (2012). The *unifying hypothesis* ought to explain the different NAADP target receptors identified; NAADP does not directly bind to its respective target receptors but rather to a NAADP binding protein, followed by binding of this complex to the respective target and induces a small Ca^{2+} release from the targeted organelles (Guse 2012; Walseth and Guse 2021). In the recent years, three NAADP binding proteins have been identified, namely ASPDH (He et al. 2022), HN1L/JPT2 (Roggenkamp et al. 2021; Gunaratne et al. 2021) and LSM12 (Zhang et al. 2021), which will be further discussed in the following sections.

1.3.2.1 NAADP-binding proteins

ASPDH

He and coworkers identified a 32 kDa member of amino acid dehydrogenases - aspartate dehydrogenase domain-containing protein (ASPDH) - as a NAADP binding protein from pig liver homogenates (He et al. 2022). These proteins use NAD or NADP as the electron acceptor and due to the high structural similarity between NADP and NAADP they proposed binding of NAADP to ASPDH. Using isothermal titration calorimetry (ITC), they demonstrated NAADP binding to recombinantly expressed mouse ASPDH with a K_d of 445 nM, but observed no binding of NAADP to LSM12 or HN1L/JPT2 using the same experimental setup (He et al. 2022). Albeit binding of NAADP to ASPDH, the high K_d indicates only medium affinity, thus rather excludes ASPDH as the *bona fide* NAADP receptor (He et al. 2022).

LSM12

LSM12 is part of the LSm protein family, including smaller LSm proteins (LSM1-10) which only contain the N-terminal LSm domain and larger LSm proteins (e.g. LSM12) that possess a C-terminal anticodon binding domain. In eukaryotes, especially smaller LSm proteins function as chaperones for messenger RNAs (mRNA) and non-coding RNAs (Wilusz and Wilusz 2013) and LSM12 is thought to be involved in mRNA degradation (Albrecht and Lengauer 2004). According to the Protein Atlas, LSM12 is abundantly expressed in various tissues and cell lines¹.

Zhang et al. (2021) describe a novel role for LSM12 as a NAADP-binding protein. Using an affinity purification and proteomic analysis approach, they showed LSM12 complexed with NAADP, TPC1 and TPC2 (Zhang et al. 2021). Furthermore, they identified the NAADP binding site in the Lsm domain, mediating the association of NAADP to TPC2 and TPC2 containing membranes (Zhang et al. 2021). TPC activation by NAADP required LSM12 in microinjection-induced whole-cell currents, thus establishing LSM12 as a NAADP receptor (Zhang et al. 2021).

HN1L/JPT2

In 2021 two groups independently identified the 22/23 kDa protein as HN1L/JPT2 and published these findings back-to-back in *Science Signaling* (Roggenkamp et al. 2021; Gunaratne et al. 2021). *Hn1l/Jpt2* is part of a conserved gene family including three splice variants and was first associated with embryonic development and is localised in the cytosol and nuclear pore (Zhou et al. 2004). HN1L/JPT2 is an intrinsically disordered protein² and is mainly expressed in liver, kidney, prostate, testis and uterus (Zhou et al. 2004).

Upregulation of HN1L/JPT2 is associated with a variety of cancers, including adenocarcinoma of esophagogastric junction (Wang et al. 2021), tumor invasion in breast cancer (Jiao et al. 2021) and prostate cancer (Nong et al. 2022) (reviewed in Heßling et al. (2023)). Moreover, HN1L/JPT2 was recently identified to be involved in T cell infiltration into intestine in Parkinson's disease patients (Shao et al. 2023).

Both groups used PAL to identify NAADP-binding proteins in Jurkat T cells (Roggenkamp et al. 2021), human erythrocytes and U2OS cells (Gunaratne et al. 2021), as well as using competition assays with cold NAADP to show binding of recombinant HN1L/JPT2 to NAADP (Roggenkamp et al. 2021; Gunaratne et al. 2021). Roggenkamp et al. (2021) describe HN1L/JPT2 to be involved in TCR/CD3 induced Ca²⁺ microdomain formation in Jurkat T cells and primary rat T cells, as gene silencing resulted in diminished initial Ca²⁺ microdomains. Furthermore, global Ca²⁺ signals in peak and plateau phase were reduced in *Hn1l/Jpt2* deficient T cells, which could be restored by re-expressing *Hn1l/Jpt2* in these cells (Roggenkamp et al. 2021). Using BZ194, a NAADP antagonist (Dammermann et al. 2009), resulted in a comparable Ca²⁺ signaling profile (Roggenkamp et al. 2021). Moreover, Gunaratne et al. (2021) provided further insight to the role of HN1L/JPT2 in NAADP induced Ca²⁺ signaling in other cell types by using HN1L/JPT2 knockdown HEK293 and U2OS cells. Interestingly, Roggenkamp et al. (2021) provide data for

¹<https://www.proteinatlas.org/ENSG00000161654-LSM12/summary/gene>; last accessed: 04/11/2025

²<https://www.uniprot.org/uniprotkb/Q9H910/entry>; last accessed: 04/11/2025

the association of HN1L/JPT2 with the RyR in T cells, whereas Gunaratne et al. (2021) show interaction with TPC1 in NAADP-evoked Ca^{2+} release in HEK293 cells. Recently, a side by side analysis of HN1L/JPT2 and LSM12 verified both HN1L/JPT2 and LSM12 as NAADP binding proteins (Gunaratne et al. 2023). LSM12 showed a higher selectivity for NAADP over NADP in comparison to HN1L/JPT2 and HN1L/JPT2 prefers association with TPC1 over TPC2 (Gunaratne et al. 2023).

Taken together, the evidence provided by different groups render HN1L/JPT2 and LSM12 as an essential part of the NAADP signalosome. The identification of multiple NAADP-binding proteins raises the question of the binding modalities of NAADP by these proteins. Besides, the identification of multiple binding proteins for NAADP might justify the different targets that have been proposed.

1.3.2.2 Target receptors of NAADP

Two-Pore Channels

TPCs are members of the voltage-gated ion channel superfamily and have been identified in eukaryotic cells due to their sequence identity with voltage-gated Ca^{2+} (Ca_v) and Na^+ (Na_v) channels (Ishibashi et al. 2000; Furuichi et al. 2001; Patel 2015). Due to their property of being non-selective cation channels localizing in acidic organelles like endosomes and lysosomes (Brailoiu et al. 2009), they open up a novel class of voltage-gated ion channels (Patel 2015). Three isoforms of TPCs have been identified, in humans, primates and mice, only two of those isoforms are expressed: TPC1 and TPC2 (Patel 2015; Zhang et al. 2021). Further, both channels are ubiquitously expressed in mice with higher expression levels of TPC1 (Zong et al. 2009), whereas high expression of human TPC2 in liver and kidney has been observed (Calcraft et al. 2009). Structurally, TPCs are present as homo- or hetero-dimers forming pseudo-tetrameric complexes (Zong et al. 2009; Rietdorf et al. 2011). Each subunit consists of 6 transmembrane (TM) domains, with a pore-forming region between segment 5 and 6 and are connected by a cytoplasmic loop (Patel and Kilpatrick 2018). The dimeric structure allows for a pore to be formed that is permeable for Ca^{2+} , Na^{2+} , but also K^+ and H^+ ions (Fig. 4) (Pitt et al. 2014).

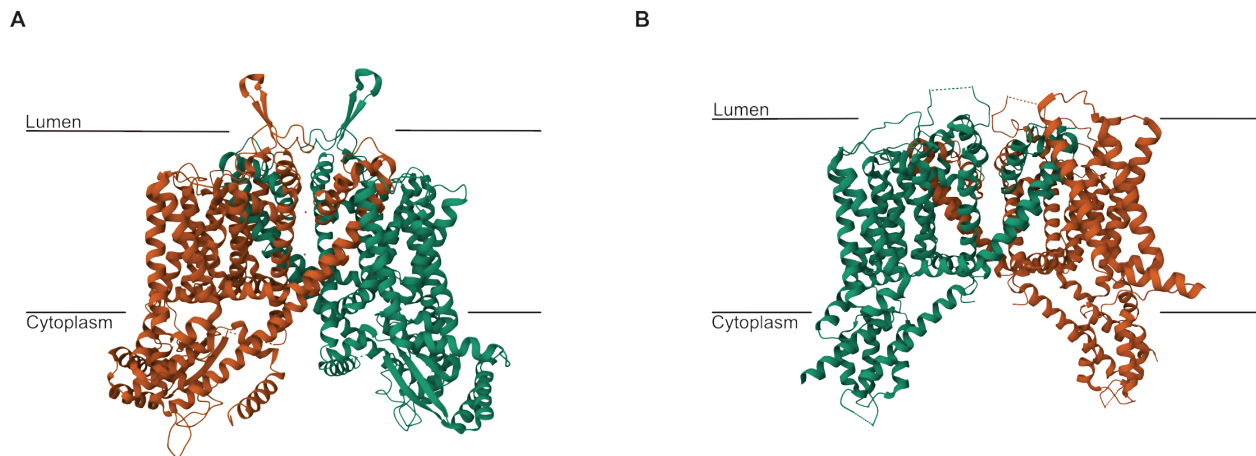


Figure 4: Cryo-EM structure of mammalian TPC1 and TPC2. (A) Cryo-EM structure of mouse TPC1 in apo state. (pdb_00006c96), modified after She et al. (2018). (B) Cryo-EM structure of human TPC2 in apo state (pdb_00006nq1), modified after She et al. (2018). Figure created with BioRender.

First evidence for TPCs on endo/lysosomes as NAADP-dependent Ca^{2+} release channels was published by three groups independently in 2009 (Calcraft et al. 2009; Brailoiu et al. 2009; Zong et al. 2009). Zong et al. (2009) used overexpressing TPC1 or TPC2 constructs in HEK293 cells, where injection of 30 nM NAADP via a patch-pipette was sufficient to evoke a Ca^{2+} response in TPC2 overexpressing HEK293 cells but not in the TPC1 constructs. Calcraft et al. (2009) confirmed TPC2 involvement in whole cell patch clamp experiments, perfusing with 10 nM NAADP. Moreover, using Bafilomycin A1, a H^{+} -ATPase inhibitor, blocked NAADP-induced Ca^{2+} signals in HEK293 cells overexpressing TPC2 (Zong et al. 2009). Interestingly, overexpression of TPC1 in SKRB3 cells lead to increased Ca^{2+} responses upon microinjection of 10 nM NAADP (Brailoiu et al. 2009). Gene silencing in WT SKRB3 cells by shRNA targeting TPC1 resulted in a diminished Ca^{2+} signal upon microinjection of 10 μM NAADP (Brailoiu et al. 2009), already highlighting the complexity of the NAADP signalosome. In the following years, multiple studies focused on deciphering the role of NAADP-dependent Ca^{2+} release from lysosomes, including *tpcn1/2* deficient mice, gene silencing by siRNA, or overexpression in multiple cell culture lines (Calcraft et al. 2009; Brailoiu et al. 2009; Zong et al. 2009; Lin-Moshier et al. 2014; Ruas et al. 2015; Yuan et al. 2022).

Controversially, another study using endolysosomal patches in *tpcn1/2* deficient mice revealed TPCs to be rather activated by phosphatidylinositol-3,5-bisphosphate ($\text{PI}(3,5)\text{P}_2$) and also showed that these are sodium-releasing channels (Wang et al. 2012). Recent structural analysis of hTPCs by cryo-EM revealed the $\text{PI}(3,5)\text{P}_2$ binding sites and gating mechanism (She et al. 2018, 2019). Moreover, in T cells, depletion of lysosomal stores by Bafilomycin A1 did not affect global Ca^{2+} signals evoked by TCR/CD3 stimulation or NAADP microinjection (Steen et al. 2007). Further, no changes in Ca^{2+} microdomain formation were found in *tpcn1/2* deficient CD4^{+} T cells (Diercks et al. 2018).

This duality of two second messengers activating the same channel is rather unusual and

the physiological importance remains elusive (Morgan et al. 2022). Studies in macrophages showed that PI(3,5)P₂ is responsible for TPC2 activation in macropinosome resolution (Freeman et al. 2020), whereas NAADP regulates TPC-dependent phagocytosis (Davis et al. 2020) in the same cell type. The recent discovery of NAADP-binding proteins HN1L/JPT2 and LSM12 and their preferred association with either TPC1 or TPC2 further adds to the complexity. In T cells, the role of lysosomes and NAADP-dependent Ca²⁺-release via TPCs needs to be further elucidated.

Ryanodine receptors

RyR and especially type 1 RyR have been associated with NAADP-dependent Ca²⁺-release from SR in muscle cells or the ER in other cell types. They belong to the largest ion channel family, with an approximate size of 2.2 MDa and form homotetrameric structures (Fig. 5). There are three mammalian isoforms, whose expression pattern varies from type 1 and type 3 primarily in skeletal muscle and type 2 in cardiac muscle, but are also found in lower levels in many other cell types e.g. kidney, liver and thymus (Lanner et al. 2010). Structural analysis of RyRs revealed various domains, e.g. several SPRY domains, TM domain, EF-hand domain, as well as co-structures with modulators, e.g. small cytosolic protein, ATP, Ca²⁺ and caffeine give rise to regulation of RyR1 by conformational changes (Georges et al. 2016; Woll et al. 2021; Weninger et al. 2024).

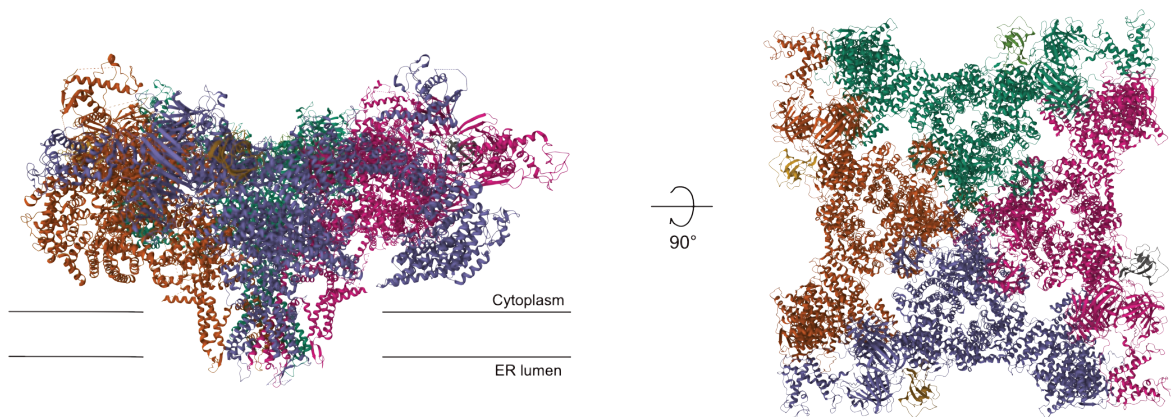


Figure 5: Cryo-EM structure of RyR1.(pdb_00008sen), modified after Cholak et al. (2023). Figure created with BioRender.

Early indications of NAADP targeting RyR were established in rat heart microsomes where microinjection of low micromolar concentrations of NAADP induced Ca²⁺ release events (Mojžišová et al. 2001). Further, the same group showed that NAADP is needed for EC-coupling in the cardiac muscle by RyR2 in the dog heart by lipid planar bilayer measurements, albeit using an apparent non-physiological NAADP (physiological: low nanomolar range) concentration of up to 10 μ M (Berg et al. 2000; Mojžišová et al. 2001). The following year, Hohenegger et al. (2002) reported channel opening, with an physiological EC₅₀ of 30 nM NAADP, in lipid planar

bilayer recordings using purified RyR1 from rabbit skeletal muscle, which could be blocked by ryanodine and ruthenium red. Furthermore, ruthenium red as well as knockdown of RyR3 and RyR1 resulted in diminished Ca^{2+} responses upon microinjection of NAADP and Ca^{2+} entry was essential for the amplification of the initial Ca^{2+} peak and sustained Ca^{2+} phase induced by NAADP in T cells (Langhorst et al. 2004). The same group later revealed that low nanomolar concentrations of NAADP (30 - 100 nM) are sufficient to evoke NAADP-dependent Ca^{2+} signals, that NAADP is formed upon TCR/CD3 stimulation and functional RyR are crucial (Dammermann and Guse 2005; Gasser et al. 2006b). Interestingly, the development of a small molecule inhibitor of NAADP (BZ194) revealed the importance of early NAADP-evoked Ca^{2+} signals for downstream signaling events, e.g. NFAT translocation, interleukin-2 (IL2) production and proliferation to effector T cells (Dammermann et al. 2009). Subcellular Ca^{2+} signals occurred 1.5 seconds after microinjection of NAADP (Dammermann and Guse 2005), similarly to the signals observed localised in close proximity to the PM after TCR/CD3 stimulation with anti-CD3 antibodies (Kunerth et al. 2003). The advancement in high-resolution Ca^{2+} imaging revealed that RyRs are crucial for Ca^{2+} microdomain formation in the first 15 seconds after TCR/CD3 stimulation (Wolf et al. 2015). Furthermore, these initial local signals were diminished in T cells lacking RyR microinjected with NAADP (Wolf et al. 2015). Additionally, using BZ194 and *ryr1* knockouts in CD4^+ T cells, Diercks et al. (2018) further strengthen the role of RyR1 in NAADP-mediated Ca^{2+} microdomain formation. However, overexpression of RyR1 and RyR3 in HEK293 failed to evoke Ca^{2+} responses upon NAADP microinjection, but was triggered by cADPR (Ogunbayo et al. 2011). Further, microinjection of NAADP in HEK293 cells overexpressing TPC1 or TPC2 induced Ca^{2+} transients (Ogunbayo et al. 2011). While Gerasimenko et al. (2015) reported both TPC1/2 and RyR1/3 as necessary to trigger NAADP-induced Ca^{2+} release from intracellular Ca^{2+} stores in pancreatic acinar cells. They propose a model where NAADP-elicited Ca^{2+} release by TPCs is necessary to further trigger Ca^{2+} induced Ca^{2+} release via RyR on the ER (Gerasimenko et al. 2015).

Consequently, the role of RyRs, especially RyR1, as the receptor targeted by NAADP needs to be further elucidated.

1.3.3 The NAADP toolbox

Since its discovery, the role of NAADP and its mode of action as a Ca^{2+} releasing second messenger has been controversially discussed. Directly studying the effect of NAADP induced Ca^{2+} signaling events remains challenging, as NAADP is not membrane-permeant due to its charge. Early studies used laborious tools like NAADP microinjection, permeabilised cell assays or lipid planar bilayer measurements to study NAADP evoked Ca^{2+} release (Lee and Aarhus 1995; Chini et al. 1995; Cancela et al. 1999; Berg et al. 2000; Hohenegger et al. 2002). Further, photocaged NAADP probes have been developed and used in microinjection studies (Lee et al. 1997). In 2008, Parkesh et al. (2008) developed a promising novel chemical

tool, using acetoxymethyl (AM) ester groups to create cell-permeant NAADP-AM and provided data for its biological activity in primary neurons. However, others could not reproduce a successful synthesis and subsequently NAADP-AM did not evoke Ca^{2+} release (Zhang et al. 2013; Ruthenbeck 2018; Dekiert 2021). Consequently, our collaboration partners developed a membrane permeant analog of NAADP, which was able to induce $[\text{Ca}^{2+}]_i$ elevations in Jurkat T cells (Dekiart 2021). Albeit its Ca^{2+} releasing capacity, the longterm stability of the analog was not given due to 2' to 3'-phosphate migration in the base (Dekiart 2021). Introducing a fluorine atom at the 3' position resulted in the synthesis of Membrane permeable, STabilized, bio-rEversibly protected precursor of NAADP (MASTER-NAADP) with similar Ca^{2+} releasing capacity (Dekiart 2021) (Publication 2.3). Others reported the synthesis of TPC2-A1-N (1, 2-cyano-3-(3,5-dichlorophenyl)-3-oxo-N-(4-(trifluoromethyl)phenyl)propanamide) and TPC2-A1-P (2, 5-(5-bromo-2-(trifluoromethoxy)phenyl)-1-(cyclohexylmethyl)-2-methyl-1H-pyrrole-3-carboxylic acid), two lipophilic small-molecule agonists of TPC2, where TPC2-A1-N seems to act as a NAADP mimic inducing Ca^{2+} release via TPC2 from lysosomes (Gerndt et al. 2020). Furthermore, to antagonise NAADP-dependent signaling two inhibitors were developed. One, BZ194, specifically blocked NAADP-stimulated $[\text{H}^3]$ ryanodine binding to RyR1 in a cell free assay, as well as Ca^{2+} signaling events evoked by NAADP, but not IP_3 or cADPR, in T cells (Dammermann et al. 2009; Zhang et al. 2018). Further, NFAT translocation, IL2 production and proliferation of CD4^+ effector T cells were diminished in cells treated with BZ194 (Dammermann et al. 2009). In experimental autoimmune encephalomyelitis (EAE), an experimental model for multiple sclerosis (MS), NAADP antagonism using BZ194 resulted in significantly lower effector T cell infiltration and an overall ameliorated clinical symptoms score (Cordiglieri et al. 2010). Second, by ligand-based virtual screening, Naylor et al. (2009) identified Ned19; antagonising NAADP-induced Ca^{2+} release in sea urchin homogenates and in intact sea urchin eggs. In memory CD4^+ T cells, Ned19 decreased TCR/CD3 dependent Ca^{2+} release, coupled with altered downstream effector functions (Chakraborty et al. 2024).

1.4 Visualisation of Ca^{2+} signals

Ca^{2+} indicators

The visualisation of intracellular Ca^{2+} changes by (extracellular) triggers is crucial to understand the underlying physiology. Therefore, over the last decade many Ca^{2+} indicators have been developed to visualise these changes. They can be classified into two larger groups I) chemical Ca^{2+} indicators and II) genetically encoded fluorescent Ca^{2+} indicators (GECI). The first chemical Ca^{2+} indicator was developed in the early 1980s by Roger Tsien using an AM group fused to a fluorescent protein, hence rendering the indicator membrane permeable (Tsien 1981). Esterases in the cytosol enable cleaving of the AM-group, therefore trapping the fluorophore inside the cell and allowing for accumulation in the cytosol (Tsien 1981). Since then, many chemical Ca^{2+} indicators have been developed, which can be classified further into three categories,

as one-wavelength (e.g. Fluo4-AM, FuraRed), dual-excitation (e.g. Fura2-AM) or dual-emission (e.g. Indo1) Ca^{2+} dyes (Grynkiewicz et al. 1985). Their common properties are the spectral shifts due to Ca^{2+} sensitivity or fluorescence intensity changes, with high affinity Ca^{2+} dyes ($K_d < 50 \text{ nM}$) to detect cytosolic $[\text{Ca}^{2+}]$ changes and low affinity indicators ($K_d > 50 \mu\text{M}$) allowing to detect $[\text{Ca}^{2+}]$ shifts in subcellular compartments (Paredes et al. 2008). Furthermore, chemical Ca^{2+} dyes may act as Ca^{2+} buffers, hence interfering with the desired detectable Ca^{2+} changes, therefore the concentration should be kept as low as possible (Paredes et al. 2008). GECI are based on green fluorescent protein (GFP) or other fluorescent proteins, attached to a moiety of a protein with Ca^{2+} binding properties, like CaM (e.g. GCaMP) and can be targeted to organelles (Miyawaki et al. 1997). Recent advances in development of new GECI, mNG-based Ca^{2+} indicators, named NEMO, ameliorate the dynamic range and improve sensitivity of previous GCaMP based sensors (Li et al. 2023). Nevertheless, to analyse subcellular Ca^{2+} dynamics the usage of Ca^{2+} dyes remains the preferred choice.

Image acquisition

Subcellular Ca^{2+} signals can be classified as four dimensional due to their spatial distribution and their temporal resolution. Especially for the initial, local and spatially restricted Ca^{2+} microdomains using the well established dual-emission or dual-excitation indicators is not suitable due to their rapid and dynamic formation (Wolf et al. 2015). Detection of Ca^{2+} microdomains comes with its challenges, as high-resolution microscopy requires low photon doses, due to phototoxicity and photobleaching of the fluorophores used (Ettinger and Wittmann 2014). Further, the transient nature of Ca^{2+} microdomains, as well as out-of-focus light add to the challenging nature of capturing Ca^{2+} microdomains, as they result in low signal-to-noise ratio (SNR) images (Ettinger and Wittmann 2014; Woelk et al. 2021). Adjustments in the experimental setup, by reducing exposure time, frame rate and spatial resolution can mitigate phototoxicity and photobleaching (Ettinger and Wittmann 2014; Miyazaki et al. 2015; Weigert et al. 2018), but would result in lower quality image sequences, hence not allowing to detect Ca^{2+} microdomains. Accordingly, the combination of two single wavelength Ca^{2+} indicators, Fluo-4 and FuraRed, combines the advantages of the possibility of rationing, thus allowing for Ca^{2+} calibration and the photostability and brightness of Fluo indicators (Wang et al. 2014; Wolf et al. 2015).

Image quality is further influenced by noise, either signal-dependent (photon-shot noise; Poisson distributed) or system-inherent (Gaussian distribution), and blurring resulting from the point-spread-function (PSF) (Young 1989; Ettinger and Wittmann 2014; Weigert et al. 2018). This distortion of an image can be represented as a convolution of two functions:

$$(h * x) + \epsilon = y,$$

where h describes the distortion function (e.g. blur, PSF), $*$ is the convolution operation, x is the original function of image to be restored, ϵ represents additive noise and y is the final image (Makarkin and Bratashov 2021).

The PSF describes the X, Y, Z coordinates of each point source of light of the object in the blurred image and needs to be determined either experimentally or analytically for the optics used (McNally et al. 1999; Makarkin and Bratashov 2021). Deconvolution algorithms try to restore the convoluted image by using (modified) inverse transformation of the above function to obtain the original image (x) (Makarkin and Bratashov 2021). Conventional deconvolution methods can be classified into linear (e.g. Wiener deconvolution (Sibarita 2005)), non-linear (e.g. Lucy-Richardson (LR) (Richardson 1972; Lucy 1974)), statistical or cluster analysis algorithms (Makarkin and Bratashov 2021).

In their approach to improve image quality by reducing SNR to detect Ca^{2+} microdomains, Wolf et al. (2015) applied different deconvolution algorithms to their image sequences. Wiener deconvolution algorithm failed to improve SNR, whereas applying LR algorithm with an analytically derived PSF resulted in clear separation of signal and noise in the image sequences (Schetelig et al. 2015; Wolf et al. 2015). Furthermore, this technique reaches a (calculated) spatial resolution of $\sim 0.368 \mu\text{m}$ and temporal resolution of 40 frames per second (fps), enabling the detection of highly dynamic local Ca^{2+} microdomains possible (Wolf et al. 2015; Diercks et al. 2018). The analysis workflow for post-processing includes 4 steps. Starting with cell selection/ ROI (region of interest) extraction and background correction, where the cell is automatically detected by spherical Hough transformation generating a cell mask. Afterwards, an additive frame-by-frame bleaching correction is only applied to FuraRed, as bleaching of Fluo-4 is negligible. A LR deconvolution algorithm is applied to reduce the image blur due to out of focus light. To be able to perform rationing of Fluo-4 and FuraRed channel data, the data images are aligned by an automated registration on the first ten frames of the image sequences (Schetelig et al. 2015). Comparison of cell subsets is achieved by using a dartboard projection, wherein the bead contact of each cell in the subset is normalised to the same point of origin and aggregated into one dartboard (Diercks et al. 2019). Of note, the dartboard projection approach is limited by the assumption of a circular cellular shape, neglecting cell motion and deformation during stimulation (Diercks et al. 2019). Hence, the dartboard projection can only be applied to round cells (e.g. primary murine T cells).

1.5 Aim of the study

Tight regulation of extra and intracellular Ca^{2+} signaling is crucial for cell survival, proliferation and differentiation. In T cells, Ca^{2+} dependent signaling pathways regulate their effector functions, leading to pro- or anti-inflammatory responses depending on the duration of $[\text{Ca}^{2+}]_i$ elevation. Upon TCR/CD3 complex engagement, the Ca^{2+} mobilising second messenger NAADP is formed rapidly, resulting in Ca^{2+} microdomains via RyR1, eventually merging into a global Ca^{2+} response, enhanced by SOCE and further amplified by IP_3 and later cADPR signaling. In addition, autocrine release of ATP by PANX1 and subsequent purinergic signaling via P2X4 and P2X7 further shape these initial Ca^{2+} microdomains in CD4^+ T cells.

Moreover, NAADPs capacity to release Ca^{2+} from intracellular stores like the ER and (endo-)lysosomes has been controversially discussed in the last decade. Recent identification of NAADP binding proteins HN1L/JPT2 and LSM12 and their respective binding receptors on the ER (RyR1) or lysosomes (TPCs) further add to the need of a tool to study NAADP-dependent signaling directly in different cell types. Furthermore, Ca^{2+} microdomains are highly-dynamic, subcellular and short-lived, hence analysis of these Ca^{2+} signaling events remains challenging. To further disentangle adenine nucleotide-mediated Ca^{2+} signaling this thesis aims to:

- optimize the current high resolution live cell Ca^{2+} imaging and analysis pipeline, specifically implementing shape normalization to the postprocessing pipeline and transferring the pipeline to an open source based program (Python)
- investigate the role of purinergic channels, e.g. P2X4 and P2X7, in Ca^{2+} microdomain formation, as well as downstream effects in CD8^+ T cells
- characterization of the novel compound MASTER-NAADP and its respective control as a new tool to study NAADP-induced Ca^{2+} signaling in high resolution live cell Ca^{2+} imaging

2 PUBLICATIONS

2.1 Publication I: DARTS: an open-source Python pipeline for Ca^{2+} microdomain analysis in live cell imaging data

The publication “DARTS: an open-source Python pipeline for Ca^{2+} microdomain analysis in live cell imaging data” was published in January 2024 in Frontiers in Immunology (Impact Factor 5.9 [2024], among top-33% journals).

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DARTS: an open-source Python pipeline for Ca^{2+} microdomain analysis in live cell imaging data

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Ca^{2+} microdomains play a key role in intracellular signaling processes. For instance, they mediate the activation of T cells and, thus, the initial adaptive immune system. They are, however, also of utmost importance for activation of other cells, and a detailed understanding of the dynamics of these spatially localized Ca^{2+} signals is crucial for a better understanding of the underlying signaling processes. A typical approach to analyze Ca^{2+} microdomain dynamics is live cell fluorescence microscopy imaging. Experiments usually involve imaging a larger number of cells of different groups (for instance, wild type and knockout cells), followed by a time consuming image and data analysis. With DARTS, we present a modular Python pipeline for efficient Ca^{2+} microdomain analysis in live cell imaging data. DARTS (Deconvolution, Analysis, Registration, Tracking, and Shape normalization) provides state-of-the-art image postprocessing options like deep learning-based cell detection and tracking, spatio-temporal image deconvolution, and bleaching correction. An integrated automated Ca^{2+} microdomain detection offers direct access to global statistics like the number of microdomains for cell groups, corresponding signal intensity levels, and the temporal evolution of the measures. With a focus on bead stimulation experiments, DARTS provides a so-called dartboard projection analysis and visualization approach. A dartboard projection covers spatio-temporal normalization of the bead contact areas and cell shape normalization onto a circular template that enables aggregation of the spatiotemporal information of the microdomain detection results for the individual cells of the cell groups of interest. The dartboard visualization allows intuitive interpretation of the spatio-temporal microdomain dynamics at the group level. The

application of DARTS is illustrated by three use cases in the context of the formation of initial Ca^{2+} microdomains after cell stimulation. DARTS is provided as an open-source solution and will be continuously extended upon the feedback of the community.

Code available at: [10.5281/zenodo.10459243](https://zenodo.org/record/10459243).

KEYWORDS

intracellular signaling, Ca^{2+} microdomains, live cell imaging, image analysis, shape normalization, Python, open source

1 Introduction

The activation of T cells, and thus the initial adaptive immune response, is mediated by an increase of the free cytosolic Ca^{2+} concentration. These signals are highly dynamic and spatially localized in the form of Ca^{2+} microdomains (1). There have been numerous studies investigating the molecular mechanisms and the role of Ca^{2+} signaling in early T cell activation in primary murine T cells (2–6) as well as Jurkat cells (2, 7). The studies show that the second messenger nicotinic acid adenine dinucleotide phosphate (NAADP), its binding protein named hematological and neurological expressed 1-like protein (HN1L) [also known as Jupiter microtubule-associated homolog 2 (JPT2)], and the type 1 ryanodine receptor (RyR1) are involved in the formation of subplasmalemmal Ca^{2+} signals that occur in the first seconds after T cell stimulation (7). Another essential component of the innate immune system are natural killer (NK) cells that contribute to the control of intracellular pathogens and cancer cells through direct cytotoxicity and the release of cytokines. To name a recent example, the NK cell status correlates with a decline in viral load in COVID-19 and thus can control SARS-CoV-2 replication by recognizing infected target cells (8). It has been shown that educated human NK cells have increased global Ca^{2+} signals compared to uneducated human NK cells following antibody stimulation (9). Similar to T cells, it is assumed that localized Ca^{2+} signals also arise after NK cell activation, but these Ca^{2+} microdomains have not been visualized so far. In line, in both cell types (and not only them), many questions about the molecular mechanisms behind the activation and signaling response are unsolved, and a better understanding of Ca^{2+} microdomain formation and dispersion is indispensable.

Fluorescence microscopy, and in particular live cell imaging, is an invaluable tool to study Ca^{2+} microdomains. As the relevant time frame is in the very early seconds after stimulation, high spatial and temporal resolution is crucial to obtain meaningful results. Even state-of-the-art imaging setups, however, encounter severe biological and physical constraints. Phototoxicity and photobleaching demand low photon doses, which, combined with out-of-focus light, lead to an intrinsically low signal-to-noise ratio

(SNR). In addition, there is a trade-off between exposure times and imaging speed. Computational restoration of the acquired images, that is, a reduction of the noise level and correction for out-of-focus light, therefore has the potential to increase the SNR and to improve the analysis of Ca^{2+} microdomains in high-resolution live cell imaging data (10–13).

However, even when using computational image restoration approaches and deconvolution techniques, a key challenge remains for the comprehensive analysis of Ca^{2+} microdomains in live cell imaging data: It requires consistent and reliable, ideally automated, localization and quantification of Ca^{2+} microdomains across large ensembles of cells that often exhibit significant morphological changes between different cells and even within the same cell over time.

To address this challenge, in the present work, the modular pipeline DARTS (Deconvolution, Analysis, Registration, Tracking, and Shape normalization) for Ca^{2+} microdomain analysis in live cell fluorescence microscopy imaging data is introduced. The pipeline tackles imaging-related issues by providing problem-tailored image postprocessing routines (for instance, denoising and deconvolution, bleaching correction). Adapting a shape normalization technique introduced in (14), DARTS is able to compensate for morphological cell shape changes typically observed in the given application context. Moreover, DARTS offers automated detection of the microdomains in the live cell imaging data, the aggregation of the analysis results across cell ensembles, and intuitive dashboard visualization of the aggregated analysis results.

The individual DARTS parts and modules are explained in the following Method section. The Results section illustrates the use of DARTS with three application examples:

- The first use case illustrates the application of DARTS to the analysis of Ca^{2+} microdomains after stimulation of primary murine T cells, focusing on the comparison of wild type (WT) and RyR1 and RyR3 knockout (KO) cells (3).
- The second use case is based on a set of Jurkat T cells, in which the NAADP-binding protein HN1L/JPT2 is genetically deleted, resulting in a reduced formation of Ca^{2+} microdomains upon activation (7).

- As the third use case, DARTS is applied to the analysis of an NK cell line (KHYG-1) to illustrate that Ca^{2+} microdomains indeed arise after the activation of NK cells.

2 Methods

DARTS is implemented using an object-oriented approach in Python 3 and follows the open-source idea. The code as well as the software are provided under the most flexible license model (Apache 2.0 license). DARTS was tested for different operating systems (Windows, MacOS, Linux). The source code and a corresponding documentation can be found at github.com/ipmi-icns-uke/DARTS and ipmi-icns-uke.github.io/DARTS.

DARTS is intended both as a complete solution that can automatically process and analyze large volumes of cellular data “overnight” and as a toolkit from which individual modules can be selected, applied, and modified as needed. The intended and expected users are experimentalists with little or no programming experience. To support users without programming experience, a graphical user interface (GUI) is developed to help with parameter input. DARTS supports all common image input and output formats by integration of the Bio-Formats library (15).

The general DARTS pipeline is illustrated in Figure 1 and can be roughly divided into three main parts: live cell microscopy imaging data postprocessing; cell shape normalization; and Ca^{2+} microdomain detection and visualization.

2.1 DARTS image postprocessing modules

DARTS offers four image postprocessing modules: general image setup; cell detection and tracking; bleaching correction; and image denoising/deconvolution.

2.1.1 General image setup: channel registration, ratio image computation, background subtraction

Input: temporal image sequence(s)

Output: temporal image sequence(s), same size as input sequences

Ratiometric imaging is a standard technique in fluorescence microscopy for imaging intracellular calcium signals. Depending on the imaging setup, the images obtained for the two calcium indicators are provided as imaging data of two often imperfectly aligned channels. As a default first processing step for ratiometric imaging, DARTS improves the alignment by an intensity-based affine registration of the images of the two channels, making use of the SimpleElastix library of the SimpleITK image analysis toolkit (16). Depending on the user's preference, only the channel images of the first frame are registered and the resulting transformation is applied to all frames of the acquired time series data (time efficient solution, default) or all frames of the time series are individually registered and aligned (accurate alignment). Based on the aligned channel data, the desired ratio image is calculated (details vary, depending on the used calcium indicators) and, if desired, lower and upper thresholds are introduced to restrict the ratio image intensity values to the ratio value range of interest. Moreover, background subtraction can be applied to compensate for background fluorescence.

2.1.2 Cell detection and tracking

Input: temporal image sequence, usually containing multiple cells

Output: temporal image sequence(s), each representing a region-of-interest containing a centered single cell

To allow analysis of Ca^{2+} microdomains at the single cell level and speed up subsequent analysis, DARTS performs automated detection and tracking of cells in the input live cell imaging sequence. The cells are identified in the individual frames of the input using the StarDist algorithm (17), a state-of-the-art algorithm that utilizes deep learning techniques to detect and segment cells in microscopy data. The result is a cell instance segmentation of the input image series at the frame level (cell representation: binary map, polyline of cell boundary). The detected individual cells are further tracked across time using the trackpy toolkit (18) in order to correctly position the regions of interest (ROI) and only retain the frames in which the cell is visible. For each cell that is successfully tracked (visible in a pre-defined number of frames, no large positional changes between consecutive frames), a new image time series is generated representing a rectangular region of interest (ROI) with the cell centered within the ROI, thus compensating translational movements of the cell.

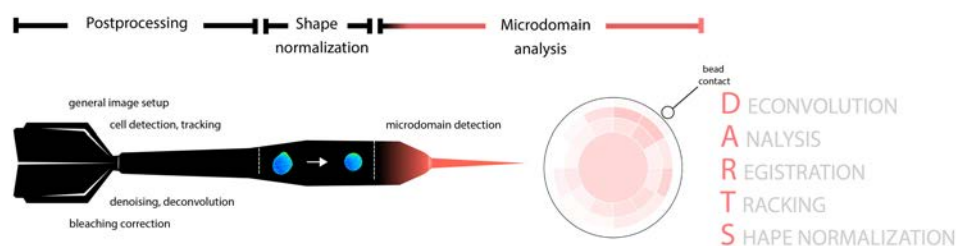


FIGURE 1

Schematic representation of the DARTS pipeline and its modules. The pipeline consists of three main parts: general image postprocessing; cell shape normalization; and Ca^{2+} microdomain detection, aggregation and visualization.

2.1.3 Bleaching correction

Input: temporal image sequence

Output: temporal image sequence, same size as image sequence

Calcium indicators often exhibit a relevant decrease of fluorescence intensity over time, commonly referred to as bleaching. DARTS provides an additive and a multiplicative bleaching correction that results in a frame-by-frame normalization of the mean cell pixel intensity to a constant value, and a biexponential fit-based additive correction (19). For ratiometric imaging, bleaching correction can be applied separately to the individual channel data.

2.1.4 Image denoising/deconvolution

Input: temporal image sequence

Output: temporal image sequence, same size as image sequence

For reduction of out-of-focus light and image noise, two algorithms are provided: the standard Lucy-Richardson (LR) algorithm (20, 21) and the in-house developed time-dependent entropy (TDE) deconvolution (12). TDE can also be used as a re-implementation of the entropy-based image restoration approach by Arigovindan et al. (22), which specifically addresses the restoration of fluorescence signals in microscopy images with low signal-to-noise ratio (SNR). TDE extends the idea to time-series data, employing temporal consistency of signals over time as opposed to temporally independent noise patterns, and is particularly suited for live cell imaging data with low SNR, for instance due to short exposure times and high temporal resolution. For image data with reasonable SNR, however, the faster and parameter-free (excepting specification of the PSF) LR algorithm might be the more efficient choice.

2.2 DARTS shape normalization

Input: temporal image sequence, representing a ROI containing a centered single-cell

Output: temporal image sequence, representing a ROI containing the shape-normalized cell

Ca²⁺ microdomain analysis at a population level requires a spatially consistent evaluation of intracellular Ca²⁺ dynamics at the level of the individual cells of the population (for example, a consistent analysis of only the assembly of localized Ca²⁺ signals close to the contact point of a cell-activating bead). This is, depending on the variation in shape between different cells and also the degree of cell distortion over time, not always an easy task, because the relative position of intracellular signals, for instance with regard to the plasma membrane or the bead contact point, cannot always be directly described in a similar way for all cells and video frames. DARTS follows an approach introduced in (11): to map the individual cells to a common coordinate system and template of circular shape, respectively. For nearly circular cells like primary murine T cells, this step involves only a translation and scaling step. For other cells, the required transformation is more complex. DARTS adapts and modifies a shape normalization approach presented in (14). For each frame of the input image

sequence (assumed to represent a ROI that contains a single cell to be analyzed), the coordinate system is converted from Cartesian to polar coordinates, $(x, y) \rightarrow (r, \theta)$, where r is the radius and θ the polar angle. The centroid of the cell serves as the origin of the polar coordinate system. The coordinates of the cell border $(r_o, \theta)_{i=1,\dots,N}$, for instance, computed by the DARTS cell detection and tracking module, are then mapped onto a circle with a radius determined by the mean radius value of the outline $r_{av} = \sum_{i=1,\dots,N} r_{o,i}/N$, scaled by a constant factor. This corresponds to a coordinate transformation $(r_o, \theta) \rightarrow (r_o', \theta) = T(r_o, \theta)$, with T being the transformation. To apply this transformation to the entire space of the considered ROI image, the transformation is interpolated using the nearest neighbor method and the inverse $T^{-1}(r', \theta) = (r, \theta)$ is used to obtain the pixel intensity values of the transformed image $J(r', \theta)$ from the corresponding locations in the source image $I(r, \theta)$.

2.3 DARTS Ca²⁺ microdomain analysis and dartboard projection

Input: temporal image sequences, each representing a ROI containing a centered single-cell

Output: analysis results at the single cell and the cell population level (text or Excel format)

The Ca²⁺ microdomain module workflow consists of two parts: frame-by-frame identification of the microdomains at the single-cell level and aggregation of the results for a cell population using a so-called dartboard projection.

Ca²⁺ microdomains are spatially localized areas of high Ca²⁺ concentration inside a cell. In DARTS, they are defined at a frame level and as small, connected sets of cell pixels with relative intensity values (normalized by the mean intensity of the cell pixels of the frame) above a predefined threshold. Both an appropriate definition of the size of pixel sets as well as the intensity threshold depends on the imaging setup (spatial and temporal microscopy resolution) and the applied postprocessing routines (for instance, deconvolution methods and additional image filters). It has to be carefully selected by the user, ideally combined with a comprehensive noise analysis of the setup. DARTS default values are chosen in agreement with (11). The single-cell microdomain analysis results (for instance the localization of the microdomains in each frame) can be exported as tables for further external signal analysis.

The dartboard projection analysis aims to aggregate and visualize the single-cell Ca²⁺ microdomain analysis results, in particular for the scenario of cell activation with a stimulatory bead. First, for each cell, the bead contact time and position relative to the center of the cell on a clock scale (1-12) needs to be defined by the user. DARTS provides a GUI for this step, where position and time point for each bead contact can be selected. To facilitate batch processing of larger data sets, the bead contact selection process is launched for all selected files before further postprocessing starts; all remaining processing steps are then fully automated and can be run “overnight”. Alternatively to the GUI-based bead contact selection, user input can be completely removed by providing a text file with the required information, for example generated by a previous run of the DARTS bead contact selection. To analyze and compare the

microdomain locations and their appearance times relative to the contact within the different cells, the shape-normalized cells (output of the shape normalization module) are rotated such that the bead contact points of the cells are spatially aligned and temporally shifted such that the bead contacts are also temporally aligned. The structure of the dartboard is illustrated in [Figure 1](#). The middle of the dartboard is defined as the frame-specific centroid coordinates of a shape-normalized cell image, and the outer border of the dartboard is aligned with the cell border. The identified Ca^{2+} microdomains are assigned to the different dartboard areas, depending on their angle relative to the bead contact position and the relative distance to the board center. The granularity of the dartboard analysis, that is, the number of dartboard rings and segments as well as the length of time periods used to aggregate the signals can be selected by the user, depending on the intended analysis scenario and cell lines. The radii of the rings of the dartboard decrease from inner to outer to ensure intuitive interpretation of the final results, that is, Ca^{2+} microdomain frequency within cell areas of identical size. To ensure comparability of all dartboard segments, results within a segment are normalized with respect to the individual segment area, thus compensating for the larger dartboard center (“bullseye”).

In principle, an averaging of the mere pixel intensities across the different cells of the considered cell set would also be possible after shape normalization. However, this would result in an unintended bias in the case of a global Ca^{2+} signal increase. In addition, the aggregation of local Ca^{2+} signals within larger spatial areas (the dartboard segments) allows a quick overview of the spatio-temporal distribution of Ca^{2+} microdomains even for medium-sized cell sets and an easy visual comparison of different data sets.

3 Results

The following application examples demonstrate the application of DARTS for three different cell types and lines.

3.1 Role of RYR1 and RYR3 in activation of primary murine T cells

The first application example corresponded to the Ca^{2+} microdomain analysis described in (3). It was based on primary murine WT and knockout CD4^{+} T cells. From the biological perspective, the imaging experiments were performed to investigate the role of type 1 ryanodine receptors 1 and 3 (RYR1, RYR3) in the formation of initial Ca^{2+} microdomains upon T cell stimulation. From a technical point of view, the application and imaging data were selected to illustrate the validity of the proposed DARTS pipeline in terms of reproducing the published observations and effects, despite the completely new implementation of the analysis parts compared to (11). In particular, primary murine T cells have a circular shape in the imaging data. Therefore, this example focused on the general workflow and not on specifics and potential problems encountered during shape normalization.

3.1.1 Data set description

Primary murine CD4^{+} WT T cells as well as *Ryr1*^{-/-} (2) and *Ryr3*^{-/-} (23) on C57BL/6 J background (*Mus musculus*) were freshly isolated by negative selection using the EasySep Mouse CD4^{+} T Cell Enrichment Kit (STEMCELL Technologies Inc.). Image data were acquired by ratiometric Ca^{2+} imaging. Therefore, the freshly isolated T cells were loaded with Fluo4-AM (10 μM) and Fura Red-AM (20 μM) for 50 min at room temperature. The T cells were stimulated with protein G beads (Merck Millipore), coated with antibodies against CD3 and CD28 to mimic an immune synapse. Imaging was carried out with a Leica IRBE2 microscope using 100-fold magnification, a Sutter DG-4 as a light source and an electron-multiplying charge-coupled device (EMCCD) camera (Hamamatsu). Temporal resolution was 25 ms (40 frames/s) in 14-bit mode with twofold binning resulting in a spatial resolution of 368 nm. The emission wavelengths were split with a Dual-View module (Optical Insights, PerkinElmer Inc.) and the following filters were applied for the acquisition of initial Ca^{2+} microdomains (excitation (ex), 480/40; beam splitter (bs), 495; emission 1 (em1) 1, 542/50; em2, 650/57).

3.1.2 Illustration of DARTS application

Since the data was acquired within a ratiometric imaging setup, all DARTS postprocessing modules were applied: channel registration, background subtraction, cell detection and tracking, deconvolution, bleaching correction, and ratio computation. Deconvolution was performed separately for the two-channel data using LR deconvolution, to be consistent with the procedure in (3). Since bleaching of Fluo4 was negligible, the bleaching correction was only applied to the Fura Red-channel data. Being interested in only Ca^{2+} microdomains, an additive correction was applied that normalizes the mean Fura Red intensity inside the cells for each frame to a constant value.

Following the application of the postprocessing modules, the ratio image series underwent the implemented shape normalization. Since primary T cells inherently exhibit a relatively circular shape, the shape-normalized ratio images closely resembled the original input ratio images and the original signal distribution ([Figure 2A](#)).

The results of the subsequent Ca^{2+} microdomain analysis are summarized in [Figures 2B–E](#). The threshold for the detection of Ca^{2+} microdomains was based on a comprehensive noise analysis (3). Focusing on microdomain formation after the bead contact, analysis was performed for all frames from 1 second before and up to 15 seconds after bead contact.

The evaluation of the detected microdomains already allowed computation of global statistics such as the fraction of activated cells (cells that show microdomains from the bead contact on) and the number of signals per frame for each cell ([Figures 2B, C](#)). This information can be exported from DARTS to Excel or text format for subsequent detailed analysis in external programs. From the biological perspective, the global statistics revealed a significant difference between the number of microdomains per frame for the WT and the *Ryr1*^{-/-} cells after bead contact ([Figure 2B](#)). The corresponding evaluation, but time-resolved and only for the period from 1 second before to 1 second after bead contact, is

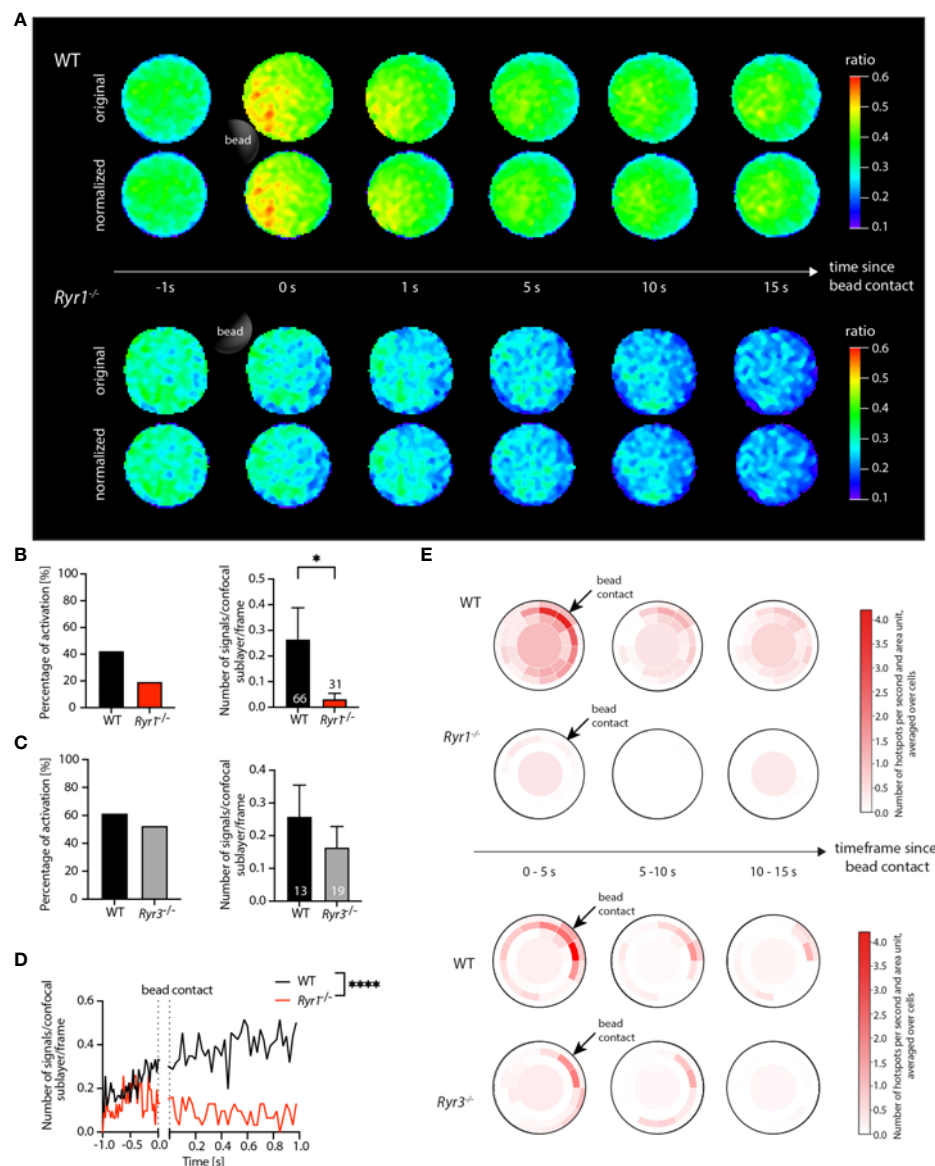


FIGURE 2

Application of DARTS to Ca^{2+} microdomain analysis in primary WT and $Ryr1^{-/-}$ and $Ryr3^{-/-}$ murine T cells. **(A)** Ca^{2+} signals in an exemplary WT T cell (top) and a $Ryr1^{-/-}$ T cell (bottom) upon the first seconds after bead contact. For both cells, the upper row shows the original and the lower row the shape-normalized cell representation. **(B, C)** Percentage of activated cells and number of Ca^{2+} microdomains within the first 15 s after bead stimulation; comparison for WT and $Ryr1^{-/-}$ and WT and $Ryr3^{-/-}$, respectively. Data are presented as means $\pm$ SEM (WT, $n = 66$ cells, $Ryr1^{-/-}$, $n = 31$ cells; WT, $n = 13$ cells, $Ryr3^{-/-}$, $n = 19$ cells; statistical testing by Mann-Whitney-U-test, $*p < 0.05$). **(D)** Number of Ca^{2+} microdomains as a function of time for WT (averaged across $n = 66$ cells) and $Ryr1^{-/-}$ cells ($n = 31$ cells); two-tailed Mann-Whitney-U-test, $****p < 0.0001$. **(E)** Dartboard projection plots of the WT cells in comparison to $Ryr1^{-/-}$ as well as WT in comparison to $Ryr3^{-/-}$, showing the number of microdomains per second and dartboard segment area unit, averaged over the intervals 0-5 s, 5-10 s, and 10-15 s after bead contact and the same cells used for the global analysis shown in **(B, C)**. Bead contact location and time were aligned for all cells.

shown in Figure 2D. In contrast, no significant differences of the averaged number of signals between WT and $Ryr3^{-/-}$ cells for the present dataset was detected. The findings are consistent with the results in (3), illustrating that DARTS was able to reproduce the previous analysis results. Since it is a completely new and extended open-source implementation of the previous proprietary Matlab analysis workflow, perfect quantitative agreement is not expected. The new workflow now includes additional analysis steps like shape normalization to allow application to other use cases.

The dartboard projections are shown in Figure 2E; related dartboard information can also be exported from DARTS for subsequent analysis or visualization in external programs. Here, three dartboards for five-second intervals (0-5 s, 5-10 s, 10-15 s after bead contact) were generated. Representing the average number of hotspots per area unit and second, the dartboards exhibited a clear visual difference between WT and $Ryr1^{-/-}$ cells in the three time intervals. The $Ryr1^{-/-}$ cells barely showed any signals, especially close to the bead contact site, while the Ca^{2+} microdomain density in the

WT cells was elevated in particular close to and directly after bead contact. In contrast, the patterns for WT and *Ryr3^{-/-}* were similar, with a relatively high microdomain density close to the bead contact site, demonstrating that the detected Ca^{2+} signals that were summarized in the diagram in **Figure 2C** were indeed related to the bead stimulation of the cells. This information, not shown in (3), illustrates the additional value of spatially and temporally local analysis and information provided by the dartboard projections.

3.2 Human Jurkat cells: WT vs. *Hn1l/Jpt2* knockout

Continuing with the formation of initial Ca^{2+} microdomains in T cells, it has been reported that RYR1 is activated by nicotinic acid adenine dinucleotide phosphate (NAADP) (24). In (7), hematological and neurological expressed 1-like protein (HN1L), also known as Jupiter microtubule-associated homolog 2 (JPT2), was identified as a NAADP-binding protein. The conclusions drawn in (7) were (at least partly) based on initial Ca^{2+} microdomain analysis in Jurkat T cells. Compared to the primary murine T cells in section 3.1, Jurkat T cells exhibited considerable shape variation and deformation over time. Therefore, a direct application of a dartboard projection was not possible, and only global Ca^{2+} microdomains statistics (for instance, average number of signals for the entire cells) for WT and *Hn1l/Jpt2^{-/-}* cells were presented in (7). Now, the DARTS workflow was applied to corresponding human Jurkat T cells to demonstrate that the shape normalization integrated in DARTS enables local spatiotemporal Ca^{2+} microdomain analysis even for cells with more complex shape and shape changes over time.

3.2.1 Data set description

WT Jurkat T cells and *Hn1l/Jpt2^{-/-}* Jurkat T cells (clone C2) were loaded with Fluo4-AM (10 μM) and Fura Red-AM (20 μM) for ratiometric single-cell Ca^{2+} imaging. The cells were stimulated with protein G beads (Merck Millipore) coated with antibodies against the T cell receptor CD3 (OKT-3) to mimic an immune synapse. Acquisition set-up for initial Ca^{2+} microdomains in Jurkat T cells was identical as described in section 3.1.

3.2.2 Illustration of DARTS application

The data processing pipeline used to analyze the Jurkat T cell data set was identical to the workflow described for the primary T cells in section 1. The analysis results are shown in **Figure 3**. **Figure 3A** illustrates the successful application of the shape normalization to the ratio images for both WT and C2 cells. The distribution of the visible Ca^{2+} signals and signal patterns inside the cells was preserved after normalization, and the microdomain size and morphology differed in accordance with the deformation of the cells and their local deviation from an optimal circle before shape normalization.

The global Ca^{2+} microdomain statistics shown in **Figures 3B, C** agree with the corresponding results in (7): The average number of signals after bead contact was significantly higher in the WT

cells compared to the C2 cells. In addition, for the WT cells, a peak of the Ca^{2+} microdomain number can be seen at around 4 to 5 s after bead contact (**Figure 3C**). Different from the data shown by Roggenkamp et al., the present analysis was based on the shape-normalized ratio images, supporting that the shape normalization does not negatively affect Ca^{2+} microdomain representation and detection.

However, the shape normalization enabled dartboard projections and, therefore, the analysis of local spatiotemporal Ca^{2+} signal distributions in the considered cell groups. In **Figure 3D**, the detected Ca^{2+} microdomains were aggregated over the first 5 seconds after bead contact. For the knockout cells, the dartboard showed that the detected signals were not located close to the bead contact area. For the WT cells, two main compartments were visible: Ca^{2+} microdomains in the dartboard regions close to the bead contact and microdomains in and near the dartboard bullseye, that is, in the cell centers. Increasing the temporal resolution of the dartboard projections, as shown in **Figure 3E**, finally illustrated for the WT cells that the microdomains close to the bead contact were primarily identified directly after bead contact, while the microdomains in the cell centers correlated to the described peak around 4–5 s in **Figure 3C**. For the knockout cells, no such observations were made.

3.3 Human NK cells: time-dependent study of signal

The examples in sections 3.1 and 3.2 focused on Ca^{2+} microdomain formation in T cells. However, Ca^{2+} microdomain formation is also relevant in other cell types and contexts, and the application of DARTS is not restricted to T cells. In this third application example, DARTS was applied to Ca^{2+} microdomain analysis in NK cells, a cell type, for which such localized Ca^{2+} signals have not been visualized and comprehensively analyzed so far.

3.3.1 Data set description

KHYG-1 cells, a human NK cell line, were loaded with Fluo4-AM (10 μM) and Fura Red-AM (20 μM) for ratiometric single-cell Ca^{2+} imaging. KHYG-1 cells were stimulated with protein G beads (Merck Millipore), coated with a combination of antibodies (anti-NKp46 and anti-2B4) for a localized activation. The imaging set-up was identical to the setup described in section 3.1.

3.3.2 Illustration of DARTS application

The analysis workflow applied to the KHYG-1 cells was the same as the one used for the Jurkat T cells. As visible for the NK cell shown in the top row of **Figure 4**, aggregation of localized Ca^{2+} signals in a NK cell set for subsequent dartboard projections required shape normalization for the cells. The central scientific questions in the present analysis scenario were: Is it possible to visualize initial Ca^{2+} microdomains in NK cells stimulated with antibody-coated beads? If so, can the Ca^{2+} microdomains be

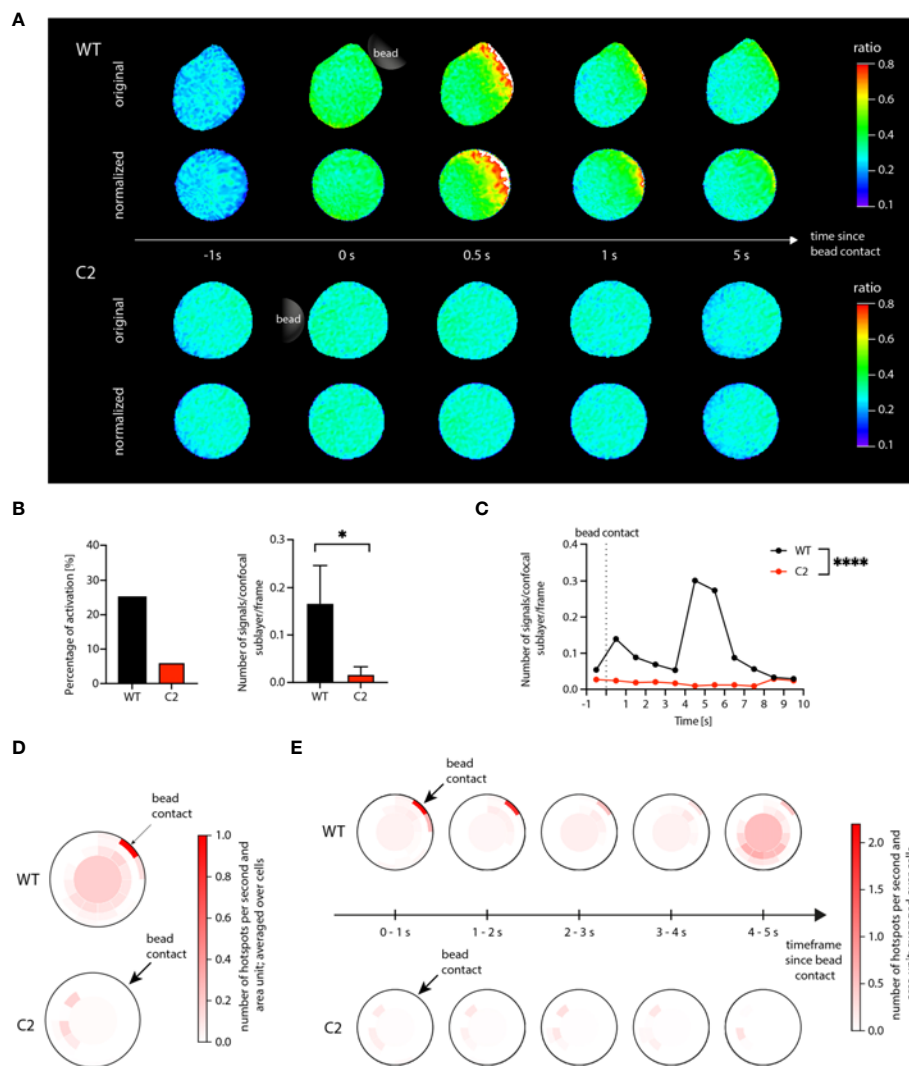


FIGURE 3

Ca²⁺ microdomain and dartboard analysis in Jurkat T cells. (A) Ca²⁺ signals in a WT Jurkat cell (top) and a *Hn1l/Jpt2^{-/-}* (C2 clone) Jurkat cell (bottom) within the first seconds after stimulatory bead contact. For both cells, the upper row shows the original and the lower row the shape-normalized cell representation. (B) Global statistical analysis: percentage of activated cells and number of Ca²⁺ microdomains within the first 15s after bead stimulation. Data are presented similarly to Figure 2 (means ± SEM; WT, n = 24 cells; C2, n = 33 cells). Statistical testing by two-tailed Mann-Whitney-U-test, *p<0.05. (C) Number of signals as a function of time for WT and C2 Jurkat T cells, averaged across the 24 WT and the 33 C2 clone data. Statistical testing by two-tailed Mann-Whitney-U-test, ****p<0.0001. (D, E) Dartboard projection plots for the WT (top) and C2 clone cell sets [bottom; same cells as used for (B) and (D)], showing the location and frequency Ca²⁺ microdomains within the first 5 seconds after bead contact (D) and at a refined time scale [(E); moving average computation with a 1s interval].

spatially and temporally associated with the stimulating bead contact?

To answer the first question, global microdomain statistics would have been sufficient. However, the dartboard projection provides the means to simultaneously answer both questions. Here, a microdomain analysis was performed in three dartboard areas: (1) close to the bead contact site, (2) for the bullseye, and (3) for the opposite side of the bead contact site (Figure 4B). The analysis results summarized in Figure 4C show that not only Ca²⁺ microdomains were detected in the NK cells, but that these were mainly located close to the bead contact. Figure 4B further illustrates that the detected bullseye microdomains arose later, approximately 10 s after bead contact. Figure 4D provides the

corresponding comprehensive dartboard projections, further strengthening the observations shown in Figures 4C, B.

Thus, both scientific questions could be answered with yes. Here, the dartboard projections could be used to define inner-cell control areas to demonstrate that the initial Ca²⁺ dynamics can be directly associated with the bead contact.

4 Discussion

In the present work, we introduced DARTS, an open-source Python pipeline to process fluorescence microscopy live cell imaging data with the specific focus on the analysis of Ca²⁺

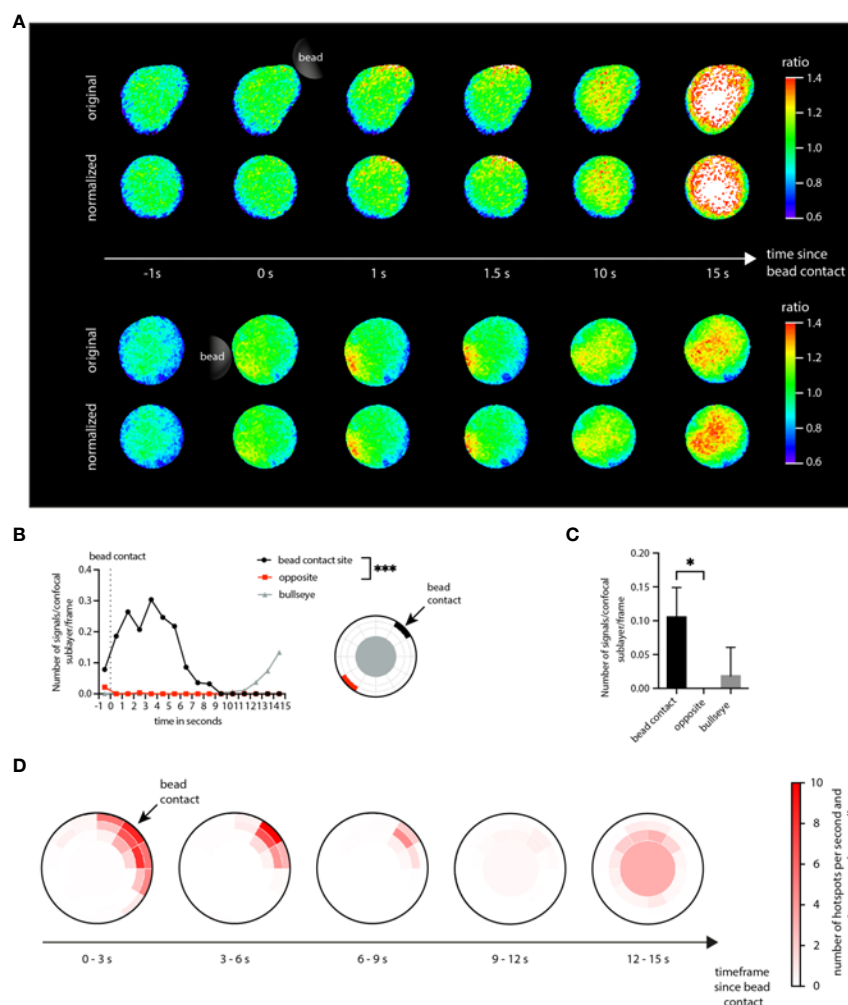


FIGURE 4

Application of DARTS to KHYG-1 cells. (A) Ca^{2+} signals in two exemplary KHYG-1 cells within the first seconds after stimulatory bead contact (top: original cell representation; bottom: shape-normalized cell representation). (B) Number of Ca^{2+} microdomains as a function of time for a dartboard area near the bead contact (black), opposite the bead contact (red), and the cell center (dartboard bullseye), averaged across $n=7$ cells. Statistical testing by two-tailed Mann-Whitney-U-test, *** $p < 0.001$. (C) The corresponding average number of selected Ca^{2+} microdomains. Statistical testing by two-tailed Mann-Whitney-U-test, * $p < 0.05$. (D) Dartboard projection plots, averaged over the $n=7$ cells and different time frames.

microdomains. DARTS integrates state-of-the-art deep learning methods for cell detection and segmentation and provides cell shape normalization and a so-called dartboard projection to facilitate intuitive visualization and comparison of Ca^{2+} microdomain dynamics within and across different cell data sets.

The application of DARTS was demonstrated by means of three examples. The examples illustrate that the dartboard analysis and visualization enables aggregation of locally resolved information that helps to compare and understand the spatio-temporal Ca^{2+} dynamics at different time points and intervals post-bead stimulation for different cell groups. This, in turn, is essential for a better understanding of the underlying cellular processes. Residual quantitative differences in, for instance, the number of hotspots compared to the corresponding quantitative results in (3, 7) are due to implementation differences of post-processing modules, such as an altered order of the deconvolution and bleaching correction and changes of the deconvolution implementation. Moreover, the shape normalization is expected

to change the results of the hotspot detection slightly, depending on the deviation from a perfect circle. However, these low-level differences were negligible for the application examples, demonstrating the robustness of the implemented methods.

As shown in the application examples, the primary use case of DARTS is to analyze Ca^{2+} microdomain dynamics for groups of bead-activated cells. However, it can also be used to define intracellular control areas, for instance to answer the question if observed Ca^{2+} dynamics are more pronounced near the bead contact than farther away (cf. application example 3). Thus, the DARTS analysis options provide a more holistic view than the related usual measures.

Despite its current application focus, different DARTS modules and analysis options are expected to be of interest for different experiments and analysis scenarios. Potential use cases are, for instance, a microdomain analysis for different cell activation approaches like microinjection. General methodical aspects of DARTS like the provided deconvolution approaches

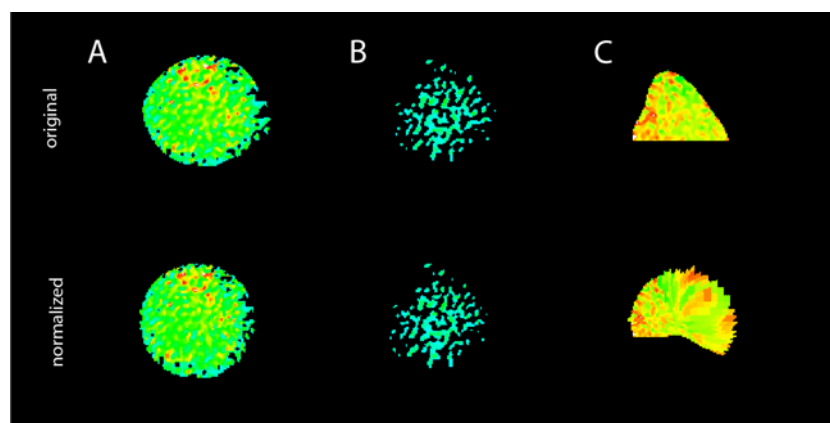


FIGURE 5

Limitations of the DARTS pipeline. Examples of failed processing: (A) frayed cell edges and (B) “vanishing” cell, both due to erroneous threshold-based background subtraction; (C) failed cell shape normalization for a cell that was not entirely within the region of interest to be processed.

and the shape normalization can also be applied to live cell imaging data acquired in an entirely different experimental setting. The application is not limited to immune cells alone, other cell types such as neurons are also expected to work with the framework. For the shape normalization, however, a minimal convexity/circularity is required. Similarly, the trigger of the Ca^{2+} release does not need to be a bead contact. The postprocessing and analysis steps can also be used without bead contact definition.

DARTS will be continuously extended. Current developments comprise the autodetection of bead contacts, adapting state-of-the-art machine learning-based methods like in (25), as well as integration of further deconvolution and denoising approaches. Moreover, options for autodetection of rare shape normalization failures (see Figure 5 for examples) will also be incorporated. In each case: Since DARTS is provided as an open-source solution, the next implementation steps and extensions will also depend on and be aligned with the needs of the community. Feedback and suggestions to optimize the DARTS pipeline and its modules are very much welcome.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

LW: Conceptualization, Formal analysis, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. DK: Formal analysis, Investigation, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. HH: Formal analysis, Software, Writing – review & editing. FF: Software, Writing – review & editing. FG: Formal analysis, Investigation, Writing – review & editing.

FM: Formal analysis, Investigation, Writing – review & editing. MA: Funding acquisition, Writing – review & editing. AG: Funding acquisition, Writing – review & editing. BD: Data curation, Formal analysis, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing. RW: Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer GD declared a past co-authorship with one of the authors B-PD, AG to the handling editor.

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2.2 Publication II: Time-resolved role of P2X4 and P2X7 during CD8⁺ T cell activation

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Time-resolved role of P2X4 and P2X7 during CD8⁺ T cell activation

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CD8⁺ T cells are a crucial part of the adaptive immune system, responsible for combating intracellular pathogens and tumor cells. The initial activation of T cells involves the formation of highly dynamic Ca²⁺ microdomains. Recently, purinergic signaling was shown to be involved in the formation of the initial Ca²⁺ microdomains in CD4⁺ T cells. In this study, the role of purinergic cation channels, particularly P2X4 and P2X7, in CD8⁺ T cell signaling from initial events to downstream responses was investigated, focusing on various aspects of T cell activation, including Ca²⁺ microdomains, global Ca²⁺ responses, NFAT-1 translocation, cytokine expression, and proliferation. While Ca²⁺ microdomain formation was significantly reduced in the first milliseconds to seconds in CD8⁺ T cells lacking P2X4 and P2X7 channels, global Ca²⁺ responses over minutes were comparable between wild-type (WT) and knockout cells. However, the onset velocity was reduced in P2X4-deficient cells, and P2X4, as well as P2X7-deficient cells, exhibited a delayed response to reach a certain level of free cytosolic Ca²⁺ concentration ([Ca²⁺]_i). NFAT-1 translocation, a crucial transcription factor in T cell activation, was also impaired in CD8⁺ T cells lacking P2X4 and P2X7. In addition, the expression of IFN-γ, a major pro-inflammatory cytokine produced by activated CD8⁺ T cells, and Nur77, a negative regulator of T cell activation, was significantly reduced 18h post-stimulation in the knockout cells. In line, the proliferation of T cells after 3 days was also impaired in the absence of P2X4 and P2X7 channels. In summary, the study demonstrates that purinergic signaling through P2X4 and P2X7 enhances initial Ca²⁺ events during CD8⁺ T cell activation and plays a crucial role in regulating downstream responses, including NFAT-1 translocation, cytokine expression, and proliferation on multiple timescales. These findings suggest that targeting purinergic signaling pathways may offer potential therapeutic interventions.

KEYWORDS

CD8⁺ T cells, Ca²⁺ imaging, activation, TCR stimulation, Ca²⁺ microdomains, NFAT, IFN-γ, proliferation

1 Introduction

CD8⁺ T cells are part of the adaptive immune system and respond to intracellular pathogens such as viruses or bacteria. They recognize pathogen-derived antigen fragments presented on major histocompatibility complex (MHC) class I molecules with their T cell receptor (TCR) on the surface of infected cells. This triggers the release of a range of effector molecules, like perforin and granzymes, but also upregulation of Fas-ligand, ultimately resulting in direct apoptosis of the infected cells (1).

The initial activation of T cells can be visualized by the formation of highly dynamic Ca²⁺ microdomains. These initial signaling events are triggered by the most potent Ca²⁺-releasing second messenger, nicotinic acid adenine dinucleotide phosphate (NAADP) (2). NAADP binds to a small cytosolic binding protein, hematological and neurological expressed 1-like protein (HN1L)/Jupiter microtubule associated homolog 2 (JPT2) (3, 4) and in turn, evokes the release of Ca²⁺ from the endoplasmic reticulum (ER) through the ryanodine receptor type 1 (RYR1) in T cells (5, 6). This promotes the activation of stromal interaction molecules 1 and 2 (STIM1/2) and thus store-operated Ca²⁺ entry (SOCE) through ORAI1 channels (7), leading to an amplification of initial Ca²⁺ microdomains (6). Sustained Ca²⁺ microdomains result in the translocation of nuclear factor of activated T cells (NFAT) (8), which promotes the secretion of pro-inflammatory cytokines such as interleukin-17 (IL-17) (9) or interferon- γ (IFN- γ) (10). Interfering with the Ca²⁺ signaling cascade has potential therapeutic effects. For example, blocking SOCE by STX140 efficiently ameliorates the course of disease in a rat model of multiple sclerosis, where it prevents immune cells from infiltrating the central nervous system (11).

It is generally accepted that CD8⁺ T cells also play a central role in mediating anti-tumor immunity, by recognizing tumor-associated antigens presented on MHCI (12). Migration of CD8⁺ T cells into the tumor microenvironment (TME) correlates with improved prognosis in multiple cancer types (13, 14). The TME is especially rich in the potent immunosuppressive factor adenosine, which is generated by the degradation of extracellular adenosine triphosphate (eATP) through ectoenzymes (15). eATP is a pro-inflammatory signaling molecule that stimulates purinergic P2X receptors, an important mechanism regulating T cell activation, homing, and function (16). The two purinergic cation channels P2X4 and P2X7 were shown to play a role in T cell function (17) and amplify global Ca²⁺ signaling during T cell activation (18–20). Furthermore, in murine CD4⁺ T cells, P2X4 and P2X7 are essential to evoke initial Ca²⁺ microdomains in the first seconds after TCR stimulation (21). These signals were reduced by blocking the release of ATP through pannexin-1 or by degrading eATP using apyrase (21). However, the role of P2X4 and P2X7 on initial activation as well as the downstream effector function(s) in CD8⁺ T cells remains to be investigated.

Therefore, in the present study, we analyzed *P2rx4*^{-/-} and *P2rx7*^{-/-} CD8⁺ T cells across different timescales following TCR stimulation. We investigated the formation of initial Ca²⁺

microdomains during the first milliseconds – seconds, NFAT-1 translocation to the nucleus over 15 min, the expression of pro-inflammatory cytokines, like TNF- α and IFN- γ , and effector mediators like granzyme B up to 24 h post-TCR stimulation as well as the proliferation after 3 days. Taken together, our data suggest that purinergic signaling enhances initial Ca²⁺ events during CD8⁺ T cell activation and plays an essential role in fine-tuning the downstream activity of T cell activation.

2 Materials and methods

2.1 Reagents

Fluo4-AM and FuraRed-AM were purchased from Life Technologies and reconstituted in DMSO. Aliquots were stored at -20°C until assayed. Fura2-AM was obtained from Merck. Apyrase was dissolved in Ca²⁺ buffer in a final concentration of 100 U/mL, aliquoted, and stored at -20°C until used. Anti-mouse CD3 and anti-mouse CD28 monoclonal antibodies were obtained from BD Biosciences.

2.2 Animal models

P2rx4^{-/-} (*P2rx4*^{tm1Rass}; MGI (Mouse Genome Informatics): 3665297) and *P2rx7*^{-/-} mice (*P2rx7*^{tm1Gab}; MGI:2386080) were backcrossed onto the BALB/c background as previously described (21) and analyzed together with WT BALB/c mice. They were bred at the animal facility of the University Medical Centre (UKE). The regulatory committee (Hamburger Behörde für Gesundheit und Verbraucherschutz, Veterinärwesen/Lebensmittelsicherheit, ORG 941 and 1115) approved the experiments, which included tissues of animal origin.

2.3 Isolation of primary murine CD8⁺ T cells

Spleens and lymph nodes from WT or knockout BALB/C mice were passed through a 40 μ m strainer and erythrocytes were lysed in ammonium-chloride-potassium (ACK) buffer (4.3 g ammonium chloride, 0.5 g KHCO₃, 0.0186 g Na₂-EDTA in 400 mL H₂O, pH 7.2–7.4) for 3–5 min. CD8⁺ T cells were separated from other cells using a negative selection kit (EasySep Mouse CD8⁺ T Cell Enrichment Kit, STEMCELL Technologies Inc.). Cell purity of 97–98% was validated by FACS.

2.4 Imaging initial Ca²⁺ microdomains in primary murine CD8⁺ T cells

CD8⁺ T cells, isolated on the day of the measurement, were loaded with the Ca²⁺ dyes Fluo4 (10 μ M) and FuraRed (20 μ M), and Ca²⁺ imaging was carried out as described in detail by

Diercks et al., 2019 (22). Cells were imaged in Ca^{2+} buffer [140 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1 mM CaCl_2 , 20 mM Hepes (pH 7.4), 1 mM NaH_2PO_4 , 5 mM glucose] for three minutes on coverslips coated with 5 μL BSA (5 mg/mL) and 5 μL Poly-L-Lysine (0.1 mg/mL) to increase adhesion of the cells. To remove extracellular ATP, 10 U/mL apyrase was added to the cells 3 minutes prior to imaging. T cells were stimulated with anti-CD3/anti-CD28 coated beads after the first minute of measurement. Small, compact connected pixel sets with high $[\text{Ca}^{2+}]_i$ values were defined as Ca^{2+} microdomains. The signals occurring after stimulation were detected in an automated MATLAB script (22). The location and time of the bead contact of all individual cells were normalized and an average value for all cells of a condition was formed and displayed in a dartboard projection for a specific time window, e.g. the first second after the bead contact.

2.5 Global Ca^{2+} imaging in primary murine CD8^+ T cells

CD8^+ T cells, which were isolated on the day of the experiment, were loaded with 4 μM Fura2-AM for 35 min at 37°C. Global Ca^{2+} imaging was performed as described by Weiß and colleagues, 2023 (8). Cells were imaged in Ca^{2+} buffer [140 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1 mM CaCl_2 , 20 mM Hepes (pH 7.4), 1 mM NaH_2PO_4 , 5 mM glucose] for 8 min on coverslips coated with 5 μL BSA (5 mg/mL) and 5 μL Poly-L-Lysine (0.1 mg/mL). Monoclonal Anti-CD3 antibody (1 $\mu\text{g}/\text{mL}$) was used to stimulate the cells one minute after the measurement started. Images were acquired in 16-bit mode with Volocity software (PerkinElmer). FIJI software (version 1.53f) was used for post-processing like background correction, splitting of the fluorescence channels, and selection of the regions of interest (ROI).

2.6 Super-resolution NFAT1 translocation

The preparation of slides was performed as described in 2.4. Next, CD8^+ T cells were seeded on the slides, fixed, and permeabilized. Cells were treated with biotin anti-CD3 (553060, BD Biosciences)/anti-CD28 (553296, BD Biosciences) coated transparent streptavidin beads (TP190902KOM5, PolyAn) for either 7.5 min or 15 min. Overnight incubation was performed with 10% (v/v) fetal calf serum (FCS) to block non-specific binding sites. Primary antibody staining to visualize NFAT-1 (rabbit anti-mouse NFAT-1, 1:200 (5861S, Cell Signaling Technology) was applied for 1 hour at room temperature. Furthermore, the cells were washed four times with 3% (v/v) FCS to remove the primary antibody. The samples were again stained for 1 hour at room temperature with a secondary antibody (anti-rabbit Alexa Fluor 488, 1:400 (A21206, Life technologies). The nucleus was labeled by DAPI staining (62247, ThermoFisher, 1:1000) for 10 min at room temperature. The glass slides were mounted overnight with Abberior Mount Solid (Abberior). Image acquisition was carried out using a high-resolution spinning disk (Visitron) containing a

CSU-W1 SoRa Optic (2.8x, Yokogawa), a 100x magnification objective (Zeiss) and a sCMOS camera (Orca-Flash 4.0, C13440-20CU Hamamatsu). The ratio of the nuclear NFAT-1 signal divided by the NFAT-1 signal in the cytoplasm was calculated as a percentage to analyze the translocation of NFAT-1. Spots were marked by pixel-based segmentation using the trainable weka (Waikato Environment for Knowledge Analysis) segmentation plugin on Fiji (version 1.53f). The area (sum of detected spots) of all NFAT-1 spots in the nucleus and cytoplasm was calculated using a MATLAB (version R2018b) script (8). To distinguish between the nucleus and the cytoplasm DAPI staining was used. Our results were normalized using the dynamic range (baseline to maximum response) determined by the 15 min bead stimulation of our NFAT-1 translocation assay, which included a baseline of 39% and a maximum response of approximately 70%, as noted.

2.7 Expression analysis by flow cytometry

Spleen cells were isolated and erythrocytes were depleted with ACK buffer as described above. Spleen cells were incubated in 500 μL of Iscove's modified Dulbecco's medium (IMDM). Fetal calf serum, glutamine, gentamicin, and 2-mercaptoethanol were added to the medium. Stimulation of the cells was performed using anti-CD3e mAb (1 $\mu\text{g}/\text{mL}$; clone 145-2C11, BioLegend, San Diego, CA). For some assays, anti-CD28 mAb (1 $\mu\text{g}/\text{mL}$; clone 37.51, BioLegend) was added, as indicated in the figure legends. Brefeldin A (Sigma-Aldrich) was added during the last 4 hours of culture for cytokine production assays. For extracellular antibody staining, cells were incubated in PBS with 1% rat serum and anti-Fc receptor mAb (10 $\mu\text{g}/\text{mL}$; clone 2.4G2, BioXCell, West Lebanon, NH) for extracellular antibody staining. A fixable dead cell stain (Alexa Flour 750 carboxylic acid, succinimidyl ester, Invitrogen, Eugene, OR), V450-conjugated anti-CD8 mAb (clone 53-6.7, BD Biosciences) and BV605-conjugated anti-CD69 mAb (clone H1.2F3, BioLegend) were then added to the cells for 20 minutes on ice. Intracellular antibody staining was performed using the Foxp3/Transcription Factor Staining Buffer Set (Invitrogen) following the manufacturer's protocol. Cells were stained with PE-conjugated anti-NUR77 mAb (clone 12.14, Invitrogen), PE-Cy7-conjugated anti-IRF4 mAb (clone 3E4, eBioscience, San Diego, CA), FITC-conjugated anti-Granzyme B mAb (clone GB11, BioLegend), AF647-conjugated IFN- γ mAb (clone XMG1.2; eBioscience), and FITC-conjugated anti-TNF- α mAb (clone MP6-XT22, BioLegend). A FACSCelesta flow cytometer (BD Biosciences, Franklin Lakes, NJ) and FlowJo software (Tree Star, Ashland, OR) were used for cell analysis.

To evaluate proliferation, spleen cells were treated with carboxyfluorescein diacetate succinimidyl ester (CFSE, 5 μM ; Invitrogen) for 10 minutes at room temperature and then washed twice. They were stimulated with anti-CD3e mAb (1 $\mu\text{g}/\text{mL}$; clone 145-2C11, BioLegend) and anti-CD28 mAb (1 $\mu\text{g}/\text{mL}$; clone 37.51, BioLegend) in supplemented IMDM. After 3 days, cells were stained with V450-conjugated anti-CD8 mAb (clone 53-6.7, BD Biosciences) and a fixable dead cell stain (Alexa Flour 750

carboxylic acid, succinimidyl ester, Invitrogen). Cell analysis was performed using FACS. Proliferation is given as the Expansion index (EI) indicating the overall expansion of the culture (23), which was calculated with the FlowJo software.

2.8 Statistics

All data are presented as the mean $\pm$ SEM of independent experiments, which were performed as a minimum in triplicate. Data were analyzed by MATLAB software (MathWorks), Prism 9 (GraphPad Software), and Excel (Microsoft). Data were tested for normality using the Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilliefors P-value. Normal distributed data sets were compared by using ordinary one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. Data sets lacking normal distribution were analyzed using Kruskal-Wallis tests and Dunnett's multiple comparisons tests. Data from flow cytometry experiments were compared with two-way ANOVA and Tukey's multiple comparisons test. A p value below 0.05 was considered to be significant.

3 Results

3.1 P2X4 and P2X7 are involved in the formation of initial Ca^{2+} microdomains in primary CD8^+ T cells during the first milliseconds to seconds after stimulation

CD8^+ T cells were freshly isolated from wild-type (WT), $P2rx4^{-/-}$ and $P2rx7^{-/-}$ mice on a BALB/c background (24) and stimulated with anti-CD3/anti-CD28-coated beads in order to imitate an immune synapse. Initial Ca^{2+} microdomains were analyzed 0.5 s prior to and up to 15 s after bead contact using high-resolution Ca^{2+} live-cell imaging (5, 6) (Figure 1A). Previously, such Ca^{2+} microdomains were defined by a signal area of $0.216 \pm 0.004 \mu\text{m}^2$ (mean $\pm$ SEM) and an average amplitude of $325 \pm 11 \text{ nM}$ (mean $\pm$ SEM) (5, 6, 25). Directly after bead contact in the first 500 ms, in CD8^+ WT T cells (Figure 1A, top) single local Ca^{2+} microdomains at the bead contact site were detectable, spreading through the whole cell within 15 s. In contrast, in CD8^+ T cells from $P2rx4^{-/-}$ and $P2rx7^{-/-}$ mice (Figure 1A, middle and bottom) these initial Ca^{2+} microdomains directly after bead contact were absent. In 80% of CD8^+ WT cells, initial Ca^{2+} microdomains were detected, with a mean amplitude of $319 \pm 22 \text{ nM}$ (mean $\pm$ SEM, $n=32$) and 0.46 signals/confocal plane/frame, which corresponds to approximately 18 Ca^{2+} microdomains/confocal plane/second (Figure 1B). Only 42% of $P2rx4^{-/-}$ cells and 49% of $P2rx7^{-/-}$ cells responded with initial Ca^{2+} microdomains, with significantly decreased numbers of Ca^{2+} signals/confocal plane/frame of 0.04 ($P2rx4^{-/-}$; $n=35$) and 0.1 ($P2rx7^{-/-}$; $n=23$), and mean Ca^{2+} amplitudes of $259 \pm 15 \text{ nM}$ (mean $\pm$ SEM, $n=16$) and $283 \pm 29 \text{ nM}$ (mean $\pm$ SEM, $n=10$) (Figure 1B). Already in the first milliseconds after bead contact the numbers of initial Ca^{2+} microdomains directly at the artificial immune synapse was significantly reduced (Figure 1C). While cells lacking the P2X4

channel showed nearly no increase of Ca^{2+} microdomains in the first milliseconds after TCR stimulation, a slight increase was observed for cells lacking the P2X7 channel. This slight increase was lower and delayed compared to the Ca^{2+} response of WT T cells at the artificial immune synapse (Figure 1C). Furthermore, if the extracellular ATP (eATP) was hydrolysed by recombinant apyrase the number of initial Ca^{2+} microdomains was significantly reduced in WT CD8^+ T cells by 99% (Figure 2). Only in one of the 21 T cells treated with extracellular apyrase Ca^{2+} microdomains could be detected, while 30% of the untreated cells responded (Figure 2). Taken together, the initial Ca^{2+} response milliseconds after T cell stimulation in CD8^+ T cells is promoted by eATP stimulating the purinergic cation channels P2X4 and P2X7.

3.2 Global Ca^{2+} signals in the first minutes are impaired in primary CD8^+ $P2rx4^{-/-}$ and $P2rx7^{-/-}$ T cells

The transition of early, spatiotemporally restricted Ca^{2+} microdomains into a global Ca^{2+} response indicates regular T cell function. Thus, we analyzed global Ca^{2+} signals in the first 8 mins after TCR stimulation with soluble antibodies against CD3. The overall Ca^{2+} response in WT ($n=217$) compared to $P2rx4^{-/-}$ ($n=164$) and $P2rx7^{-/-}$ ($n=135$) CD8^+ T cells appeared similar (Figure 3A). However, compared to WT T cells the signal onset velocity was 2.6-fold reduced in $P2rx4^{-/-}$ T cells while there was no difference in $P2rx7^{-/-}$ CD8^+ T cells (Figure 3B). In contrast the time to reach a 200 nM threshold was significantly delayed in $P2rx4^{-/-}$ and $P2rx7^{-/-}$ T cells (Figure 3C). T cells lacking either the P2X4 or P2X7 channel reached the threshold after $226 \pm 8 \text{ s}$ (mean $\pm$ SEM, $n=135$) or $234 \pm 5 \text{ s}$ (mean $\pm$ SEM, $n=164$) compared to WT cells reaching the threshold already after $119 \pm 6 \text{ s}$ (mean $\pm$ SEM, $n=217$). Furthermore, there was no significant difference in the area under the curve (AUC) (Figure 3D). In summary, the global Ca^{2+} response during the first 8 mins after TCR stimulation is altered in CD8^+ T cells from $P2rx4^{-/-}$ and $P2rx7^{-/-}$ mice but in different aspects.

3.3 NFAT-1 translocation after 15 minutes is reduced in primary CD8^+ $P2rx4^{-/-}$ and $P2rx7^{-/-}$ T cells

NFAT-1 is one of the major transcription factors for T cell activation. The translocation of NFAT-1 is regulated by Ca^{2+} and the phosphatase calcineurin (26). NFAT-1 is present in naïve CD8^+ T cells and its dephosphorylation is detectable already 15 min after stimulation with ionomycin (27). Hence, we analyzed the translocation of NFAT-1 in primary murine CD8^+ WT, $P2rx4^{-/-}$ and $P2rx7^{-/-}$ T cells after 7.5 and 15 min of bead stimulation. Unstimulated T cells from WT ($n=61$) or knockout mice ($P2rx4^{-/-}$ $n=38$; $P2rx7^{-/-}$ $n=56$) showed an equal distribution of NFAT-1 protein (indicated in green) outside the nucleus (indicated in blue) in the cytoplasm (Figure 4A, upper row). There was no difference in the percentage of nuclear NFAT-1 signal in CD8^+ T cells without stimulation (basal). After 7.5 min bead stimulation, moderate

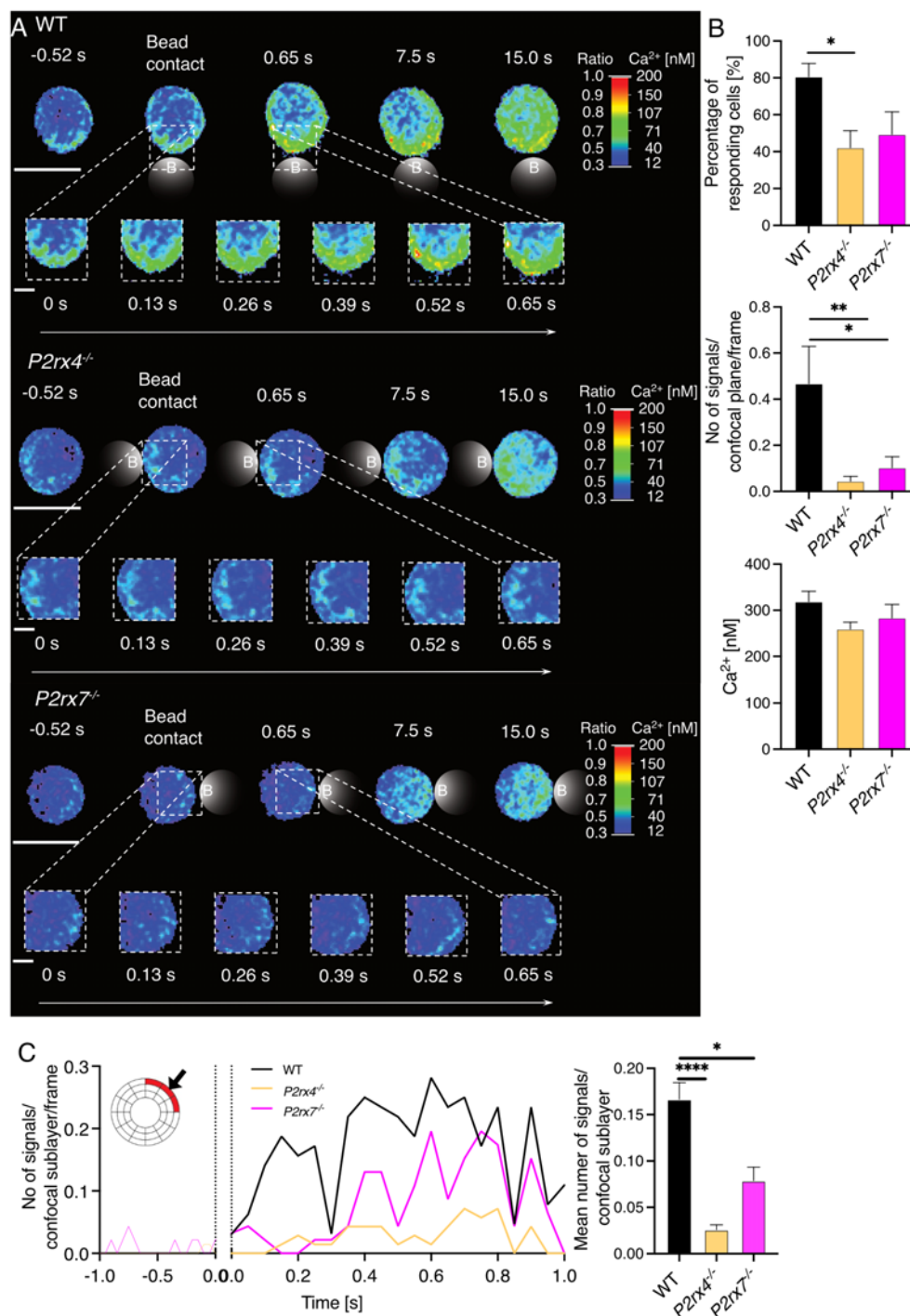


FIGURE 1

P2X4 and P2X7 influence the formation of Ca²⁺ microdomains in the first seconds after TCR stimulation. Initial Ca²⁺ microdomain imaging of CD8⁺ T cells of WT, *P2rx4*^{-/-} and *P2rx7*^{-/-} mice was performed with the Ca²⁺ indicators Fluo4 and FuraRed using anti-CD3/anti-CD28 coated beads. For each condition, 7 mice were used. (A) A representative cell of WT, *P2rx4*^{-/-} and *P2rx7*^{-/-} is shown for a time window of -0.52 s to 15 s after bead engagement (scalebar 5 μm) and a magnified region of the bead contact site from 0 s - 0.65 s (scalebar 1 μm). B-C) Data are mean ± SEM, WT n = 32 cells, *P2rx4*^{-/-} n = 35 cells, *P2rx7*^{-/-} n = 23 cells. N = 7 per group. Results were analyzed using the Kruskal-Wallis test and Dunnett's multiple comparisons test (*p < 0.05; **p < 0.01, ****p < 0.0001). (B) Quantification of the initial Ca²⁺ microdomains in CD8⁺ T cells 15 s after bead engagement. The percentage of responding cells (upper panel), the number of Ca²⁺ microdomains per frame for whole cells (confocal plane, middle panel) and the average Ca²⁺ amplitude of these signals are shown (lower panel). (C) Analysis of the initial Ca²⁺ microdomains in a time window -1 s to 1 s after bead engagement for contact site sublayer of a dartboard projection (as indicated in red, left panel) and quantification of these signals (right panel).

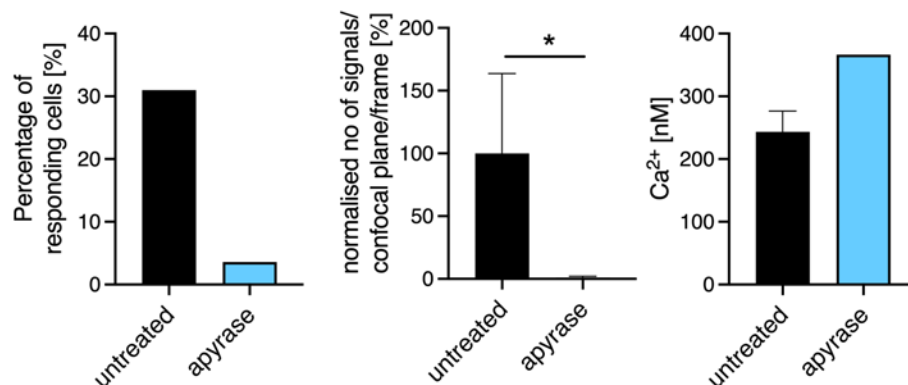


FIGURE 2

Extracellular ATP (ATP) influences the formation of Ca^{2+} microdomains in the first seconds after TCR stimulation. Initial Ca^{2+} microdomain imaging of CD8^{+} T cells of WT with and without addition of apyrase to the Ca^{2+} measurement buffer was performed with the Ca^{2+} indicators Fluo4 and FuraRed using anti-CD3/anti-CD28 coated beads. Bar charts show the percentage of responding cells (left panel), the normalized number of Ca^{2+} microdomains per frame for whole cells (per confocal plane, middle panel), the average Ca^{2+} amplitude of these signals are shown (right panel) 15s after bead engagement. Data are mean $\pm$ SEM, untreated $n = 20$ cells, apyrase $n = 21$ cells from 7 independent experiments. Results were analyzed using the Mann-Whitney test (* $p < 0.05$).

NFAT-1 translocation of $37 \pm 12\%$ (mean $\pm$ SEM, $n = 29$) was visible in WT CD8^{+} T cells but only marginally detected in $P2rx4^{-/-}$ with $0.8 \pm 8\%$ (mean $\pm$ SEM, $n = 29$) and $P2rx7^{-/-}$ T cells with $24 \pm 9\%$ (mean $\pm$ SEM, $n = 33$) (Figure 4B right panel). The differences

between WT and $P2rx4^{-/-}$ or $P2rx7^{-/-}$ T cells increased to a highly significant level after 15min. In comparison to the WT (normalized ratio signal of $100 \pm 8\%$, $n = 35$), only $29 \pm 8\%$ (mean $\pm$ SEM, $n = 36$) and $19 \pm 7\%$ (mean $\pm$ SEM, $n = 24$) of nuclear NFAT-1 protein

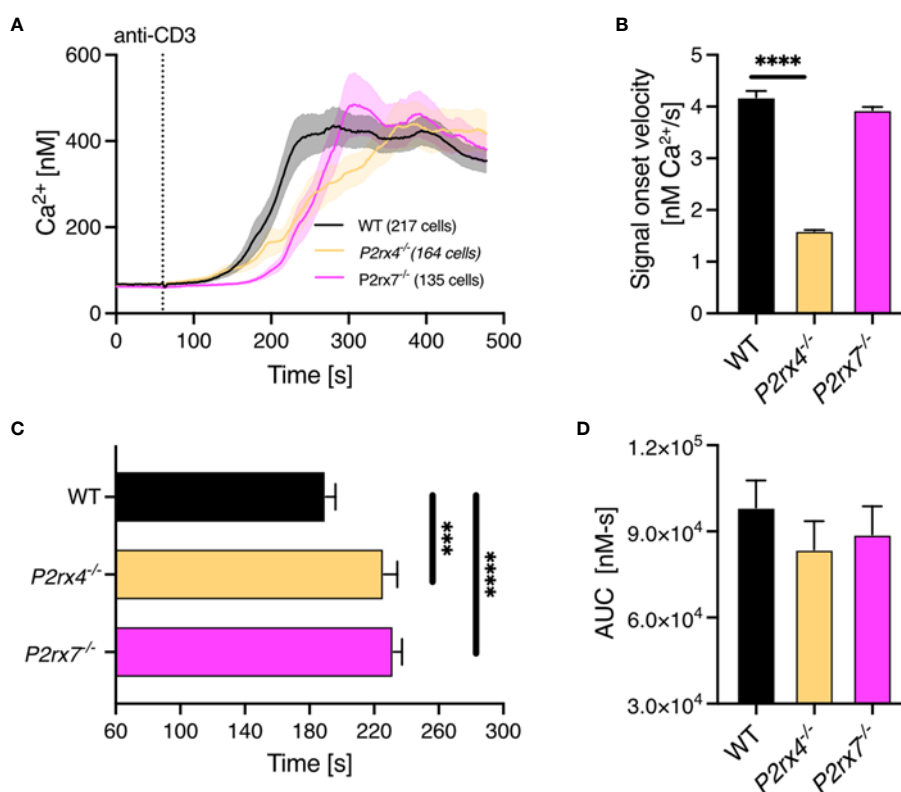


FIGURE 3

Impaired global Ca^{2+} response in CD8^{+} T cells from $P2rx4^{-/-}$ and $P2rx7^{-/-}$ mice. Ca^{2+} imaging of CD8^{+} T cells of WT, $P2rx4^{-/-}$ and $P2rx7^{-/-}$ mice was performed with the Ca^{2+} indicator Fura-2 for 8 min. (A) Cells were stimulated with monoclonal antibodies against CD3 ($10\mu\text{g}/\text{mL}$) after the first min of measurements. For each condition, 3 mice were used ($N = 3$ per group). Data are mean $\pm$ SEM, WT $n = 217$ cells, $P2rx4^{-/-}$ $n = 164$ cells, $P2rx7^{-/-}$ $n = 135$ cells. Results were analyzed using Kruskal-Wallis test and Dunnett's multiple comparisons test (** $p < 0.001$, **** $p < 0.0001$). The data were quantified by analyzing the signal onset velocity (B), the time to reach $200\text{ nM } \text{Ca}^{2+}$ (C) and the area under the curve (D).

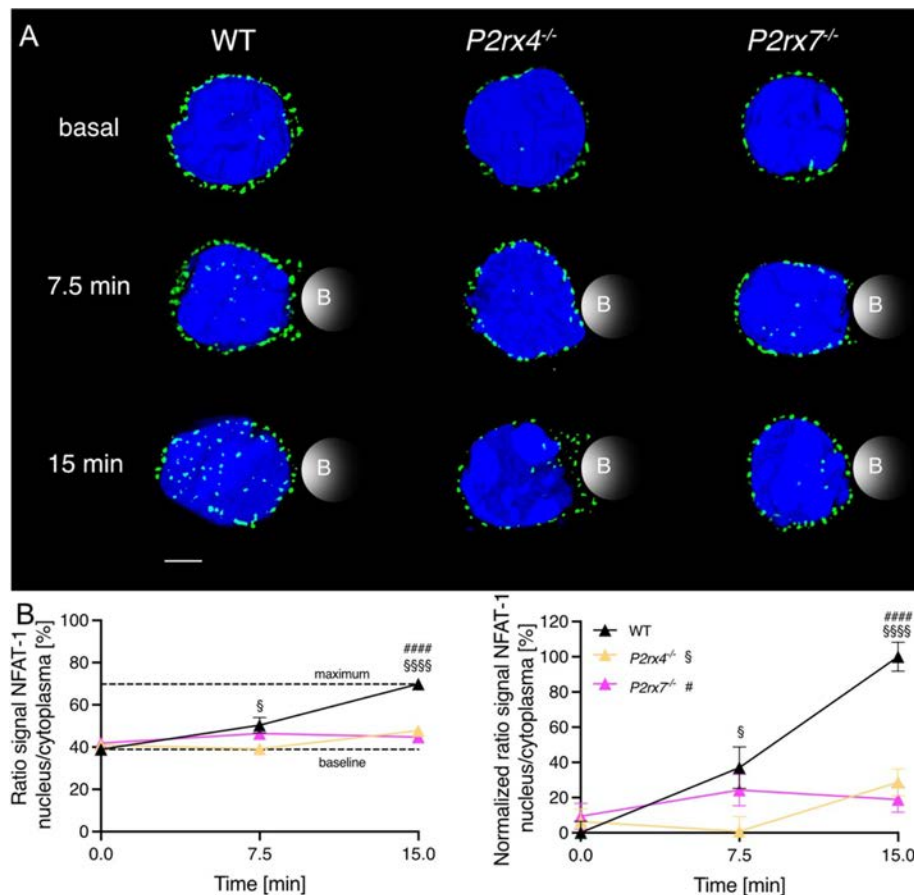


FIGURE 4

The nuclear translocation of NFAT-1 is influenced by P2X4 and P2X7 minutes after TCR stimulation. CD8⁺ T cells of WT, *P2rx4*^{-/-} and *P2rx7*^{-/-} mice were activated with anti-CD3/anti-CD28 coated beads for 7.5 min and 15 min and NFAT-1 protein and the nucleus were stained. Three spleens per condition were used. (A) A representative cell for WT, *P2rx4*^{-/-} and *P2rx7*^{-/-} without stimulation (basal), and after 7.5 min and 15 min stimulation are shown. NFAT-1 is presented in green, the nucleus in blue. Bead contact is indicated schematically. Scalebar 2 μ m. (B) The translocation of NFAT-1 is quantified by the ratio of the signal of NFAT-1 in the nucleus/cytoplasm (right panel). Approximation of the dynamic range using the maximal response, which was obtained with 15 min of bead stimulation in WT T cells and data were normalized to this dynamic range (left panel). Data are means $\pm$ SEM. Basal WT $n = 61$ cells, *P2rx4*^{-/-} $n = 38$ cells and *P2rx7*^{-/-} $n = 56$ cells. 7.5 min WT $n = 29$ cells, *P2rx4*^{-/-} $n = 29$ cells and *P2rx7*^{-/-} $n = 33$ cells. 15 min WT $n = 35$ cells, *P2rx4*^{-/-} $n = 36$ cells and *P2rx7*^{-/-} $n = 24$ cells. Data were compared using ordinary one-way ANOVA with Dunnett's multiple comparisons test (P-values § for *P2rx4*^{-/-}, # for *P2rx7*^{-/-}, p §/§ < 0.05, and p §§§/#### < 0.0001).

signal was detected in *P2rx4*^{-/-} and *P2rx7*^{-/-} T cells (Figure 4B right panel). The separated individual channels, as well as brightfield images with beads, are shown in Supplementary Figure 1. On the other hand, if proximal TCR signaling is bypassed with the addition of thapsigargin (positive control) a comparable NFAT-1 translocation in all genotypes was induced (Supplementary Figure 2). These results indicate the involvement of P2X4 and P2X7 in the signal transduction following TCR stimulation of CD8⁺ T cells from early Ca²⁺ signals to the transcriptional response.

3.4 P2X4 and P2X7 are involved in cytokine and activation marker expression at 18–24 hours and proliferation at days in primary CD8⁺ T cells

Previously, it was shown that the key regulator of IFN- γ production is NFAT-1 (28). The expression of IFN- γ occurs

rapidly after T cell stimulation and is linked to several cell functions, like cytotoxicity (29, 30). Since NFAT-1 translocation is reduced in CD8⁺ T cells from *P2rx4*^{-/-} and *P2rx7*^{-/-} mice (Figure 4) and NFAT-1 is the major regulator of IFN- γ production (28), we analyzed the expression of IFN- γ after stimulation in the WT and both knockout cells. After 4 h, only 1–2% of the CD8⁺ T cells also express IFN- γ , with a little higher expression in *P2rx4*^{-/-} and *P2rx7*^{-/-} T cells (Figure 5A). An increase in T cells expressing IFN- γ was observed after 18 h for WT and *P2rx4*^{-/-} and *P2rx7*^{-/-} mice. At this time point, a significantly lower percentage of *P2rx4*^{-/-} and *P2rx7*^{-/-} T cells of 10 $\pm$ 1% (mean $\pm$ SEM, $N = 3$) and 7 $\pm$ 1% (mean $\pm$ SEM, $N = 3$) express IFN- γ compared to 21 $\pm$ 2% (mean $\pm$ SEM, $N = 3$) of WT T cells (Figure 5A; Supplementary Figure 4). Another important proinflammatory cytokine whose expression is regulated by NFAT-1 is tumor necrosis factor alpha (TNF- α) (31). Analyzing TNF- α positive T cells with and without stimulation after 4 h revealed only a significant reduction in the percentage of *P2rx4*^{-/-} T cells (11 $\pm$

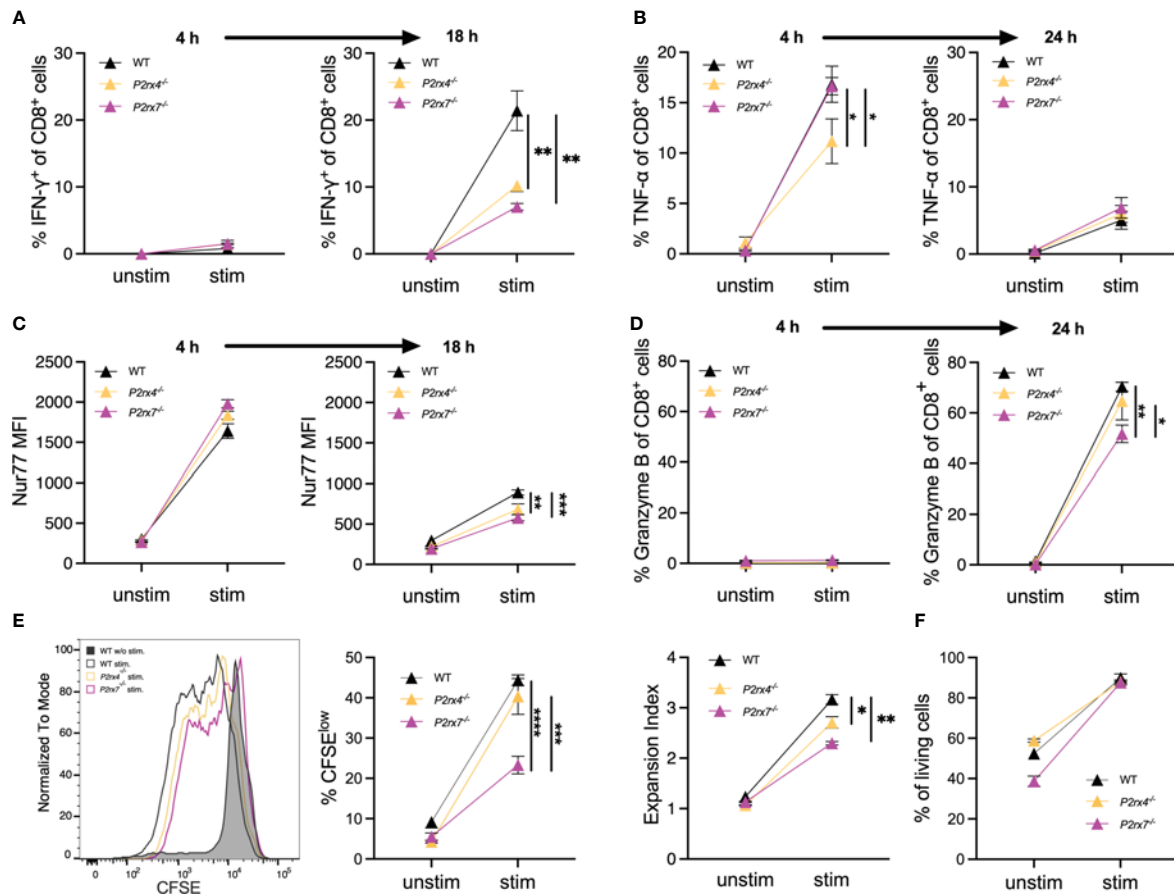


FIGURE 5

P2X4 and P2X7 are involved in the expression of cytokines and activation markers as well as the induction of proliferation. (A–D) Spleen cells from WT, *P2rx4*^{-/-} and *P2rx7*^{-/-} mice were incubated with and without anti-CD3 mAb and anti-CD28 mAb (A, C) or only anti-CD3 mAb (B, D). After 4 h and 18 h or 24 h, the expression of IFN-γ (A), TNF-α (B), Nur77 (C) and granzyme B (D) was analyzed by FACS given as % of CD8⁺ T cells (A, B, D) or mean fluorescence intensity (MFI) of antibody staining (C). (E) Spleen cells from WT, *P2rx4*^{-/-} and *P2rx7*^{-/-} mice were labeled with CFSE and incubated with and without anti-CD3 mAb and anti-CD28 mAb. Representative results for CFSE staining of CD8⁺ T cells is shown in the histogram. The strength of proliferation is given as % CFSE^{low} and as expansion index which takes into account the number of divisions. (F) Survival of cells was estimated by % of living cells after culture for 72 h with and without stimulation. Mean ± SEM, N = 3 per group. (F) Results were analyzed with two-way ANOVA and Tukey's post-test (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

2%; mean ± SEM, N = 3), while the percentage of *P2rx7*^{-/-} (17 ± 1%; mean ± SEM, N = 3) and WT T cells (17 ± 2%; mean ± SEM, N = 3) are almost identical (Figure 5B). Interestingly, after 24h the induced expression of TNF-α was generally much weaker in CD8⁺ T cells from mice of all genotypes (Figure 5B; Supplementary Figure 4). Critical for the effector function of CD8⁺ T cells is the expression and release of granzyme B (32). Without stimulation and after stimulation for 4h, there was hardly any expression of granzyme B in CD8⁺ T cells from mice of all genotypes (between 0–1%) but granzyme B was strongly induced after stimulation for 24h (Figure 5D). For granzyme B, we also observed weaker induction in CD8⁺ T cells from *P2rx4*^{-/-} (65 ± 8%; mean ± SEM, N = 3) and particularly from *P2rx7*^{-/-} (52 ± 4%; mean ± SEM, N = 3) mice compared to WT (70 ± 2%; mean ± SEM, N = 3) (Figure 5D; Supplementary Figure 4). To further assess the effects of P2X4 and P2X7 on downstream signaling in CD8⁺ T cells, the expression of Nur77, IRF4, and CD69 were measured after 4h and 18h of TCR stimulation. The transcription factors Nur77 and IRF4 are expressed immediately after TCR stimulation and their expression

has been associated with the strength of TCR signal (33, 34). CD69 is an early surface marker of activated T cells. Following TCR stimulation, we observed the induction of all three proteins in WT CD8⁺ T cells (Figure 5C, Supplementary Figures 3A, B). Compared to WT CD8⁺ T cells, *P2rx4*^{-/-} and *P2rx7*^{-/-} T cells showed similar expression of IRF4 (Supplementary Figures 3B, 4). Expression of Nur77 and CD69 was reduced in *P2rx4*^{-/-} and *P2rx7*^{-/-} T cells, but only for Nur77 in *P2rx4*^{-/-} and *P2rx7*^{-/-} T cells reduction reached a level of significance. Compared to the WT T cells (893 ± 30 mean fluorescence intensity (MFI); mean ± SEM, N = 3), T cells lacking P2X4 (684 ± 68 MFI; mean ± SEM, N = 3) or P2X7 (579 ± 38 MFI; mean ± SEM, N = 3) showed a significant reduced Nur77 expression 18h after stimulation. Representative histograms and plots of all flow cytometry stainings are shown in Supplementary Figure 4. Next, the proliferation of cells was assessed by dilution of CFSE staining 3 days after incubation with anti-CD3 mAb (Figure 5E, Supplementary Figure 5). Compared to WT CD8⁺ T cells, CD8⁺ T cells of *P2rx4*^{-/-} mice showed slightly lower and of *P2rx7*^{-/-} mice significant lower frequencies of CFSE^{low} cells after

stimulation. When the expansion index (EI) was calculated, which takes into account the number of divisions, both *P2rx4^{-/-}* and *P2rx7^{-/-}* CD8⁺ T cells showed significantly reduced proliferation which was again slightly more pronounced for *P2rx7^{-/-}* T cells (EI after stimulation: WT 3.1 ± 0.10 , $N = 3$), for *P2rx4^{-/-}* of 2.7 ± 0.12 , $N = 3$ and *P2rx7^{-/-}* of 2.3 ± 0.04 , $N = 3$). To control if these effects were not due to different cell survival the percentage of living cells was measured (Figure 5F). No difference between T cells from the different genotypes after stimulation for 72h was observed (Figure 5F). As a control, T cells were stimulated with Phorbol 12-myristate 13-acetate (PMA) and the Ca^{2+} ionophore ionomycin. With this stimulus, which bypasses proximal TCR signaling, there was similar upregulation of Nur77 and CD69 in CD8⁺ T cells from all genotypes (Supplementary Figure 5). In conclusion, these results indicate that P2X4 and P2X7 differentially affect the expression of TCR-induced proteins and the proliferation of CD8⁺ T cells.

Consistently, the results from the current study demonstrate the influence of the purinergic cation channels P2X4 and P2X7 on CD8⁺ T cell activation across timescales, starting with initial signals in the first milliseconds after TCR stimulation up to T cell functions after days. These results are summarized in Figure 6 as a percentage of signals compared to WT (100%; dashed line). Initial Ca^{2+} microdomains in the first milliseconds after TCR stimulation were reduced in *P2rx4^{-/-}* (85%) and *P2rx7^{-/-}* (54%) T cells (Figures 1, 6), leading to a delayed global Ca^{2+} response in the cells lacking either P2X4 (16%) or P2X7 (19%) in the first minutes while the overall Ca^{2+} signal was not impaired. Still, the translocation of the nuclear factor NFAT-1 few minutes post-stimulation was significantly reduced (*P2rx4^{-/-}* (71%) and *P2rx7^{-/-}* (81%)) (Figure 6). Furthermore, T cell function is still impaired in *P2rx4^{-/-}* and *P2rx7^{-/-}* CD8⁺ T cells hours after stimulation resulting in impaired cytokine (IFN- γ : *P2rx4^{-/-}* (52%) and *P2rx7^{-/-}* (67%)) and granzyme B expression (*P2rx4^{-/-}* (8%) and *P2rx7^{-/-}* (26%)) as

well as reduced proliferation of these cells after 3 days (*P2rx4^{-/-}* (15%) and *P2rx7^{-/-}* (25%)) (Figures 5A–C, 6). Only in CD8⁺ T cells from *P2rx4^{-/-}* mice, the expression of TNF- α was significantly reduced by 33%.

4 Discussion

In this study, we investigated the role of the purinergic cation channels P2X4 and P2X7 in the signal transduction of CD8⁺ T cells from very early cytosolic events to downstream responses. Upon TCR stimulation, Ca^{2+} microdomains are induced within milliseconds by the production of NAADP that binds to HN1L/JPT2 and engages RYR1, as well as the interaction of STIM1/2 with ORAI1 (3, 5, 6). Recently, we reported the influence of the purinergic channels P2X4 and P2X7 on the formation of initial Ca^{2+} microdomains in CD4⁺ T cells (21). In line with these results, Ca^{2+} microdomains were significantly reduced in the first millisecond after TCR stimulation in CD8⁺ T cells from *P2rx4^{-/-}* and *P2rx7^{-/-}* mice (Figure 1) or by addition of extracellular apyrase (Figure 2). Ca^{2+} microdomains are one of the first signaling events in a wide range of cell types, affecting different downstream pathways depending on the cell function and the source of the Ca^{2+} microdomains (35). While Ca^{2+} microdomains induced by the STIM-ORAI system are associated with ER Ca^{2+} refilling, PMCA, and adenylyl cyclase activity, the activation of calcineurin, and the translocation of NFAT (36), lysosomal loading by ER-derived Ca^{2+} microdomains affects the endolysosomal system and dynamics (37). A similar fine-tuning mechanism for signal transduction represents the purinergic signaling system via P2X channels. While activation of the P2X1 channel via ATP concentrations in the nanomolar range maintains the mitochondrial metabolism (38), signaling through P2X4 regulates T cell migration (39), and the expression

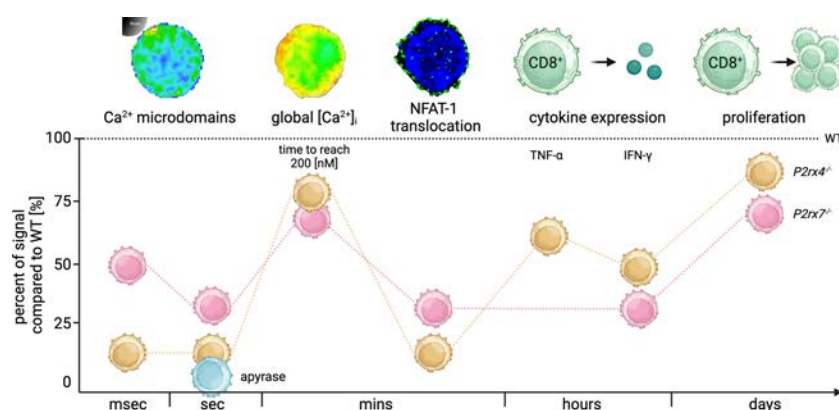


FIGURE 6

Summary of the time-resolved role of P2X4 and P2X7 during CD8⁺ T cell activation. Summary of the significant signal reduction in CD8⁺ T cells from *P2rx4^{-/-}* and *P2rx7^{-/-}* compared to the WT (dashed line) in milliseconds (msec), seconds (sec), minutes (mins), hours and days after TCR stimulation. In the first msec Ca^{2+} microdomains were reduced in *P2rx4^{-/-}* and *P2rx7^{-/-}* T cells by 85% and 54%. Seconds after stimulation the Ca^{2+} microdomains were reduced by 91% and 78%. Moreover, the hydrolysis of eATP by the addition of apyrase (blue) reduces the number of Ca^{2+} microdomains by 99% in the first 15 seconds in WT CD8⁺ T cells. The global Ca^{2+} response in the first mins after stimulation was delayed and the cells lacking either P2X4 or P2X7 showed only 16% or 19% of the time to reach 200 nM Ca^{2+} compared to the WT. The translocation of the nuclear factor NFAT-1 was decreased in *P2rx4^{-/-}* and *P2rx7^{-/-}* T cells by 71% and 81% after mins, whereas cytokine expression of IFN- γ was decreased by 52% and 67%. Only in CD8⁺ T cells from *P2rx4^{-/-}* mice, the number of TNF- α expressing cells was significantly reduced by 33%. Furthermore, the proliferation of the cells was affected by a reduction of 15% and 25% compared to WT cells. (Created with BioRender.com).

P2X7 is associated with the generation of memory cells following acute infections (40). Recently, it was shown that the ATP-releasing channel pannexin-1 promotes CD8⁺ T cell effector and memory responses through several intracellular pathways (41).

In the current study, we not only emphasized one aspect of T cell activity but tried to understand the whole cascade from initial to downstream responses. Thus, as the next step in the signaling cascade (after the formation of initial Ca²⁺ microdomains), we analyzed the global Ca²⁺ response in CD8⁺ T cells from *P2rx4*^{-/-} and *P2rx7*^{-/-} mice. The overall global Ca²⁺ response looked quite similar between WT and both knockout cells. The signal onset velocity was significantly reduced in T cells from *P2rx4*^{-/-} mice, whereas T cells from *P2rx4*^{-/-} and *P2rx7*^{-/-} mice showed delayed responses to reach 200 nM Ca²⁺. CD4⁺ WT T cells respond to anti-CD3⁺ within approx. 115 s to reach 200 nM Ca²⁺ (20), whereas CD8⁺ WT T cells showed slower responsiveness of approx. 140 s (Figure 3C). The delayed response observed in CD8⁺ T cells could be attributed to a higher activation threshold, which serves to avoid inappropriate activation and subsequent tissue damage resulting from their cytotoxicity (42). The strength and duration of a Ca²⁺ signal can shape the cellular response and determine between a certain proliferation program or cell death. Cytosolic and nuclear proteins translate the diverse Ca²⁺ signaling cascade into transcriptional activity (7).

One of these proteins is NFAT-1 (24). After dephosphorylation and translocation into the nucleus, NFAT-1 interacts with various other transcription factors and associated proteins, like activator protein 1 (AP1), forkhead box protein 3 (FOXP3), or Ikaros, in a stimulus-dependent manner. This cooperative DNA binding subsequently shapes immune cell response, like T cell differentiation or proliferation (43) and transcription of numerous genes, e.g. IFN- γ gene (IFNG) (28) and TNF- α (TNF) (31). Previous studies reported the impact of eATP and the P2X7 receptor on NFAT activation in Jurkat T cells and rat pheochromocytoma (PC12) cells (19, 44). Thus, we analyzed NFAT-1 translocation after TCR stimulation in CD8⁺ T cells from *P2rx4*^{-/-} and *P2rx7*^{-/-} mice (Figure 2). Strikingly, no or just a slight increase in NFAT-1 translocation was observed for T cells from *P2rx4*^{-/-} and *P2rx7*^{-/-} mice compared to the WT T cells after 7.5 min and 15 min of bead stimulation (Figure 4B). These results highlight the role of the purinergic channels P2X4 and P2X7 on the activation of CD8⁺ T cells by influencing not only the initial Ca²⁺ signals but also the translocation of the transcription factor NFAT-1.

Activated CD8⁺ T cells start to produce TNF- α and IFN- γ after TCR stimulation. We observed an increase in TNF- α expression of 17% of WT CD8⁺ T cells after 4h of stimulation and an increase IFN- γ expression of WT CD8⁺ T cells (20%) after 18 h of stimulation. The increase in CD8⁺ T cells expressing IFN- γ is regulated at the transcriptional level (45) and IFN- γ as well as TNF- α production is dependent on NFAT-1 (27, 31). The current study also highlights this relationship between NFAT-1 translocation and the production of pro-inflammatory cytokines. Both, NFAT-1 translocation minutes after TCR stimulation as well as CD8⁺ T cells expressing TNF- α and IFN- γ within hours of stimulation, were significantly reduced in T cells from *P2rx4*^{-/-} and

P2rx7^{-/-} mice (Figures 4, 5). The synthesis of granzyme B is tightly regulated at both transcriptional and translational levels and the transcriptional activation is induced by the stimulation of the T cell receptor as well as co-stimulation with cytokines (46). Furthermore, the release of granzyme B via granule exocytosis at the immunological synapse is dependent on the rises of free cytosolic Ca²⁺ (47). Hence, if the Ca²⁺ response is impaired in T cells from *P2rx4*^{-/-} and *P2rx7*^{-/-} mice this will lead to a decreased expression of granzyme B which is in line with our experiments (Figure 5D).

Moreover, TCR signaling is linked to the immediate expression of the NR4A family of nuclear orphan receptors and their transcripts can be used as biomarkers for TCR signaling (48). One member of the NR4A family is Nur77 (NR4A1). Nur77 is a negative transcriptional regulator of T cell activation, cell cycle progression, and proliferation that is upregulated within hours of TCR signaling (48–50). Therefore, Nur77 is an ideal tool for analyzing TCR signaling-dependent differences in the first hours after stimulation in our WT and knockout T cells. In line, we observed significantly reduced Nur77 expression in T cells from *P2rx4*^{-/-} and *P2rx7*^{-/-} mice 18h after stimulation. It is important to note that this only refers to TCR-induced Nur77 expression, as basal expression is unchanged in CD8⁺ T cells from WT mice compared to those from *P2rx4*^{-/-} and *P2rx7*^{-/-} mice.

T cell proliferation is regulated by various transcription factors, like NFAT, AP-1, NFkB, or Nur77 (45, 51, 52). We showed a significantly decreased proliferation in CD8⁺ T cells from *P2rx4*^{-/-} and *P2rx7*^{-/-} mice (Figure 5C). Previous reports indicate the influence of the duration and amplitude of Ca²⁺ signals on the activation of the pro-inflammatory transcriptional regulators NFAT, AP-1, NFkB (52). Further reports again highlighted the role of short-lived local Ca²⁺ signals on the translocation of NFAT-1 (53, 54). Here, we clearly demonstrate the association of Ca²⁺ signaling, transcription factor, and cytokine expression with the proliferation of CD8⁺ T cells. Impaired Ca²⁺ microdomain formation in *P2rx4*^{-/-} and *P2rx7*^{-/-} CD8⁺ T cells directly after T cell stimulation results in significantly delayed global Ca²⁺ signaling (Figure 3C), while the overall Ca²⁺ response was not changed (Figure 3D). Furthermore, in cells lacking either P2X4 or P2X7 channel NFAT-1 translocation several minutes after TCR stimulation and TNF- α , IFN- γ , Nur77 and granzyme B expression were impaired within hours of stimulation and, finally, slightly reduced proliferation of these cells after days was demonstrated (Figure 6). Previous work has discussed the importance of the duration and amplitude of a Ca²⁺ signal to the downstream response. While it is claimed that oscillation frequency influences gene transcription of certain transcription factors (52, 55) and NFAT-1 translocation depends on short-lived Ca²⁺ microdomains (53), the proliferation of cells is controlled by a full and global Ca²⁺ signal (56). This is in line with our data. In cells lacking either P2X4 or P2X7 channels, there are profound effects on Ca²⁺ microdomain formation, affecting NFAT-1 translocation as well as TNF- α and IFN- γ expression, whereas the global Ca²⁺ response is only slightly affected and, consequently, cell proliferation.

Although the affinities of P2RX4 and P2RX7 for eATP are different, with P2RX4 having a higher affinity (1–10 μ M EC₅₀) as P2RX7 (>100 μ M EC₅₀) for ATP (57), the knock-out of either

receptor still had similar outcomes. The effects of P2RX4 were greatest on initial Ca^{2+} signals, which could be explained by the higher affinity for ATP especially during the first milliseconds to seconds after TCR stimulation, NFAT-1, which is known to be dephosphorylated upon local Ca^{2+} rises, and TNF- α expression. So, in line with small local rises in ATP trigger Ca^{2+} responses and NFAT translocation. In contrast, P2RX7 has stronger effects in IFN- γ and granzyme B expression as well as proliferation, which is at a time point (18h to 72h) where higher eATP concentrations are present due to release from activated immune cells (57) and/or dying of virus-infected cells (58). Previously we described that there is co-localization of P2RX4 and P2RX7 in CD4^+ T cells at basal state which is significantly increased 5 mins after TCR stimulation (21) hinting to the possible formation of heterotrimers. The formation of heterotrimers in CD4^+ T cells and hence possibly also in CD8^+ T cells could explain these overall quite similar effects in T cells from *P2rx4^{-/-}* and *P2rx7^{-/-}* mice. If either of the two receptors is absent possibly no fully functional receptors are expressed in CD8^+ T cells from *P2rx4^{-/-}* and *P2rx7^{-/-}* mice.

Taken together, our data suggest that purinergic signaling enhances the initial Ca^{2+} events during CD8^+ T cell activation and plays an essential role in the refinement of the downstream effector function, providing advanced possibilities for clinical interventions, for example, in the tumor microenvironment.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the regulatory committee (Hamburger Behörde für Gesundheit und Verbraucherschutz, Veterinärwesen/Lebensmittelsicherheit, ORG 941 and 1115) which included tissues of animal origin. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

VB: Conceptualization, Data curation, Validation, Visualization, Writing – original draft, Writing – review & editing, Formal analysis, Investigation, Methodology. NL: Formal analysis, Investigation, Writing – review & editing. FM: Formal analysis, Investigation, Visualization, Writing – review & editing. MB: Formal analysis, Investigation, Writing – review & editing. TS: Formal analysis, Investigation, Writing – review & editing. L-MW: Methodology, Software, Writing – review & editing. MJ: Investigation, Writing – review & editing. JH: Supervision, Project administration, Funding acquisition, Writing – review & editing. FK-N: Funding acquisition, Project administration, Supervision, Writing – review & editing. BR:

Funding acquisition, Project administration, Supervision, Writing – review & editing. H-WM: Funding acquisition, Project administration, Supervision, Writing – review & editing. AG: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. RW: Funding acquisition, Project administration, Software, Supervision, Writing – review & editing. B-PD: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1258119/full#supplementary-material>

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2.3 Publication III: MASTER-NAADP: a membrane permeable precursor of the Ca^{2+} mobilizing second messenger NAADP

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MASTER-NAADP: a membrane permeable precursor of the Ca^{2+} mobilizing second messenger NAADP

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Upon stimulation of membrane receptors, nicotinic acid adenine dinucleotide phosphate (NAADP) is formed as second messenger within seconds and evokes Ca^{2+} signaling in many different cell types. Here, to directly stimulate NAADP signaling, MASTER-NAADP, a Membrane permeable, STabilized, bio-rEversibly pRotected precursor of NAADP is synthesized and release of its active NAADP mimetic, benzoic acid C-nucleoside, 2'-phospho-3'-F-adenosine-diphosphate, by esterase digestion is confirmed. In the presence of NAADP receptor HN1L/JPT2 (hematological and neurological expressed 1-like protein, HN1L, also known as Jupiter microtubule-associated homolog 2, JPT2), this active NAADP mimetic releases Ca^{2+} and increases the open probability of type 1 ryanodine receptor. When added to intact cells, MASTER-NAADP initially evokes single local Ca^{2+} signals of low amplitude. Subsequently, also global Ca^{2+} signaling is observed in T cells, natural killer cells, and Neuro2A cells. In contrast, control compound MASTER-NADP does not stimulate Ca^{2+} signaling. Likewise, in cells devoid of HN1L/JPT2, MASTER-NAADP does not affect Ca^{2+} signaling, confirming that the product released from MASTER-NAADP is a bona fide NAADP mimetic.

Nicotinic acid adenine dinucleotide phosphate (NAADP) was discovered as an impurity of commercial NADP preparations¹ and turned out to be the most potent Ca^{2+} mobilizing 2nd messenger known to date (reviewed in refs. 2–5). NAADP is formed rapidly within seconds

upon stimulation of plasma membrane receptors^{6–8}. Currently, two major models for formation of NAADP exist: either NAADP is formed by the 'base-exchange' reaction through either CD38⁹ or SARM1¹⁰, or via oxidation of its reduced derivative NAADPH¹¹. The base-exchange

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reaction catalyzed by CD38 requires special conditions, e.g. acidic pH and excess of nicotinic acid⁹, although it was recently reported that SARM1 may catalyze the same reaction also at neutral pH¹². Further, the acidic pH present in the endo-lysosomal lumen allows for NAADP formation by CD38 using NADP and NAAD as substrates to generate NAADP via the base-exchange reaction. Solid evidence for this endo-lysosomal model of NAADP production was obtained in lymphokine activated killer cells stimulated by interleukin-8¹³. Recently, NAADPH oxidation by plasma membrane spanning NADPH oxidases was demonstrated as alternative pathway of NAADP generation; in contrast to the endo-lysosomal model, this process proceeds at neutral pH in the cytosol just below the internal leaflet of the plasma membrane and requires both NAADPH and O₂ as substrates¹¹.

The Ca²⁺ release mechanism operated by NAADP requires NAADP receptor/NAADP binding proteins, as demonstrated by Lin-Moshier et al.¹⁴ and Walseth et al.¹⁵ Based on these results the unifying hypothesis that links NAADP to activation of Ca²⁺ channels was published¹⁶. Two such NAADP receptors/NAADP binding proteins were recently identified as HN1L/JPT2^{17,18} or Lsm12¹⁹. These NAADP receptors/NAADP binding proteins activate different ion channels, e.g. type 1 ryanodine receptor (RYR1)¹⁷, two-pore channel 1 (TPC1)¹⁸, or TPC2¹⁹, resulting in release of Ca²⁺ either from endoplasmic reticulum (ER) through RYR1 or from endo-lysosomes through TPC1 and/or TPC2. Primary release of Ca²⁺ via these NAADP-directed mechanisms results in local Ca²⁺ release, e.g. Ca²⁺ microdomains in T cells^{20,21}. However, very rapid amplification mechanisms enlarge the initial local signals finally resulting in global Ca²⁺ signaling; among these amplification mechanisms are (i) Ca²⁺ induced Ca²⁺ release through RYR²², (ii) enhancement of IP₃ evoked Ca²⁺ release through IP₃Rs²³, (iii) store-operated Ca²⁺ entry (SOCE)²¹, or amplification via purinergic signaling involving purinergic P2X4 and P2X7²⁴.

To study NAADP signaling, a couple of chemical biology and pharmacological tools have been developed. Ned-19²⁵ and BZ194²⁶ were both introduced in 2009 as NAADP antagonists and have been used to block signaling of endogenously formed NAADP in different cell types. The known Ca²⁺ mobilizing second messengers, including NAADP, are charged at physiological pH and thus are not membrane-permeant. Therefore, to study effects of NAADP directly in intact cells, microinjection²⁰ or infusion via patch-clamp pipette²⁷ must be carried out. However, these methods are laborious and have disadvantages, e.g. the risk to contaminate the injected volume by traces of extracellular buffer containing high Ca²⁺ concentration during microinjection, or dilution of relevant cytosolic proteins into the patch-clamp pipette in whole-cell mode measurements. Thus, NAADP-acetoxymethyl ester (NAADP-AM) was synthesized and characterized as membrane permeant precursor for NAADP^{28,29}. NAADP-AM was, among other cell types, successfully used to evoke global Ca²⁺ signals in cytotoxic T cells³⁰.

Since it is known that acetoxymethylation of pyrophosphate moieties in small molecules such as dinucleotides may result in unstable products³¹, here we develop a Membrane permeable, STabilized, bio-reversibly pRotected precursor of NAADP (MASTER-NAADP). Synthesis and full chemical characterization of MASTER-NAADP and a similar lipophilic precursor for the inactive NADP, MASTER-NADP, are described. Further, MASTER-NAADP and MASTER-NADP are evaluated regarding their ability to evoke local and/or global Ca²⁺ signaling in T cells, natural killer cells, and a neuronal cell line. In addition, the deprotected MASTER compounds are directly characterized regarding biological activity in permeabilized T cells and by lipid planar bilayer measurements using RYR1.

Results

Synthesis of MASTER-NAADP

First, the “southern” adenosine-2',5'-phosphate derivative was prepared. Xylose **1** (Fig. 1) was first protected in the 1,2-position with an

isopropylidene group (99%). In solution xylose **1** exists in the thermodynamically favored pyranose form over the furanose form. This equilibrium can be shifted if the sugar is forced into the furanose form by the introduction of the isopropylidene protecting group³². The 5'-protecting group was introduced following standard reaction conditions using benzoyl chloride³³. To obtain the fluorinated compound **5**, the isopropylidene group was cleaved and the 1'-position was protected with a methoxy group (86%). Next, the 3'-position was fluorinated with diethylaminosulfur trifluoride (DAST). Instead of using 6.0 eq. as described before³⁴, the fluorination was carried out by adding only 1.5 eq. of DAST because using more than 1.5 eq. did not result in increased product formation. Acetyl protecting groups were introduced according to literature procedures to obtain the desired β-configured product **7**³⁵ in the following step. The *N*-glycosylation was carried out according to the Vorbrüggen method³⁶ following by ester cleavage of the protecting groups under basic conditions, yielding compound **8**. After the 5'-position was *tert*-butyldimethylsilyl (TBDMS)-protected, the bisacyloxybenzyl (AB)-masked phosphate group was introduced into the 2'-position. Therefore, the bis(AB)-phosphoramidite **12** was synthesized starting from 4-hydroxyl benzyl alcohol **10** which was first converted into acyloxybenzyl alcohol **11**. Phosphoramidite **12** was prepared using the alcohol **11** and subsequently reacted with the 2'-position using acid-catalysis³⁷ to give the silyl-protected 2'-phosphate **13** in a good yield of 87%³⁸. After cleavage of the TBDMS group with triethylamine-trihydrofluoride to give the 2'-phosphate **14**, the monophosphorylation was carried out by the Yoshikawa phosphorylation method to yield the 2',5'-diphosphate **15**³⁹.

The “northern” C-nucleoside **19** was synthesized according to Krohn et al.⁴⁰ (Fig. 2). The Yamaguchi esterification of the carboxylic acid **19**⁴¹ was carried out using the carbonate mask **22**. After complete debenzoylation, the monophosphorylation was carried out at the 5'-position of the carbonate-masked C-nucleoside **24** (42%)³⁹. MASTER-NAADP **26** was successfully obtained in a yield of 57 % by coupling the two monophosphates **25** and **15**⁴².

Synthesis of MASTER-NADP

The synthesis of the amide comprising C-nucleoside monophosphate **32** (“northern” part) follows the synthesis route of Supplementary Fig. 1. In contrast to the esterification step (Fig. 2A), the amide **30** is synthesized using HBTU⁴³.

Supplementary Fig. 2 summarizes the synthesis of the “southern” building block **40** and the NADP-derivative **42**. The 5'- and 3'-hydroxyl group of adenosine **35** were protected with the tetra-isopropylidisiloxan-1,3-diyl (TIPDS) group⁴⁴ to allow selective phosphoramidite coupling at the 2'-position to obtain compound **38**³⁸. The synthesis of the masking moiety is analogous to the phosphoramidite protocol in Fig. 1³⁷. To prevent 2'- to 3'-phosphate group migration and to allow subsequent coupling to the NADP-derivative **40**, the 5'-position was selectively deprotected⁴⁵. Direct monophosphorylation of the 5'-position was not possible because a competing reaction to the silanol phosphate was observed. Therefore, the 5'-*H*-phosphonate (**39**; 89%) was synthesized first, which was oxidized to give the monophosphate **40** in a yield of 99%⁴⁶. By coupling the two monophosphates **32** and **40**, the protected MASTER-NADP **41** was obtained in a yield of 64% (Supplementary Fig. 2)⁴². To obtain the NADP-derivative **42**, the silyl protecting group at the 3'-position was cleaved off using tris(dimethylamino)sulfonium difluorotrimethylsilicate (TASF)⁴⁷.

Deprotected MASTER-NAADP releases Ca²⁺ from permeabilized cells and activates RYR1 channel opening in lipid planar bilayers

The dinucleoside diphosphate liberated by endogenous esterase from MASTER-NAADP is a very close derivative of NAADP, benzoic acid C-nucleoside, 2'-phospho-3'-F-adenosine-diphosphate. In fact, the major product of *in vitro* digestion of MASTER-NAADP by porcine liver esterases was purified by HPLC and identified by mass spectrometry as

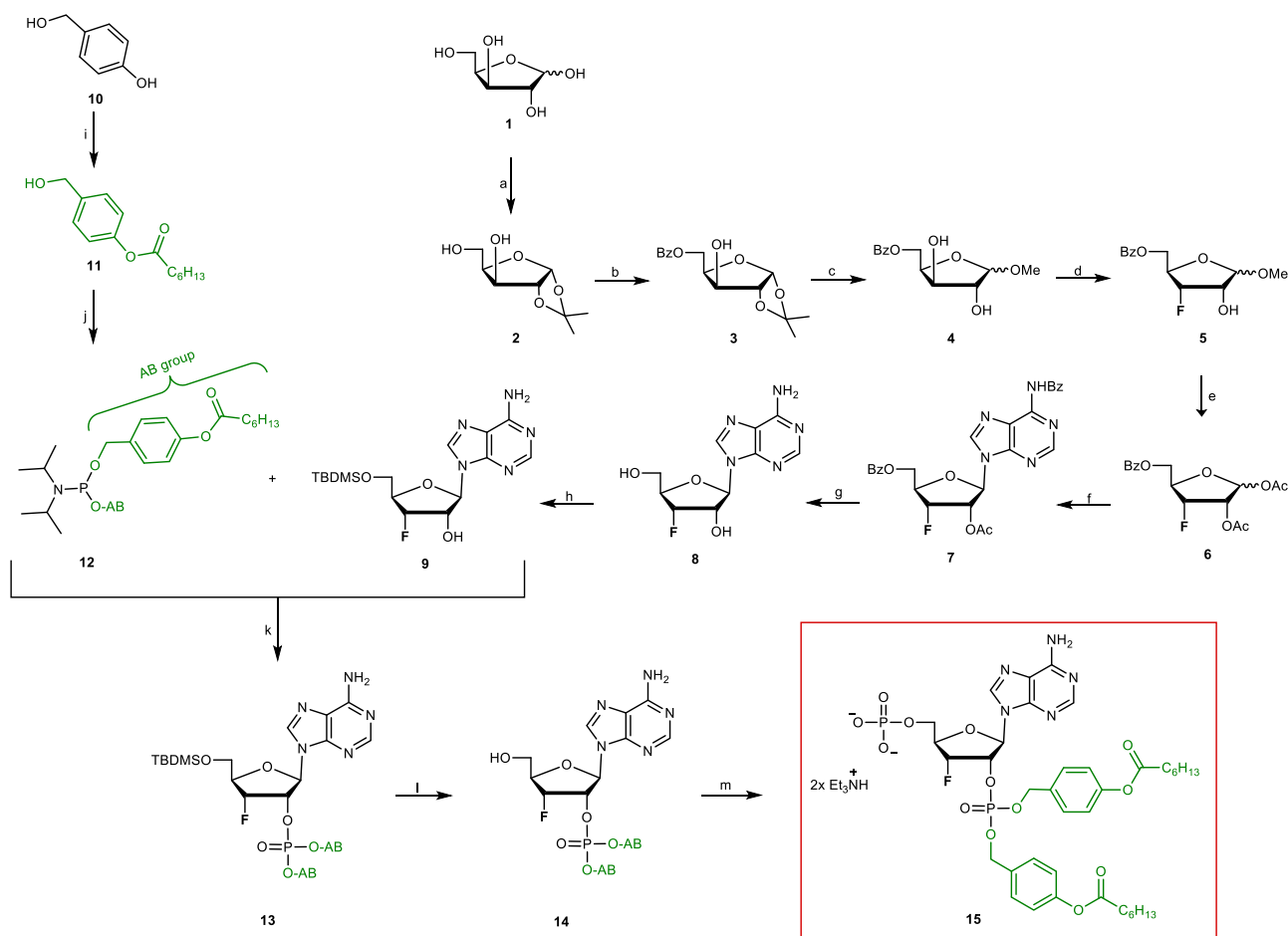


Fig. 1 | Synthesis of MASTER-NAADP and derivatives | Reagents and conditions.

[a] (i) 0.10 eq. CuSO_4 , 2.8 eq. conc. H_2SO_4 , acetone, 3 h, rt, (ii) HCl-solution (0.2 %), 6 h, rt, 99 %; [b] 1.5 eq. Et_3N , 1.1 eq. BzCl , CH_2Cl_2 , 1 h, 0 °C, 92 %; [c] 0.2 eq. I_2 , MeOH, 4 h, 70 °C, 86 %; [d] 1.5 eq. DAST, dry CH_2Cl_2 , 20 h, -78 °C $\rightarrow$ rt, 43 %; [e] 4.7 eq. Ac_2O , 4.7 eq. conc. H_2SO_4 , AcOH, 19 h, rt, 93 %; [f] (i) 1.5 eq. *N*-benzoyladenine, 1.2 eq. BSA, $\text{C}_2\text{H}_4\text{Cl}_2$, 1 h, 90 °C, (ii) 1.0 eq. **6**, 4.0 eq. TMSOTf, $\text{C}_2\text{H}_4\text{Cl}_2$, 5.5 h, 90 °C, 61 %; [g] ammonia 7 N in MeOH, 28 h, 70 °C, 84 %; [h] 1.5 eq. TBDMSCl, dry pyridine, 24 h,

rt, 71 %; [i] 1.2 eq. 4-hydroxyl benzyl alcohol **10**, 1.2 eq. Et_3N , 1.0 eq. heptanoyl chloride, THF, 2 h, 0 °C, 87 %; [j] 1.0 eq. dichloro-*N,N*-diisopropylaminophosphoramidite, 2.2 eq. **11**, 2.3 eq. Et_3N , THF, 0 °C $\rightarrow$ rt, 16 h, 92 %; [k] (i) 1.3 eq. **12**, 1.2 eq. pyridinium trifluoroacetate, CH_2Cl_2 , 50 min, rt, (ii) 1.5 eq. *tert*-butylhydroperoxide (5.0 M in decane), 0 °C, 1 h, 87 %; [l] 5.2 eq. triethylamine-trihydrofluoride, CH_2Cl_2 , 19 h, rt, 81 %; [m] 4.0 eq. POCl_3 , TMP, 6.5 h, 0 °C, 31 %.

benzoic acid C-nucleoside, 2'-phospho-3'-F-adenosine-diphosphate (deMASTER-NAADP; Fig. 2B). The NAADP derivative released from MASTER-NAADP was also obtained by in vitro digestion using porcine liver esterases and eluted as only partially resolved double peak that represents the 2'- and 3'-phosphate isomers of benzamide C-nucleoside, phospho-adenosine-diphosphate (deMASTER-NAADP)^{48,49} (Supplementary Fig. 3A). deMASTER-NAADP lacks the essential 3-carboxyl group at the benzoic acid moiety (which replaces the nicotinic acid moiety here) and should not mimic NAADP's Ca^{2+} releasing activity. Thus, only the expected NAADP mimic deMASTER-NAADP contains all structural moieties reported to be crucial for biological activity of NAADP, the 3-carboxyl group of nicotinic acid, the 6-amino group and the 2'-phosphate of the adenosine moiety⁵⁰. Further, modeling of deMASTER-NAADP and its overlay with NAADP indicate very similar overall architecture of NAADP and its synthetic mimic released from MASTER-NAADP (Fig. 2C).

The deprotected MASTER compounds were then validated in two systems: (i) Ca^{2+} release in saponin-permeabilized Jurkat T cells, and (ii) current recordings in planar lipid bilayers containing RYR1 and NAADP receptor HN1L/JPT2 (Fig. 3). In the absence of exogenous addition of recombinant HN1L/JPT2 neither natural NAADP, nor deMASTER-NAADP, nor deMASTER-NAADP released Ca^{2+} (Fig. 3A). In contrast, in the presence of HN1L/JPT2, both NAADP and deMASTER-

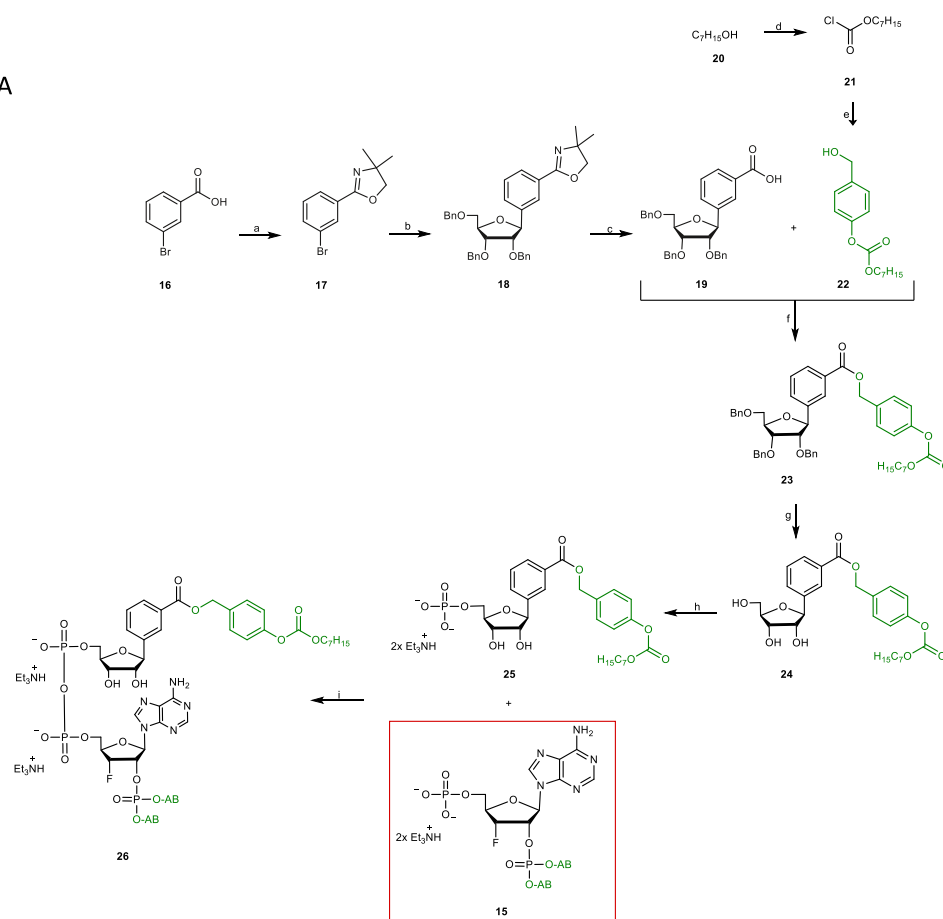
NAADP released Ca^{2+} , whereas deMASTER-NAADP or NAADP did not (Fig. 3B). In line with NAADP's target Ca^{2+} channel in T cells, RYR1^{14,17,18,23}, NAADP (Fig. 3C) or deMASTER-NAADP (Fig. 3D) evoked Ca^{2+} release in a Ca^{2+} dependent fashion. Further, RYR1 open probability (P_o) was determined in lipid planar bilayers containing highly purified RYR1 (Fig. 3E,F). Whereas 100 nM deMASTER-NAADP alone only slightly elevated P_o , markedly increased RYR1 channel opening was observed upon combination of deMASTER-NAADP and recombinant HN1L/JPT2 (Fig. 3E, F). In contrast, deprotected control compound deMASTER-NAADP in combination with recombinant HN1L/JPT2 did not increase P_o (Fig. 3F).

MASTER-NAADP evokes Ca^{2+} microdomains

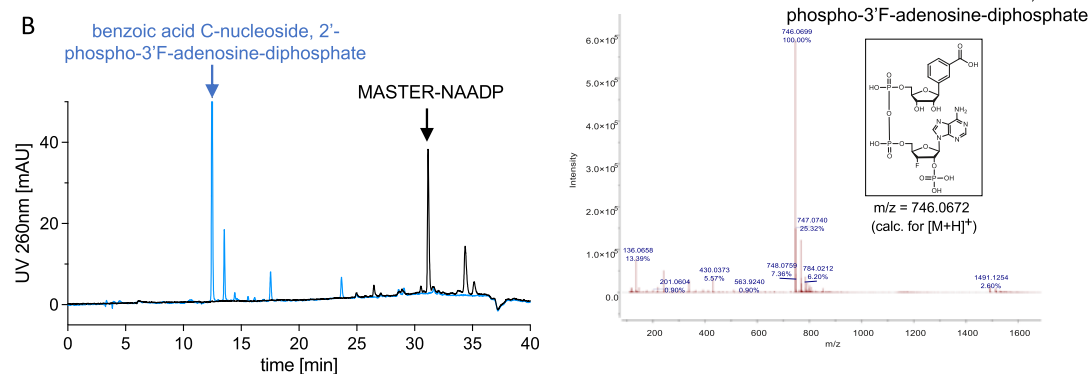
Next, we compared the two lipophilic precursors MASTER-NAADP and MASTER-NAADP regarding their Ca^{2+} mobilizing properties in different cell types.

In many cell types, NAADP has been shown, or at least has been assumed, to be involved in initializing Ca^{2+} signaling processes; in fact, increases of endogenous NAADP concentrations within few seconds upon stimulation of plasma membrane receptors were reported^{6–8}. Thus, Ca^{2+} microdomains were recorded as described previously²¹, within approx. 60 s upon extracellular addition of MASTER-NAADP or MASTER-NAADP (Fig. 4). In Jurkat WT T cells MASTER-NAADP evoked

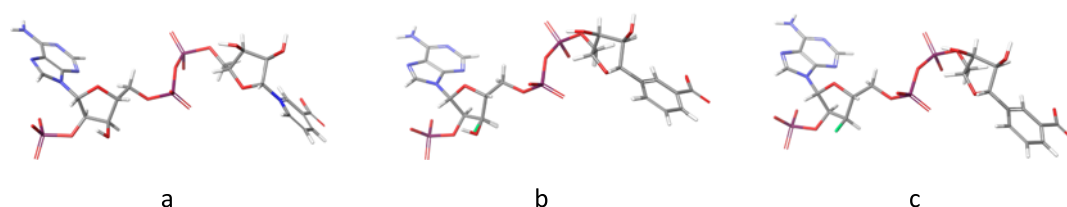
A



B



C



Ca²⁺ microdomains that initially were observed as single low amplitude signaling events (Figs. 4A, 2.5–3.8s); within few more seconds these locally resolved Ca²⁺ signals merged into a global signal (Figs. 4A, 7.5s). In contrast, control compound MASTER-NADP did not stimulate local Ca²⁺ signals in the majority of cells (Fig. 4B). A statistically significant increase of Ca²⁺ microdomains was observed for MASTER-NADP when compared to inactive MASTER-NADP (Fig. 4C). Further, in Jurkat T cells lacking expression of the NAADP receptor/binding protein

HN1L/JPT2^{17,18} only background signals for both MASTER-NADP or MASTER-NADP were recorded (Fig. 4C; Supplementary Fig. 4A, B); similar background signals were observed upon addition of vehicle DMSO (Fig. 4C). Quantitative evaluation of the spatio-temporal pattern of Ca²⁺ microdomains was obtained by averaging Ca²⁺ microdomain data in spatial segments of the cell resembling a dart board, originating from single cells stimulated under identical conditions, as described in ref. 51. For WT Jurkat T cells, a clear difference is observed between

Fig. 2 | Synthesis of MASTER-NAADP and derivatives II A) Reagents and conditions. [a] Thionylchloride, kat. DMF, 2 h, 80 °C, then 2.0 eq. amino-2-methyl-1-propanol, CH₂Cl₂, 2 h, 0 °C → rt, thionylchloride, 16 h, rt, 87 %; [b] 1.5 eq. **17**, 1.6 eq. n-BuLi (1.6 M in hexane), THF, 1 h, -78 °C, then 1.0 eq. 2,3,5-tri-*O*-benzyl-ribo-nolactone, THF, 2 h, -78 °C → -30 °C, 3.0 eq. triethylsilane, 2.5 eq. BF₃·Et₂O, CH₂Cl₂, 16 h, -78 °C → rt, 41 %; [c] CH₃NO₂/MeI (2:1) 16 h, reflux, then MeOH/KOH 20 %, 1:1, 16 h, reflux, quantitatively; [d] 0.33 eq. triphosgene, 1.0 eq. pyridine, CH₂Cl₂, 15 h, rt, 93 %; [e] 1.1 eq. 4-hydroxyl benzyl alcohol **10**, 1.0 eq. Et₃N, 1.0 eq. **21**, CH₂Cl₂, 15 min, 0 °C, then 3 h, rt, 74 %; [f] 1.0 eq. **19**, 1.1 eq. Yamaguchi-reagent, 1.2 eq. Et₃N, 1.2 eq. **22**, 0.3 eq. DMAP, THF, 3 h 70 °C, 16 h, rt, 58 %; [g] 3.5 eq. BCl₃ (1 M, CH₂Cl₂), 2 h, -78 °C, 67 %; [h] 2.0 eq. POCl₃, 2.5 eq. Bu₃N, trimethylphosphate, 16 h, 0 °C, 42 %. [i] 10.0 eq. TFAA, 16 eq. Et₃N, 1.0 eq. monophosphate **25**, CH₃CN, 10 min, 0 °C → Rt,

then 6.0 eq. NMI, 10 eq. Et₃N, CH₃CN, 10 min, 0 °C → rt, then 1.1 eq. **15**, CH₃CN, 3 h, rt, then TEAB-buffer 1 M, 15 min, rt, 57%. B) Release of MASTER-NAADP by porcine liver extract and mass spec characterization of main product. **B** MASTER-NAADP was analyzed by RP-HPLC for purity (black chromatogram). A representative out of 6 experiments is shown. Then, reaction products obtained upon incubation of MASTER-NAADP with porcine liver extract were separated by RP-HPLC (blue chromatogram). The main product was then identified by ESI-mass spectrum measured in positive ionization mode as benzoic acid C-nucleoside, 2'-phospho-3'-F-adenosine-diphosphate. **C** Molecular modeling: (a) NAADP, (b) overlay of NAADP and benzoic acid C-nucleoside, 2'-phospho-3'-F-adenosine-diphosphate, (c) benzoic acid C-nucleoside, 2'-phospho-3'-F-adenosine-diphosphate.

cells treated with vehicle DMSO, MASTER-NAADP and control compound MASTER-NADP (Supplementary Fig. 5A). *Hn1l/Jpt2*^{-/-} Jurkat T cells reacted to MASTER-NAADP in a comparable fashion as for MASTER-NADP in WT cells (Supplementary Fig. 5A). To demonstrate the specific effects of MASTER-NAADP over the first 15 s, unspecific signals evoked by MASTER-NADP were subtracted segment-wise (Supplementary Fig. 5B). Initial signals induced by MASTER-NAADP were observed in the center of the cells (Supplementary Fig. 5B, 1–3s), but rapidly spread to more peripheral parts of the cells (Supplementary Fig. 5B, 4–6s), followed by a phase of more even distribution of the Ca²⁺ microdomains throughout the cells (Supplementary Fig. 5B, 7–11s). Regarding kinetics, Ca²⁺ microdomains increased significantly over 30 s in WT Jurkat T cells upon stimulation by MASTER-NAADP, as compared to control conditions (Supplementary Fig. 6). In addition to the Jurkat T-lymphoma cell line, in naïve murine T cells, localized Ca²⁺ signals were also observed upon MASTER-NAADP addition (Fig. 4D). The number of Ca²⁺ microdomains was significantly higher as compared to MASTER-NADP addition (Fig. 4D, E), demonstrating effectiveness of MASTER-NAADP also in primary T cells. MASTER-NAADP was also assessed in KHYG-1 natural killer (NK) cells. Similar to Jurkat T cells, rapid local Ca²⁺ signals were observed (Fig. 4F); as for the other two cell types tested, the number of Ca²⁺ microdomains evoked by MASTER-NADP was significantly lower (Fig. 4G). As for Jurkat T cells (Supplementary Fig. 5), spatio-temporal analysis of Ca²⁺ microdomains using the dartboard representation was also conducted for KHYG-1 cells, demonstrating clearly elevated numbers of Ca²⁺ microdomains over the first 15 s upon MASTER-NAADP vs MASTER-NADP (Supplementary Fig. 7A). The corresponding subtraction plots demonstrate initial Ca²⁺ microdomains confined to a ring-like structure in the cytosol (Supplementary Fig. 7B, 1–4s). Then, Ca²⁺ microdomains were also observed close to the plasma membrane and in the inner part of the cell (Supplementary Fig. 7B, 5–9s), followed by a more even distribution of Ca²⁺ microdomains across the whole cell (Supplementary Fig. 7B, 10–15s).

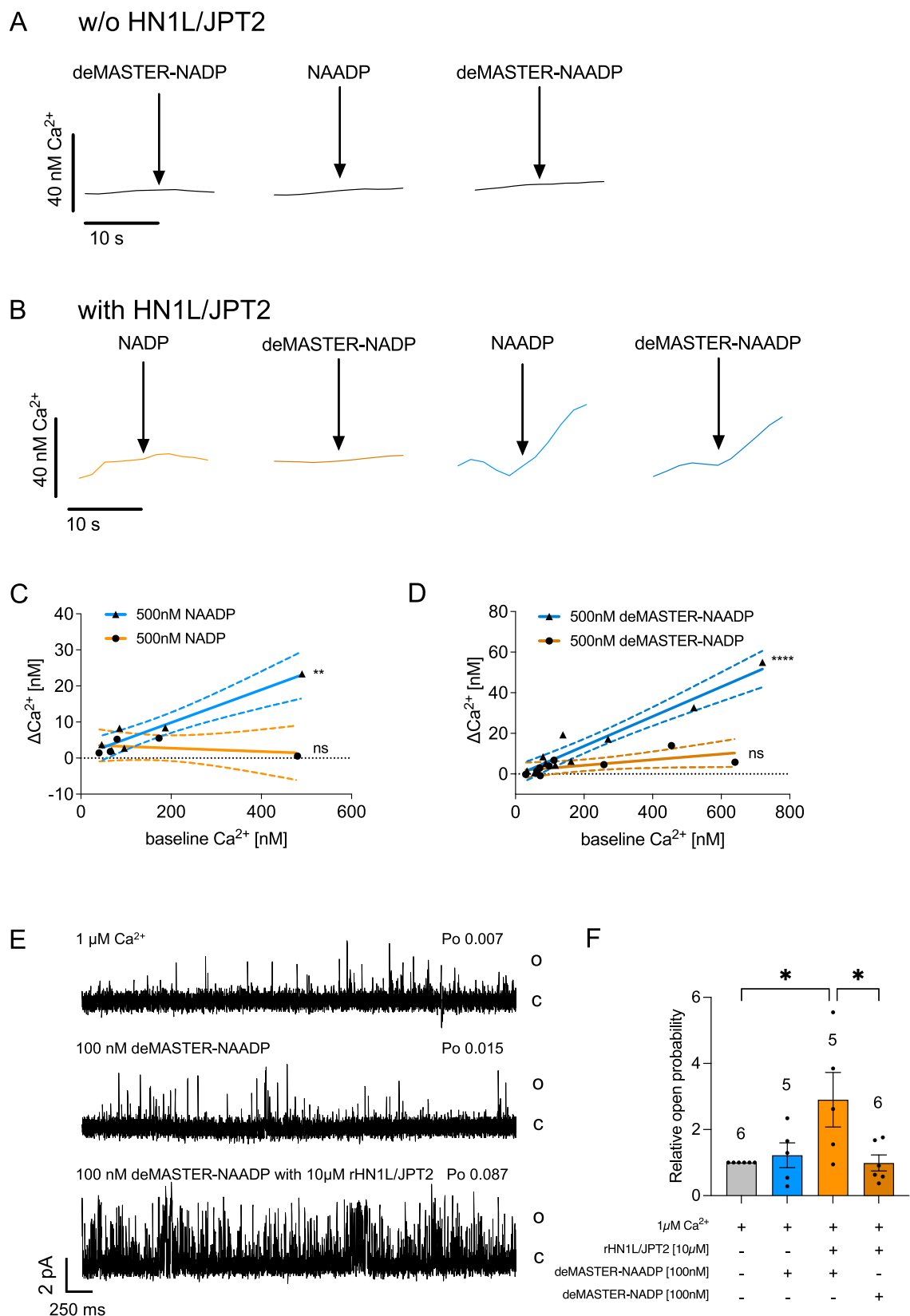
In addition to T cells and NK cells, the neuronal cell line Neuro2A was stimulated with both MASTER-NAADP or MASTER-NADP (Fig. 4H, I). MASTER-NAADP stimulated local Ca²⁺ signals; soon after an almost global, but transient Ca²⁺ signal was observed (Fig. 4H). In T cells and NK cells, MASTER-NAADP evoked an approx. 3-fold increase of the number of Ca²⁺ microdomains over background (MASTER-NADP or DMSO). Of note, in Neuro2A cells MASTER-NAADP stimulated an approx. 10-fold increase of the number of Ca²⁺ microdomains, as compared to MASTER-NADP (Fig. 4I). *Hn1l/Jpt2*^{-/-} Neuro2A cells neither responded to MASTER-NAADP nor to MASTER-NADP (Supplementary Fig. 4C, D), confirming the data obtained in Jurkat WT vs *Hn1l/Jpt2*^{-/-} T cells (Fig. 4A vs Supplementary Fig. 4A). The absence of any Ca²⁺ microdomains above background in *Hn1l/Jpt2*^{-/-} cells, together with direct effects of deprotected MASTER-NAADP shown in Fig. 3, strongly suggests that the NAADP derivative liberated from MASTER-NAADP, benzoic acid C-nucleoside, 2'-phospho-3'-F-adenosine-diphosphate, is a bona fide Ca²⁺ mobilizing NAADP mimetic acting through the NAADP receptor/NAADP binding protein HN1L/JPT2^{17,18}, while benzamide

adenine dinucleotide phosphate released from MASTER-NADP does not mimic NAADP.

Global Ca²⁺ signaling stimulated by MASTER-NAADP

In addition to local Ca²⁺ signaling observed within a few seconds upon extracellular addition of MASTER-NAADP (Fig. 4), MASTER-NAADP also evoked global Ca²⁺ signaling in Jurkat T cells (Fig. 5). In these experiments Synta66 was added to block SOCE. Due to the lack of SOCE via Orail channels, global Ca²⁺ signals evoked by MASTER-NAADP were often of transient nature (Fig. 5A left). The control compound MASTER-NADP stimulated only negligible Ca²⁺ signals in a minority of cells (Fig. 5A middle). Commercially available NAADP-AM was also tested in Jurkat T cells for its Ca²⁺ mobilizing activity as these cells have been shown in many studies to utilize NAADP as initial Ca²⁺ mobilizing 2nd messenger during T cell receptor (TCR)/CD3 evoked Ca²⁺ signaling^{11,17,20,21} and show Ca²⁺ signals upon microinjection of NAADP^{20,26,52–54}. Upon addition of NAADP-AM under identical conditions as for MASTER-NAADP (100 nM final concentration), similar results as for control compound MASTER-NADP were obtained (Fig. 5A). As for the Ca²⁺ microdomain data in Fig. 4, also for analysis of global Ca²⁺ signals *Hn1l/Jpt2*^{-/-} Jurkat T cells were employed; both MASTER-NAADP and MASTER-NADP evoked only few minor and transient Ca²⁺ signals in *Hn1l/Jpt2*^{-/-} Jurkat T cells (Fig. 5B left, middle), similar to the Ca²⁺ signals observed in WT cells stimulated by the control compound MASTER-NADP (Fig. 5A middle). Similar results were obtained for NAADP-AM (Fig. 5B right). When comparing the Ca²⁺ peak amplitudes for the six different conditions, it became clear that the mean amplitudes of Ca²⁺ transients were not significantly different (Fig. 5C). However, MASTER-NAADP evoked a significantly higher number of global Ca²⁺ oscillations as compared to MASTER-NADP, while in *Hn1l/Jpt2*^{-/-} T cells no global Ca²⁺ oscillations above background were observed (Fig. 5D). In addition, a similar situation as shown in Fig. 5D was observed for the percentage of responding cells (Fig. 5E). Further, as a more impactful parameter for the Ca²⁺ mobilizing activity, the 'mean responsiveness', was defined as product of peak number and amplitude. The mean responsiveness demonstrates that only MASTER-NAADP shows superior stimulation of Ca²⁺ signaling vs all other control conditions and also vs NAADP-AM (Fig. 5F).

Since NAADP-AM has been previously used over a wide concentration range^{30,55–58}, also higher concentrations of NAADP-AM were applied to Jurkat T cells, with no detectable Ca²⁺ signaling above background (vehicle DMSO) (Supplementary Fig. 8). Two different lots of NAADP-AM were used to exclude that a single lot was decomposed: lot #280181 shown as green tracings and lot #303795 shown in purple, both with similar results (Supplementary Fig. 8A). In contrast, subsequent stimulation of the same cells through TCR/CD3 showed rapid and sustained Ca²⁺ signaling (Supplementary Fig. 8A) confirming that Ca²⁺ signaling mechanisms were intact in these cells (Supplementary Fig. 8A). Further, 100 μM of MASTER-NAADP, MASTER-NADP, and NAADP-AM were added to intact Jurkat T cells (Supplementary Fig. 8B–E). While the peak amplitude was only different between MASTER-NAADP and vehicle DMSO (Supplementary Fig. 8B), the



number of peaks evoked by both MASTER-NAADP or MASTER-NADP was higher as compared to NAADP-AM, but not different between MASTER-NAADP and MASTER-NADP (Supplementary Fig. 8C). At 10 μ M a similar pattern among MASTER-NAADP, MASTER-NADP or NAADP-AM was observed, but at a lower level (Supplementary Fig. 8C, orange bars). Similar effects were observed for the percentage of responding cells (Supplementary Fig. 8D). Calculation of the most robust read-out, the mean responsiveness, resulted in a somewhat improved result (Supplementary Fig. 8E). However, compared to the experiments with 100 nM MASTER-NAADP or -NADP (Fig. 5), in T cells such high concentrations of the MASTER compounds are neither necessary nor useful to study NAADP signaling.

Fig. 3 | Deprotected MASTER-NAADP evokes Ca^{2+} signals in permeabilized Jurkat T cells and increases the open probability of RYR1. 3.5×10^7 Jurkat T cells were permeabilized (80 $\mu\text{g}/\text{mL}$ saponin) and transferred to a fluorimeter in 1 mL cuvettes with gentle magnetic stirring. Free Ca^{2+} concentration was measured every 2 s ratiometrically using emission wavelengths of CalRed (ex 488 nm, em 525/650 nm). Ca^{2+} uptake into the ER was stimulated by the addition of creatine phosphate, creatine kinase and adenosine triphosphate, followed by the addition of 15 μg recombinant human HN1L/JPT2 (except for control experiments) and 10 μM glucose 6-phosphate dehydrogenase inhibitor-1 (g6pdi-1). Deprotected MASTER-compounds or natural nucleotides were diluted in bidistilled water and injected using a Hamilton gas-tight 50 μL syringe. **A** Example tracings showing no response to either biological NAADP or deprotected MASTER-compounds without the re-addition of exogenous HN1L/JPT2. Representative tracings out of 4 experiments are shown. **B** Example tracings showing increases in the free Ca^{2+} concentration after the injection of NAADP or deprotected MASTER-NAADP, but not of the respective NADP controls. A representative out of at least 5 experiments is shown. **C, D** The delta Ca^{2+} of the different experiments (mean Ca^{2+} concentration during the first

10 s after injection after subtraction of the mean Ca^{2+} concentration the last 10 s before injection) was plotted against the baseline Ca^{2+} (mean Ca^{2+} concentration the last 10 s before injection) and analyzed by linear regression with two-sided F test of the slope using GraphPad Prism 10. The graph show results from the individual experiments (symbols), linear regression (line) with 95% confidence interval (dotted lines) and significance level of the slope being non-zero, i.e. the amplitude of the responses being correlated with the basal Ca^{2+} concentration (ns = not significant, ** $p < 0.01$, **** $p < 0.0001$, $N = 5$ experiments for C and $N = 8$ experiments for D).

E Single channel analysis of deMASTER-NAADP with purified HN1L/JPT2 on RYR1. Representative single-channel current traces of purified RYR1 measured under the following conditions: upper tracing: 1 μM free Ca^{2+} , middle tracing: 100 nM deMASTER-NAADP, lower tracing: 100 nM deMASTER-NAADP with 10 μM HN1L/JPT2. **F** Summarized relative open probability for each condition, presented as mean $\pm$ SEM, n = biological replications as indicated above the bars. Two-sided, mixed-effects analysis with Holm-Šidák multiple comparison test, with a single pooled variance * $p < 0.05$. Each p value is adjusted to account for multiple comparisons. Source data and exact p values are provided as a Source Data file.

The negative results with NAADP-AM prompted us to analyze by HPLC commercially available NAADP-AM that was used in the experiments shown in Fig. 5 and Supplementary Fig. S8. Of note, NAADP-AM did not elute as a single compound, but consisted of >25 peaks of different size (Supplementary Fig. 9A), suggesting that commercially available NAADP-AM is a complex mixture of undefined individual compounds. Similar results were obtained with four different lots of NAADP-AM (Supplementary Fig. 10, all left panels), demonstrating that it was not a single decomposed lot of NAADP-AM, but appears to be a more general problem with this type of lipophilic and biocleavable NAADP derivative.

Upon digestion of NAADP-AM using porcine liver esterase (PLE), the chromatogram in Supplementary Fig. 9B showed only little changes: 2 minor peaks eluting at 23.4 min and 23.7 min disappeared (Supplementary Fig. 9B, brown arrows), while 2 new, minor peaks eluted at 11.8 min and 14.1 min (Supplementary Fig. 9B, blue arrows). In NAADP-AM digested using PLE, no compound co-eluting with standard NAADP was detected, as shown by magnification of the chromatograms (Supplementary Fig. 9C). Of note, in one single batch from lot #3030795 we observed minute amounts of NAADP, both in digested and undigested NAADP-AM (Supplementary Fig. 10C, upper panel), however, NAADP did not increase upon digestion.

To control enzymatic activity of PLE, commercially available cAMP-AM was digested under same conditions, resulting in almost full conversion to cAMP (Supplementary Fig. 11).

Additionally, KHYG-1 NK cells and Neuro2A neuronal cells were also studied regarding global Ca^{2+} signaling evoked by MASTER-NAADP (Fig. 6). In KHYG-1 NK cells MASTER-NAADP evoked rapid global Ca^{2+} signals, with negligible background signaling observed with MASTER-NADP (Fig. 6A). Quantitative analysis revealed marked difference in Ca^{2+} peak amplitude and percentage of responding cells (Fig. 6B, D), and highly significant statistical differences between active MASTER-NAADP and inactive MASTER-NADP for the number of peaks (Fig. 6C), and the mean responsiveness (Fig. 6D).

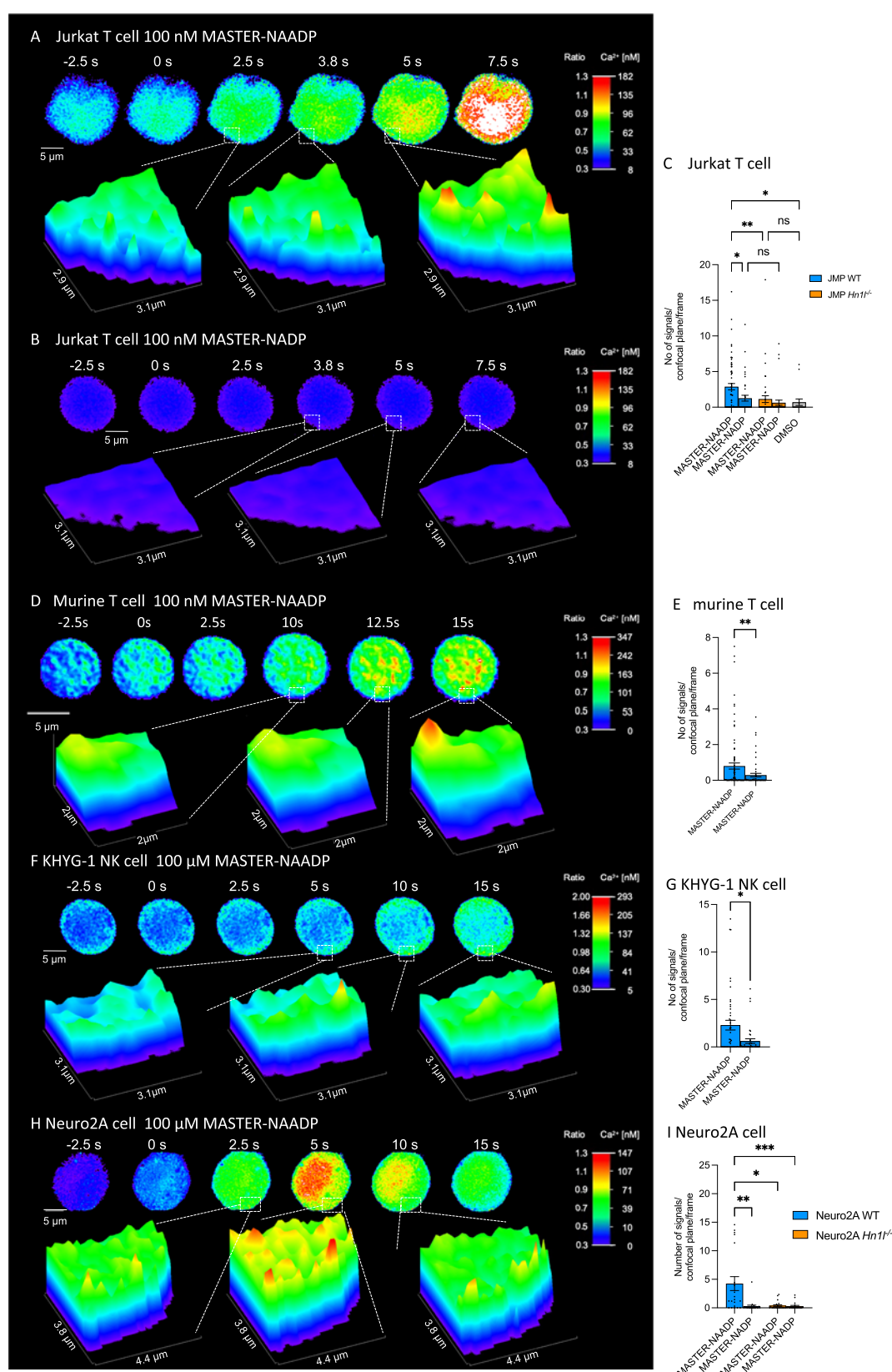
In addition to cells of the immune system, T cells or natural killer cells, also the neuronal cell line Neuro2A responded with global Ca^{2+} signaling upon addition of MASTER-NAADP (Fig. 6F–K). Qualitatively, the response to MASTER-NAADP was more similar to the NK cell line KHYG-1 (Fig. 6A left), as compared to Jurkat T cells (Fig. 5A), with rapid and relatively uniform (among individual cells) monophasic and high-amplitude Ca^{2+} signals (Fig. 6F left). Control compound MASTER-NADP did not evoke Ca^{2+} signaling in any of the cells (Fig. 6F right), also more similar to KHYG-1 cells (Fig. 6A right) as compared to Jurkat T cells (Fig. 5A). As for Jurkat T cells, *Hn1l/Jpt2*^{-/-} Neuro2A cells were created using Crispr/Cas9 technology. Putative null alleles were identified in several Neuro2A cell clones. NGS sequencing revealed frameshift mutations in all alleles in clones 1F3 and 1G4, and accordingly HN1L/

JPT2 protein was not detected in Western blot analysis (Supplementary Fig. 12A). *Hn1l/Jpt2*^{-/-} Neuro2A clone 1G4 neither responded to active MASTER-NAADP nor to inactive MASTER-NADP (Fig. 6G); likewise, MASTER-NAADP did not stimulate Ca^{2+} signaling in *Hn1l/Jpt2*^{-/-} Neuro2A clone 1F3 (suppl S12B), confirming also in this cell type that the NAADP mimic benzoic acid C-nucleoside, 2'-phospho-3'-F-adenosine-diphosphate activates Ca^{2+} signaling via the NAADP receptor/NAADP binding protein HN1L/JPT2. As for KHYG-1 cells (Fig. 6B), a markedly higher mean value of Ca^{2+} peak amplitude was observed for MASTER-NAADP, as compared to control conditions (Fig. 6H). Quantitative evaluation of number of Ca^{2+} oscillations and mean responsiveness were highly significant when compared between MASTER-NAADP and MASTER-NADP in WT cells (Fig. 6I,K). Likewise, in *Hn1l/Jpt2*^{-/-} Neuro2A cells these two parameters were extremely low and not significantly different from the values in WT cells stimulated by control compound MASTER-NADP (Fig. 6I,K). Similar results were obtained for the percentage of responding cells (Fig. 6J).

Since stimulation of the different cell types used in this study required different concentrations of MASTER-NAADP to evoke Ca^{2+} signaling, the expression of the NAADP receptor/NAADP binding protein HN1L/JPT2 was compared. Using anti-HN1L/JPT2 antibody HPA041888 (Atlas Antibodies) HN1L/JPT2 was detected as single band at approx. 21 kDa in Jurkat T cells and KHYG-1 cells, while in Neuro2A cells a double band at approx. 24 kDa was observed (Supplementary Fig. 13A); with a different antibody for detection of HN1L/JPT2 (orb1412, Biorbyt) both the double-band at approx. 24 kDa and the band at approx. 20 kDa was observed in Neuro2A cells (Supplementary Fig. 12A). Recombinant HN1L/JPT2 was used as positive control (Supplementary Fig. 13A). When normalized to α -actin no significant differences in expression of HN1L/JPT2 in the three cell types was observed (Supplementary Fig. 13B), indicating that it is not the cellular amount of HN1L/JPT2 that defines the sensitivity of the system. However, post-translational modifications, as seen in Neuro2A cells, might be involved.

Discussion

Initially, we tried to reproduce the synthesis of NAADP-AM, as described in refs. 28,29. However, this approach led to a complex mixture of compounds and to non-reproducible results, mainly caused by the non-selective reaction site at the NAADP molecule and particularly by the low stability of the AM-ester^{59,60}. Moreover, alkylation of the NAADP pyrophosphate moiety by the AM-reagent would lead to high chemical instability of the phosphate anhydride bond due to the neutralization of the negative charge(s). Observations made during synthesis of MASTER-NAADP showed that after the esterification of the carboxylic acid, the N-glycosidic bond in the nicotinic acid moiety became labile. Additionally, the esterification of the 2'-hydroxyl group



in the adenosine part by a dialkylphosphate led to a high tendency of migration of this esterified phosphate group from the 2'- to the 3'-position or even to cleavage of the ester. All these unwanted properties may also occur after AM-alkylation which finally led to a complex mixture of products. Analysis by HPLC of commercially available NAADP-AM confirmed these problems because all tested lots – some

lots even tested in different batches that were purchased at different time points – showed highly complex mixtures of compounds detected at 260 nm (Supplementary Figs. 9, 10). To our surprise, upon digestion of the commercial NAADP-AM by porcine liver esterases, traces of NAADP were identified only in one of out the four commercially available lots (Supplementary Fig. 10C, 1. batch, right panel);

Fig. 4 | Ca²⁺ microdomains evoked by MASTER-NAADP in different cell types. Representative high-resolution Ca²⁺ images of Jurkat T cells loaded with both Fluo4-AM and Fura-Red-AM after stimulation with 100 nM MASTER-NAADP (**A**) or 100 nM MASTER-NADP (**B**). Number of cells indicated in figure. **A, B** (upper panel): Pseudocolor images indicate emission ratios between Fluo-4 and Fura-Red ranging from 0.3–1.3; ratio data were then converted using external calibration corresponding to 8 to 182 nM [Ca²⁺]_i. Scale bars, 5 μm for whole cells. **A, B** (lower panel): Magnified regions as indicated as 3D surface plots. **C** Analysis across the first 15 s of stimulation shown as number of Ca²⁺ microdomains. **C** Data are displayed as mean ± SEM; Jurkat WT MASTER-NAADP, *n* = 66 cells; Jurkat WT MASTER-NADP, *n* = 40 cells; Jurkat *Hn1l/jpt2*^{-/-} MASTER-NAADP, *n* = 43 cells; Jurkat *Hn1l/jpt2*^{-/-} MASTER-NADP, *n* = 37 cells; Jurkat WT DMSO, *n* = 17 cells; nonparametric Kruskal-Wallis test and Dunn's correction for multiple testing **P* < 0.05; ***P* < 0.005. **D** (upper panel) Representative high-resolution Ca²⁺ images of primary murine T cells loaded with both Fluo4-AM and Fura-Red-AM after stimulation with 100 nM MASTER-NAADP. Pseudocolor images indicate emission ratios between Fluo-4 and Fura-Red ranging from 0.3–1.3; ratio data were then converted using external calibration corresponding to 0 to 347 nM [Ca²⁺]_i. Scale bars, 5 μm for whole cells. **D** (lower panel) Magnified regions as indicated as 3D surface plots. **E** Analysis across the first 15 s of stimulation shown as number of Ca²⁺ microdomains. **E** Data are displayed as mean ± SEM; MASTER-NAADP, *n* = 89 cells; MASTER-NADP, *n* = 58 cells; two-sided,

nonparametric Mann-Whitney U test: ***P* < 0.005. **F** (upper panel) Representative high-resolution Ca²⁺ images of human NK cells (KHYG-1) loaded with both Fluo4-AM and Fura-Red-AM after stimulation with 100 μM MASTER-NAADP. Pseudocolor images indicate emission ratios between Fluo-4 and Fura-Red ranging from 0.3–2.0; ratio data were then converted using external calibration corresponding to 5 to 293 nM [Ca²⁺]_i. Scale bars, 5 μm for whole cells. **F** (lower panel) Magnified regions as indicated as 3D surface plots. **G** Analysis across the first 15 s of stimulation shown as number of Ca²⁺ microdomains. **G** Data are displayed as mean ± SEM; MASTER-NAADP, *n* = 46 cells; MASTER-NADP, *n* = 39 cells; two-sided, nonparametric Mann-Whitney U test: **P* < 0.05. **H** (upper panel) Representative high-resolution Ca²⁺ images of Neuro2A cells loaded with both Fluo4-AM and Fura-Red-AM after stimulation with 100 μM MASTER-NAADP. **H** (lower panel) Magnified regions as indicated as 3D surface plots. Pseudocolor images indicate emission ratios between Fluo-4 and Fura-Red ranging from 0.3–1.3; ratio data were then converted using external calibration corresponding to 0 to 147 nM [Ca²⁺]_i. **I** Analysis across the first 15 s of stimulation shown as number of Ca²⁺ microdomains. **I** Data are mean ± SEM; Neuro2A WT MASTER-NAADP, *n* = 20 cells; Neuro2A WT MASTER-NADP, *n* = 23 cells; Neuro2A *Hn1l/jpt2*^{-/-} MASTER-NAADP, *n* = 21 cells; Neuro2A *Hn1l/jpt2*^{-/-} MASTER-NADP, *n* = 20 cells; nonparametric Kruskal-Wallis test and Dunn's correction for multiple testing **P* < 0.05; ***P* < 0.005; ****P* < 0.001. Source data for (**C, E, G, I**) and exact *p* values are provided as a Source Data file.

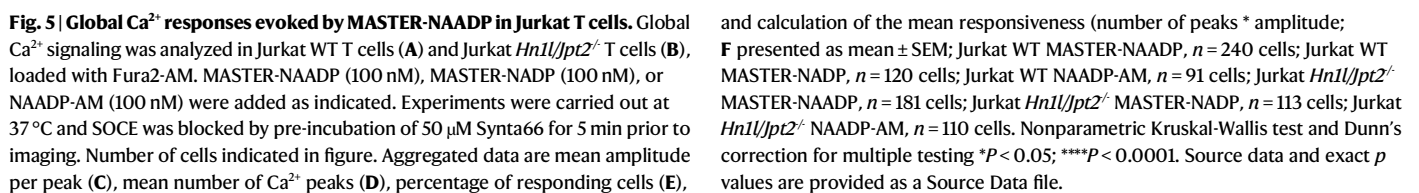
moreover, in the same lot #3030795 a very small amount of NAADP was already present before digest (Supplementary Fig. 10C, 1. batch, left panel), indicating spontaneous degradation of NAADP-AM to NAADP, or even remaining starting substrate NAADP within the synthesis product mixture. This finding may be important because, when added to the extracellular solution, NAADP activated Ca²⁺ signaling in intact cells via purinergic P2Y11 receptors was observed^{61,62}. Thus, some of the previous results in intact cells stimulated by NAADP-AM^{28,30,55–58} may be explained by Ca²⁺ signaling secondary to purinergic P2Y11 receptors.

After the synthesis of the NAADP-AM esters failed in our hands, we decided to develop a total synthesis route towards the membrane-permeable NAADP precursors which are modified with our previously developed acyloxybenzyl moieties that are cleaved in the cytosol by esterases/lipases^{31,63,64}. Retrosynthetically a convergent approach was used in which the two nucleotidic parts were synthesized first and coupled at the end to form the pyrophosphate bridge. First, attempts were made to use nicotinic acid which was esterified at the carboxylic group. However, this led to a marked increase in lability of the glycosidic bond, which was incompatible with the later reaction conditions. Therefore, we decided to replace the *N*-glycosidic bond by a C-nucleosidic bond (“northern” part). Also, in the adenosine (“southern” part) the presence of the 3'-OH group and the 2'-phosphate ester group resulted in instabilities. Over time, degradation of the phosphate ester was detected by HPLC analysis due to 2',3'-phosphate group migration. Since the 2'-phosphate is essential for NAADP's Ca²⁺ mobilizing activity⁵⁰, we decided to replace the hydroxyl group at the 3'-position by the bioisosteric fluorine atom. This replacement may change the conformational properties of the molecule to some degree, but completely avoided the migration of the phosphate group in aqueous incubation studies. While the 3'-OH-comprising compound showed a marked conversion, the 3'-fluorinated derivative proved to be completely stable. In case of the control compound, MASTER-NADP, no laborious 3'-fluorination was included; this resulted in production of a mixture of the 2'-phosphate and 3'-phosphate isomers of deMASTER-NADP upon digestion with porcine liver esterases, as described^{48,49}. Since deMASTER-NADP does not contain the 3-COO⁻ group at the C-nucleoside and thus does not possess Ca²⁺ releasing activity, the 2',3'-phosphate migration can be tolerated in this control compound.

Structural activity data for NAADP were initially obtained mainly in the sea urchin egg homogenate system. Already, in 1997 Lee and Aarhus demonstrated the significance of three parts of NAADP: the

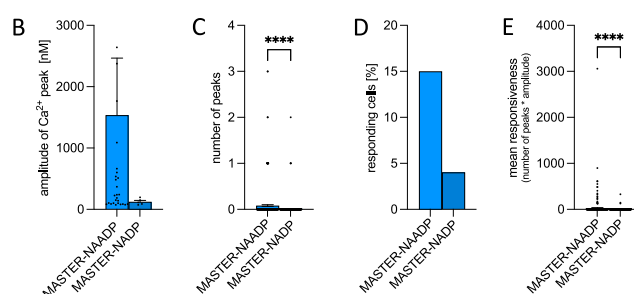
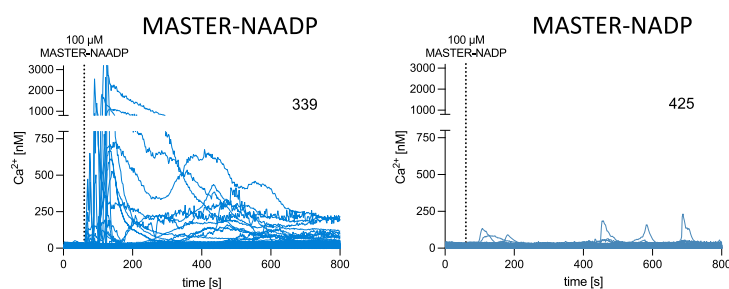
3-carboxyl group of the nicotinic acid moiety, the 2'-phosphate of the adenosine moiety, and the 6-amino/imino group of the nucleobase adenine⁵⁰. In particular, they showed that either moving the 3-carboxyl group to the 4-position of nicotinic acid or replacement by an uncharged group (3-carbinol-derivative) completely abolished Ca²⁺ release⁵⁰. Replacement of the 3-carboxyl group by a 3-sulfonic acid group resulted in a weaker agonist⁵⁰. The significance of the 2'-phosphate was demonstrated by replacement using either 2',3'-cyclic-phosphate or 3'-phosphate at the adenosine ribose; both modifications shifted the EC₅₀ towards higher agonist concentrations, but did not completely abolish Ca²⁺ release activity⁵⁰. The role of the 6-amino/imino group of the purine base adenine was assessed by using the hypoxanthine derivative of NADP, NHDP, as substrate to produce nicotinic acid hypoxanthine dinucleotide phosphate (NAHDP) via the base-exchange mechanism. NAHDP released Ca²⁺ from sea urchin egg homogenate only at concentrations ≥ 5 μM, while the EC₅₀ for NAADP was approx. 10 nM⁶⁵. When the 4-position of the nicotinic acid moiety in NAADP was substituted, EC₅₀ was shifted to higher concentrations; in contrast, different substitutions at the 5-position showed good agonist activity⁶⁵. Of note, both 4- and 5-modified caged NAADP derivatives showed Ca²⁺ mobilizing activity in mammalian SKBR cells upon microinjection and flash photolysis, suggesting differences in agonist recognition by NAADP receptors of sea urchin eggs vs higher eukaryotic cells⁶⁶. NAADP derivatives modified in the 8-position of adenine, 8-ethynyl-NADP, 8-ethynyl-NAADP, and 5-N₃-8-ethynyl-NAADP did not significantly decrease agonist activity in sea urchin egg homogenates⁶⁷.

deMASTER-NAADP contains all relevant moieties for Ca²⁺ mobilizing activity: the 3-carboxyl group of the nicotinic acid moiety, the 2'-phosphate of the adenosine moiety, and the 6-amino/imino group of adenine. However, as discussed above, specific structural variations had to be introduced: (i) the benzoic acid C-nucleoside to overcome the lability of the *N*-glycosidic bond of nicotinic acid during synthesis, and (ii) the 3'-F-adenosine to inhibit migration of the 2'-phosphate during synthesis or de-esterification. Further, modeling studies (Fig. 2C) indicate a highly similar overall structure of NAADP and deMASTER-NAADP in solution. As result, deMASTER-NAADP released Ca²⁺ in permeabilized Jurkat T cells and increased the open probability of RYR1 in lipid planar bilayer recordings (Fig. 3). Of note, both experiments required presence of NAADP receptor HN1L/JPT2. The lipophilic precursor MASTER-NAADP evoked both local and global Ca²⁺ signaling in intact cells whereas deMASTER-NADP did not (Figs. 4, 5, 6).

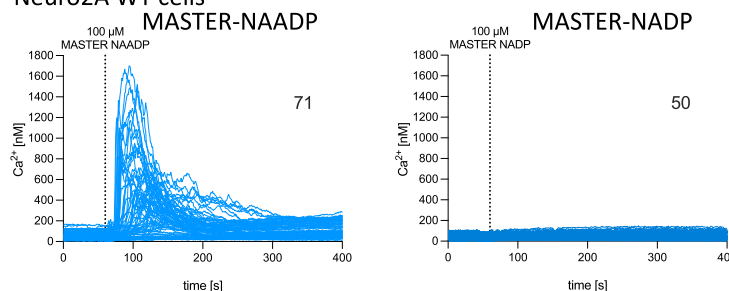


Of note, in both Jurkat T cells and Neuro2A cells, clones lacking expression of the *Hn1l/jpt2* gene encoding HN1L/JPT2, the recently discovered NAADP receptor/NAADP binding protein^{17,18}, did not respond to MASTER-NAADP, confirming that the endogenous

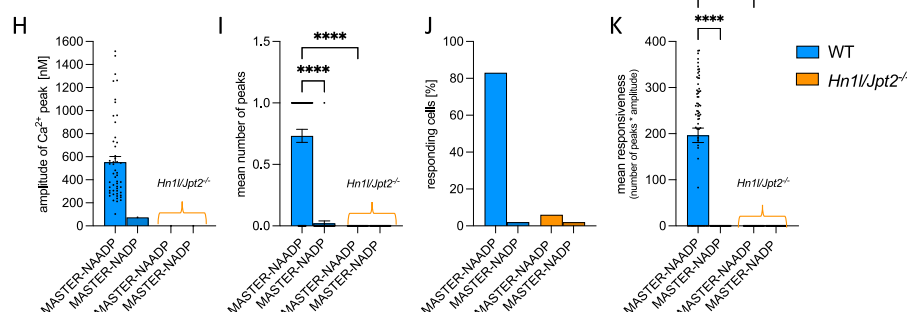
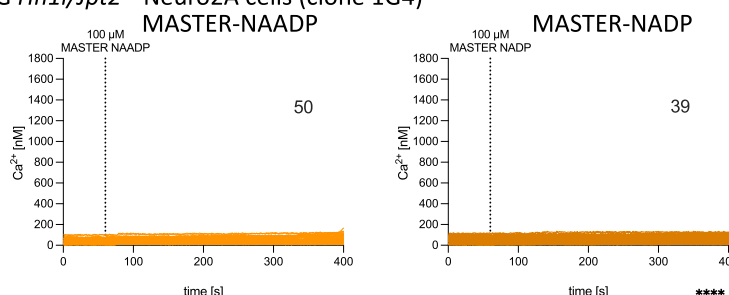
A KHYG-1 cells



F Neuro2A WT cells



G *Hn1/Jpt2*^{-/-} Neuro2A cells (clone 1G4)



signaling pathway of NAADP is mimicked by its derivative deMASTER-NAADP.

A crucial point for us was whether MASTER-NAADP can be reliably used to study NAADP mediated Ca^{2+} signaling processes in different cell types as a bona fide membrane permeable, stabilized, biocleavably protected precursor. In fact, MASTER-NAADP's chemical stability,

release of the NAADP mimetic benzoic acid C-nucleoside, 2'phospho-3'F-adenosine-diphosphate, and signaling via NAADP receptor/NAADP binding protein HN1L/JPT2 evoked Ca^{2+} mobilization in different cell types. Thus, MASTER-NAADP appears generally applicable to investigate NAADP signaling and its downstream events in cells. As a limitation, we observed that quantitatively there are differences regarding

Fig. 6 | Global Ca^{2+} responses evoked by MASTER-NAADP in KHYG-1 NK cells and Neuro2A cells. Global Ca^{2+} signaling was analyzed in Fura2-loaded KHYG-1 cells (A–E), Neuro2A WT or Neuro2A *Hn1l/jpt2^{-/-}* cells (F–K). **A** MASTER-NAADP (100 μM) or MASTER-NADP (100 μM) were added at RT as indicated. Number of cells indicated in figure. Aggregated data are mean amplitude per peak (**B**), mean number of Ca^{2+} peaks (**C**), responding cells (**D**), and calculation of the mean responsiveness (number of peaks * amplitude; **E**; as mean $\pm$ SEM; MASTER-NAADP, $n = 339$ cells; MASTER-NADP, $n = 425$ cells; two-sided, nonparametric Mann-Whitney U test: **** $P < 0.0001$. **F** and **G** MASTER-NAADP (100 μM) or MASTER-NADP (100 μM) were

added at RT as indicated to Neuro2A WT (**F**) or Neuro2A *Hn1l/jpt2^{-/-}* cells (**G**). Number of cells is indicated in figure. Aggregated data are mean amplitude per peak (**H**), mean number of Ca^{2+} peaks (**I**), responding cells (**J**), and calculation of the mean responsiveness (number of peaks * amplitude; **K**; as means $\pm$ SEM; Neuro2A WT MASTER-NAADP, $n = 71$ cells; Neuro2A WT MASTER-NADP, $n = 50$ cells; Neuro2A *Hn1l/jpt2^{-/-}* clone 1G4 MASTER-NAADP, $n = 50$ cells; Neuro2A *Hn1l/jpt2^{-/-}* clone 1G4 MASTER-NADP, $n = 39$ cells; Nonparametric Kruskal-Wallis test and Dunn's correction for multiple testing **** $P < 0.0001$. Source data are and exact p values provided as a Source Data file.

the magnitude of Ca^{2+} mobilization by MASTER-NAADP vs MASTER-NADP between the cell types studied. However, the fact that MASTER-NADP is available as relevant control allows a more precise investigation of cellular NAADP signaling in the future.

Methods

Ethics

All mice experiments were approved by the Animal Welfare Officers of the University Medical Center Hamburg-Eppendorf (UKE) and Behörde für Gesundheit und Verbraucherschutz Hamburg (ORG934).

Chemistry

Most reactions were performed in oven-dried glassware, under nitrogen atmosphere, and with dry solvents. THF, CH_2Cl_2 , acetonitrile and pyridine were dried with a MBraun (MB SPS-800) Solvent System and stored over nitrogen and molecular sieves (3 or 4 Å). Further dry solvents which are not listed were purchased from Acros Organics and dried over molecular sieves. All starting materials and reagents were purchased from Sigma Aldrich, TCI, Acros, ABCR or Carbosynth and were used without further purification. *Ultrapure Water* was obtained from a Sartorius arium pro system (Sartopore 0.2 μm , UV) and HPLC-grade CH_3CN , used for automated RP-18 chromatography and HPLC analysis, was purchased from VWR or Honeywell. *Column chromatography* was carried out with technical grade solvents, which were distilled before use, and silica gel (0.04–0.063 mm) from Macherey-Nagel. Precoated thin-layer-sheets (ALU- GRAM® Xtra SIL G/UV 254, Macherey-Nagel) were used for *thin-layer-chromatography*. The UV-active compounds were detected using a UV lamp at a wavelength of 254 nm. The non-UV-active compounds could be visualized using various staining reagents (cerium ammonium nitrate, potassium permanganate and vanillin) and followed by heat treatment. *Automated RP-18 flash chromatography* was performed using prepacked MN RS 40 C18ec columns on an Interchim Puriflash 430. For all automated *RP-18 chromatography*, a gradient from 100 % ultrapure water to 100 % CH_3CN within 20 min was used (flowrate: 20 mL/min). All NMR solvents were purchased from Euriso-Top or Deutero. *NMR spectra* were recorded on Bruker instruments Fourier HD 300, Avance I 400, DRX 500, or Avance III 600 at room temperature. All proton and carbon NMR spectra were calibrated by the respective solvent signal. *High-resolution mass spectra* were measured with an Agilent 6224 ESI-TOF instrument, detailed information can be found in the section mass spectrometry. IR spectra were obtained on a Bruker Alpha IR spectrometer.

Details regarding synthesis and characterization of chemical intermediates and final products are found in the Supplementary Methods section of the Supplementary Information File.

General synthetic procedure A: synthesis of 4-(hydroxymethyl) phenylalkanoates and 4-(hydroxymethyl) phenylalkylcarbonates

Under nitrogen atmosphere, 4-hydroxybenzyl alcohol (1.1 eq. - 1.2 eq.), dry Et_3N (1.0 eq. - 1.2 eq.), and a catalytic amount of 4-dimethylaminopyridine (DMAP) were dissolved in dry CH_2Cl_2 or dry THF and cooled to 0 °C. A solution of acid chloride or alkyl

chloroformate (1.0 eq.) in dry CH_2Cl_2 or dry THF was added dropwise, and the mixture was stirred for 2–3 h at 0 °C or room temperature. The crude product was filtered and the filtrate was concentrated in vacuo. The residue was diluted with CH_2Cl_2 and washed once with saturated sodium hydrogen carbonate solution and once with H_2O . The organic layer was dried with Na_2SO_4 and concentrated in vacuo. The crude product was purified by column chromatography.

General synthetic procedure B: synthesis of phosphoramidites

In a nitrogen atmosphere, dichloro-*N,N*-diisopropylaminophosphoramidite (1.0 eq.) was dissolved in dry THF and cooled to –20 °C – 0 °C. A solution of the respective alcohol (2.0 eq. – 2.2 eq.) and dry Et_3N (2.2 eq. – 2.3 eq.) in dry THF were added dropwise. The reaction mixture was stirred for 16 h at room temperature. The suspension was filtered, and the filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography.

General synthetic procedure C: Synthesis of 5'-phosphates

The nucleoside (1.0 eq.) was dissolved in dry trimethyl phosphate under a nitrogen atmosphere and the solution was cooled to –5 °C. The solution was mixed with tributylamine, 1,8-bis(*N,N*-dimethylamino) naphthalene, or activated mole sieve (3 Å) (2.5 eq.). After the solution was cooled to 0 °C, $\text{P}(\text{O})\text{Cl}_3$ (2.0–4.0 eq.) was added slowly. The reaction solution was then stirred for 3–16 h at 0 °C. To stop the reaction, a 1 M TEAB buffer solution was added and stirred for 15 min at room temperature. The solution was extracted three times with hexane. The aqueous layer was purified using automatic RP-18 column chromatography ($\text{H}_2\text{O}/\text{CH}_3\text{CN}$ gradient) and finally lyophilized.

General synthetic procedure D: Coupling to the NAADP analog

The nicotinic acid unit (1.0 eq.) or its analogs was dissolved in dry acetonitrile and cooled to 0 °C. In a separate flask, dry Et_3N (16.0 eq.) was added to dry CH_3CN and then TFAA (10.0 eq.) was slowly added. After stirring at 0 °C for 10 min, the solution was dropped slowly to the solution of nicotinic acid analogs. The solution was stirred at room temperature for 15 min and the solvent was removed in vacuo. The residue was then dissolved in dry CH_3CN , cooled to 0 °C, and 1-methylimidazole (NMI) (6.0 eq.) was added. The reaction solution was stirred for 15 min at room temperature. Then, the corresponding adenosine derivative (0.9 eq.) was coevaporated three times with dry CH_3CN and then added dissolved in dry CH_3CN . The mixture was stirred for 3 h at room temperature. To terminate the reaction, a 1 M TEAB buffer solution was added and stirred for 15 min at room temperature. The aqueous layer was purified by RP-18 column chromatography ($\text{H}_2\text{O}/\text{CH}_3\text{CN}$ gradient) and finally lyophilized.

Reagents for biochemistry and cell biology

Fura2-AM, Fluo4-AM, and Fura-Red-AM were obtained from Life Technologies. All dyes were dissolved in dimethyl sulfoxide (DMSO), divided into aliquots, and stored at –20 °C until required for use. Synta66 was purchased from aobius inc., dissolved and aliquoted in 50 mM stocks in DMSO and stored at –20 °C.

NAADP-AM was obtained from AAT Bioquest and stored lyophilized at –80 °C until use. MASTER-NAADP and MASTER-NADP

lyophilised aliquots were dissolved in DMSO (stock concentration 10 mM). NAADP-AM, MASTER-NAADP and MASTER-NADP were aliquoted into 'single-use aliquots' and stored at -80°C until use.

Cell culture

Wild type (WT) and *Hn1l/jpt2^{-/-}* clone 2 Jurkat T cells were cultured in RPMI (1640) medium (Gibco, Life Technologies) containing 25 mM HEPES and GlutaMAX-1, which was further supplemented with 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin and 7.5% (v/v) newborn calf serum (NCS) (Biochrom, Merck Millipore). For culturing NK cell line KHYG-1, RPMI (1640) medium (Gibco, Life Technologies) containing 25 mM HEPES and GlutaMAX-1 further supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng/mL IL-2 (PeproTech) was used. Neuro2a cells were maintained in DMEM medium including GlutaMAX-1 and additionally containing 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin and 10% (v/v) fetal calf serum (FCS) (Biochrom, Merck Millipore). All cells were kept at 37°C and 5% CO_2 in the incubator. For Jurkat T cells, as well as KHYG-1 cells, media was changed every second to third day. Cell density was maintained between 0.1 to 0.8 cells/mL for KHYG-1 cells, 0.3 and 1.3 million cells/mL for Jurkat T cells and Neuro2a cells. Neuro2a cells were split every three days.

Animals

Spleen and lymph nodes of WT mice (C57BL/6J; *Mus musculus*) were used for studies with primary murine CD4^{+} T cells. Housing was performed under standardized conditions at a 12/12 h light-dark cycle with food and water supply *ad libitum* in the animal facility of the University Medical Center Hamburg-Eppendorf (UKE). All mice experiments were approved by the Animal Welfare Officers of UKE and Behörde für Gesundheit und Verbraucherschutz Hamburg (ORG934).

Isolation of primary murine T cells

Spleens and lymph nodes were immediately used for isolation of primary murine CD4^{+} T cells after dissecting them from the mice. RPMI, 7.5% (v/v) newborn calf serum (NCS), 1% (v/v) penicillin/streptomycin was used as isolation medium. The tissue was disrupted using a syringe plunger and cell strainer (40 μm nylon). After transfer to a 50 mL tube, the suspension was centrifuged at 4°C (300 g, for 5 min) and the supernatant was then discarded. 5 mL of cold ACK buffer (4.3 g ammonium chloride, 0.5 g KHCO_3 , 0.0186 g $\text{Na}_2\text{-EDTA}$ in 400 mL H_2O , pH 7.2 to 7.4) was used for the lysis of erythrocytes, which was stopped by the addition of medium. After centrifugation (4°C , 300 g, for 5 min), the supernatant was discarded, followed by resuspension of the pellet in 2 mL PBS. Further isolation of CD4^{+} T cells was carried out using a negative selection kit (EasySep™ Mouse T Cell Enrichment Kit, STEMCELL Technologies, Germany) following instructions of the manufacturer. Cell purity was typically 95% CD4^{+} T cells and was determined by immunostaining with fluorescein isothiocyanate-conjugated anti-mouse

TCR β antibody (clone H57-597, BioLegend) and measured with a FACSCalibur flow cytometer (BD Biosciences).

Global ratiometric Ca^{2+} imaging using Fura2-AM

For ratiometric Ca^{2+} imaging, all cells except Neuro2a cells were loaded for 40 min at 37°C with the fluorescent Ca^{2+} indicator Fura2-AM (4 μM) diluted in RPMI medium. Neuro2a cells were incubated at RT for 20 min with 2 μM Fura2-AM in DMEM medium additionally containing 0.1% Pluronic F-127 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). To all cell types 2 mL of fresh medium was added after 15 min of incubation. Cells were then washed with Ca^{2+} buffer (140 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1 mM CaCl_2 , 1 mM NaH_2PO_4 , 20 mM HEPES, 5.5 mM glucose, pH 7.4, sterile filtered) before being re-suspended in Ca^{2+} buffer. For adhesion during live-cell imaging of Jurkat T cells, primary murine CD4^{+} T cells and KHYG-1 cells, coverslips were coated with bovine serum albumin (BSA) (5 mg/mL, Merck), followed by

coating with poly-L-lysine (PLL) (0.1 mg/mL, Merck). Rubber O-rings were sealed with silicone grease (Kurt Obermeier GmbH & Co. KG, Germany) and glued to the coverslips to create a chamber for seeding the cells. Approximately 100,000 cells per slide were seeded a few minutes prior to imaging. In contrast, uncoated 8 well μ -slides (IBIDI GmbH, Germany) were used to place 10,000 Neuro2a cells per well 72 h before imaging.

Measurements were either performed at RT or in an incubation chamber at 37°C . Additionally, Jurkat T cells were incubated with 50 μM Synta66 (Merck) for 5 min prior to imaging to block SOCE through inhibition of Orai1. Next, slides were mounted on a Leica IRBE microscope (40-fold magnification). Images were taken with an electron-multiplying charge-coupled device camera (EM-CCD; C9100-12, Hamamatsu) and a Sutter DG-4 was used as a light source (Filter-set (nm) excitation (ex): HC340/26, HC387/11; beam splitter (bs): 400DCLP; em: 510/84) at 0.5 ratios/s. Image acquisition was performed with Volocity software (version 6.6.2; PerkinElmer Inc.). Stimulation of cells with either commercial NAADP-AM, MASTER-NAADP, MASTER-NADP as a control compound or DMSO as a vehicle control was performed 60 s after starting the acquisition. Depending on the cell type and experimental procedure, the final concentrations of NAADP-AM, MASTER-NAADP or MASTER-NADP were either 100 nM, 10 μM or 100 μM . As a positive control either thapsigargin (1.67 μM) or OKT3 (1 $\mu\text{g/mL}$) was added after 7 min. For data processing Fiji (ImageJ Version 2.1.0/1.53c) was used. First, the ratiometric raw file was split into respectively 340 nm and 380 nm channels. Next, background correction was performed and cells were selected by drawing a round-shaped ROI (region of interest) and for both channels' fluorescence intensity was measured over time. The resulting values for each cell in the 340 nm and 380 nm channels were used to calculate the ratio 340 nm/380 nm in Excel (Microsoft, USA). To obtain Ca^{2+} concentration, calibration was performed using a high concentration of 5 μM Ionomycin (Santa Cruz Biotechnology, USA), to measure the maximal ratio value (R_{max}). Furthermore, the minimal ratio value (R_{min}) was determined by chelating free Ca^{2+} using 4 mM EGTA (Sigma-Aldrich, Germany) before incubation with Ionomycin. Calculation of Ca^{2+} concentration was performed using the equation in ref. 69. Furthermore, using Prism the mean Ca^{2+} concentration per peak, the number of peaks as well as the percentage of responding cells, defined as an increase of at least 120 nM above baseline and lasting for at least 2 time points, was calculated. Additionally, the mean responsiveness refers to the mean Ca^{2+} concentration multiplied by the number of peaks of the respective cell.

HPLC purity analysis of NAADP-AM, MASTER-NAADP, or MASTER-NADP and deprotection by esterase treatment

Commercially available NAADP-AM (AAT Bioquest), cAMP-AM (Biolog), MASTER-NAADP or MASTER-NADP were incubated with 5 U/mL of porcine liver esterase (PLE) (Merck) for 5 min at 37°C . Reversed-phase (RP)-HPLC was used to analyze the purity of educts and to separate the reaction products by using a 250 mm $\times$ 4.6 mm C8 Luna column (5 μm particle size, Phenomenex) as stationary phase. The mobile phase consisted of HPLC buffer A (20 mM KH_2PO_4 , pH 6.0) and B (10% buffer A, 90% methanol), and elution of nucleotides from the column occurred with increasing methanol content in the mobile phase (0.0 min [100.0% buffer A], 5.0 min [100.0% buffer A], 27 min [100% buffer B], 32.0 min [100% buffer B], 34.0 min [100.0% buffer A], 45.0 min [100.0% buffer A]). The flow rate was set to 0.8 mL/min. A diode-array detector (DAD, Agilent Technologies) was used to detect nucleotides at 260 nm. For each experiment different samples were analysed: (i) 250 pmol NAADP- or 1 nmol cAMP-standard (Biolog), (ii) 1 nmol cAMP-AM, 5 nmol NAADP-AM, 5 nmol MASTER-NAADP or 10 nmol MASTER-NADP, (iii) 1 nmol cAMP-AM, 5 nmol NAADP-AM, 5 nmol MASTER-NAADP or 10 nmol MASTER-NADP after PLE digest, and (iv) PLE alone.

Furthermore, after digestion of MASTER-NAADP and MASTER-NADP with PLE, product peaks were collected. Therefore, a 250 mm × 4.6 mm C8 Luna column (5 µm particle size, Phenomenex) was used as stationary phase. The mobile phase consisted of different compositions of HPLC buffer A (97% 50 mM triethylamine acetate buffer, 0.3% (v/v) acetic acid, pH 5.5 and 3% methanol) and B (3% buffer A, 97% methanol). The gradient was as follows: 0.0 min [100.0 % buffer A], 7.0 min [100.0% buffer A], 57 min [100% buffer B], 62.0 min [100% buffer B], 70.0 min [100.0% buffer A], 75.0 min [100.0 % buffer A]. The flow rate was set to 0.8 mL/min. The major peak after digestion of MASTER-NAADP by PLE was collected and subjected to mass spectrometry (see below). Data were processed by the OpenLAB CDS ChemStation Edition C.01.05 data acquisition software from Agilent.

HPLC of intermediates and end products during chemical synthesis

HPLC Chromatograms were recorded on an Agilent 1260 Infinity II with the following components: C₁₈ column (Macherey-Nagel, EC 125/3 Nucleodur 100-5), G7129A Vialsampler, G7111A Quat Pump, G7116A MCT, G7117C DAD HS. UV detection was performed at wavelengths of 240 nm, 250 nm, 260 nm and 270 nm with the following method: 0–25 min 5 % CH₃CN in 2 mL aq. tetrabutylammonium acetate buffer (pH 6.0) 5 % to 100 % CH₃CN, 1 mL/min.

High-resolution mass spectrometry (HRMS and electrospray ionization)

The samples were dissolved in water (LC-MS grade) or organic solvents and measured with electron-spray ionization-time of flight mass spectrometry (Agilent 6224 ESI-TOF instrument). The measurements were done in positive and negative mode with a mass range of *m/z* 110–3200 and a rate of 1.03 spectra/s. The gas temperature was set to 325 °C and the drying gas flow to 10 L/min. Data evaluation was performed using MestReNova (version 14.0.1-235559) software.

Molecular modeling

Molecular Modeling of NAADP and MASTER-NAADP was performed with SCHRÖDINGERS MAESTRO (Version 12.6). Ligands were generated using the LigPrep module of MAESTRO. The overlay of the structures was performed with the ligand alignment module of MAESTRO.

Ca²⁺ microdomain imaging

Detection of Ca²⁺ microdomains after addition of MASTER-NAADP or MASTER-NADP, as well as image processing and analysis of these data was performed as described in detail in ref. 70.

The loading protocol was similar for all cell types. Shortly, cells were loaded with Fluo4-AM (10 µM) and Fura-Red-AM (20 µM) (Life Technologies) for 50 min at RT. Next, cells were washed and taken up in Ca²⁺ buffer. The procedure for coating the slides and seeding the cells was performed similarly to that described for the global Ca²⁺ imaging procedure was carried out on a wide-field microscope using a Dual-View module (Optical Insights, PerkinElmer Inc.) to split the emission wavelengths (filters: excitation (ex), 480/40; beam splitter (bs), 495; emission 1 (em1) 1, 542/50; em2, 650/57). For image acquisition a 100x magnification and an exposure time of 25 ms was used. During the first min the frame rate was set to 1 frame per 5 s and the remaining 2 min were imaged at maximum frame rate (40 frames/s). After 60 s the compound was added to evoke Ca²⁺ microdomain formation. Postprocessing and detection of Ca²⁺ microdomains were performed as described in ref. 70 using MATLAB (version 2022a). The first step during the postprocessing was a frame-by-frame additive bleaching correction of FuraRed using a biexponential decay curve. To get digital confocal images, a Lucy-Richardson deconvolution with a maximum number of 4 iterations, independent of each frame, was applied to both channels using an analytically generated point spread

function (PSF). The next step was to spatially align both channels, remove the image background by semiautomated thresholding and compute a ratio image sequence by frame-by-frame pixel-wise division of Fluo4 and FuraRed intensities. Calibration is based on Grynkiewicz et al.⁶⁹ with a *K_d* of Fluo4 and FuraRed of 408 ± 12 nM²⁰. Since an additive frame-by-frame bleaching correction is applied for Fura-Red, the mean Fura-Red fluorescence remains stable throughout each measurement and is included in the calibration as Fura-Red₀ (initial mean Fura-Red fluorescence). Subcellular Ca²⁺ microdomains are defined as small, compact connected sets of pixels with high [Ca²⁺]_i values. To discriminate between signal and noise, noise was determined in a cell-free system in EGTA buffer ([Ca²⁺]_i = 100 nM) to calculate a threshold for the Ca²⁺ microdomain detection. The threshold for Jurkat cells is 72 nM and for primary T cells, N2A cells and KHYG-1 cells is 112.5 nM. Next, on a frame-by-frame basis the frame-specific mean [Ca²⁺]_i value was calculated and a Ca²⁺ microdomain is counted as one if the Ca²⁺ microdomain consists of at least 6 and maximally 20 pixels, that is limited by the spatial resolution of 368 nm, and if the [Ca²⁺]_i value of the Ca²⁺ microdomain is ≥ the frame specific mean plus the respective threshold. The starting time of cell activation after the addition of MASTER-NAADP was determined automatically and defined as the beginning of a linear ascent of the logarithmized mean cell signal (i.e., the beginning of an exponential signal increase). Furthermore, for the localization of MASTER-NAADP evoked Ca²⁺ microdomains Jurkat and KHYG-1 cells were shape-normalized and dartboard projections were generated to visualize Ca²⁺ microdomain mean data, as described in ref. 51. To analyze pure MASTER-NAADP Ca²⁺ microdomains, responses to MASTER-NADP (control compound) were subtracted from MASTER-NAADP signals. Data analysis was performed with Excel (Microsoft, USA) and Prism 10 (GraphPad Software, USA).

Recombinant production of HN1L/JPT2

HN1L/JPT2 protein was produced as described recently¹⁷ using the extracted plasmids pETM33 with His-tag and GST-tag. The *hn1l/jpt2* gene (accession number, Q9H910) was codon-optimized for bacterial expression and cloned in XL1-blue bacteria (New England Biolabs). Rosetta2 E. coli strain was transformed with plasmid DNA. The culture after isopropyl β-D-1-thiogalactopyranoside induction was further incubated at 24 °C for 24 h at 180 rpm. Bacteria were lysed by ultrasonication on ice (3 times for 3 min, 70% power, 50% cycle) using Sonopuls GM 70 (Bandelin Electronic). Recombinant HN1L/JPT2 was then purified from the supernatant using the ÄKTA pure™ 25 system (Cytiva) with HisTrap™ FF 5 mL column (Cytiva). The His-tag was cleaved using PreScission Protease (provided by Dr. Susanne Witt, CSSB, Hamburg, Germany) and removed with the previously mentioned system and column. Size exclusion chromatography was performed with the same system and with a HiLoad Superdex™ 75 pg (Cytiva) column. The protein was concentrated using an Amicon® Ultra-15 Centrifugal Filter 10 kDa MWCO (Merck). Concentrations of the purified protein were determined by measuring absorbance at 280 nm using NanoDrop OneC (Thermo Fisher Scientific). To check the purity of recombinant HN1L/JPT2 SDS-PAGE was performed and the identity was assessed using anti-HN1L antibodies (1:5000, ab200587 abcam, and 1:5000, HPA041888, Atlas Antibodies) on a Western blot.

Ca²⁺ release in permeabilized Jurkat T cells

To detect the response of deprotected MASTER-compounds in permeabilized Jurkat T cells, approx. 3.5 × 10⁷ cells per experiment were transferred into a 50 mL falcon tube and centrifuged for 5 min at 300 × *g* and room temperature. After removal of the supernatant using a suction pump equipped with a glass capillary, the cell pellet was rinsed twice with 10 mL preparation buffer (120 mM KCl, 10 mM NaCl, 1.2 mM MgCl₂, 0.533 mM CaCl₂, 1 mM EGTA, 10 mM HEPES, pH 7.2 set with 7 M KOH) and resuspended in saponin (80 µg/mL diluted in

preparation buffer) for 10 min in a water bath at 37 °C. After incubation, the cells were centrifuged (5 min at 300 × *g*, room temperature) and the supernatant was carefully removed using the suction pump. The permeabilized cells were rinsed once in 10 mL intracellular buffer (120 mM KCl, 10 mM NaCl, 1.2 mM MgCl₂, 10 mM HEPES, pH 7.2 set with 7 M KOH) and resuspended in a final volume of 1 mL intracellular buffer. Next, cells were transferred into a clean quartz cuvette, a small magnetic stirring bar was added and the cuvette was placed in the fluorimeter. Stirring was set to the slowest option. For the measurements, fluorimeter parameters were set to 488 nm excitation and emissions at 525 nm and 650 nm. The acquisition rate was set to 0.5 s⁻¹. Before starting the measurement, 25 µg/mL creatine kinase, 200 mM creatine phosphate and 1 µg/mL CalRed 525/650 were added. After the addition of CalRed, data acquisition was started and 1 mM ATP was added after a stable baseline was recorded for at least 1 min. Approximately 5 min after ATP addition 10 µM of a reversible and non-competitive glucose-6-phosphate dehydrogenase (G6PD) inhibitor (g6pdi-1) was added and after a stable baseline was recorded for 2 min, 15 µg recombinant human HN1L/JPT2 was added. Further additions were performed by injecting the compounds through a membrane in the closed lid using a needle-equipped gastight syringe.

The measurement was calibrated by stepwise injecting solutions of ionomycin (1 µM final concentration), CaCl₂ (2 mM final concentration), and Tris-EGTA (60 mM final concentration of Tris-base and 8 mM EGTA). For data analysis, the ratio of 525 nm emission divided by 650 nm emission for each timepoint was calculated. Ratio values were converted to [Ca²⁺] concentrations based on Grynkiewicz et al.⁶⁹ using a *K_d* for CalRed of 330 nM. For every injection, the mean Ca²⁺ concentration over 10 s before the addition was subtracted from the mean Ca²⁺ concentration over the following 10 s after the injection and plotted against the mean Ca²⁺ concentration over the 10 s before the injection. The resulting datasets were analyzed using linear regression.

Planar lipid bilayer measurements

RYR1 was purified from rabbit skeletal muscles. Purified RYR1 was reconstituted into liposomes as described before ref. 71. Purified RYR1 was concentrated to <0.5 mL and incubated at a molar ratio 1:1,000 with phosphatidylethanolamine and phosphatidylcholine (5:3 ratio) in 10% CHAPS for 10 min prior to loading onto a hand-packed G50 column pre-equilibrated with buffer S containing 300 mM NaCl, 10 mM HEPES (pH 7.4), 1 mM EGTA, 0.5 mM tris(2-carboxyethyl)phosphine. Liposome formation and RYR1 incorporation occurred on the column and proteoliposomal RYR1 was eluted at 0.20–0.25 mL/min in buffer S, frozen in liquid nitrogen, and stored at -80 °C.

Proteoliposomal RYR1 was fused to planar lipid bilayers formed by painting a lipid mixture of phosphatidylethanolamine and phosphatidylcholine (Avanti Polar Lipids) in a 5:3 ratio in decane (Sigma) across a 200 µm hole in polysulfonate cups (Warner Instruments) separating two chambers. The trans chamber (1.0 mL), representing the intra-SR (luminal) compartment, was connected to the head stage input of a bilayer voltage clamp amplifier. The cis chamber (1.0 mL), representing the cytoplasmic compartment, was held at virtual ground. Symmetrical solutions used were as follows: 1 mM EGTA, 250/125 mM HEPES/Tris, 50 mM KCl (pH 7.35) as *cis* solution and 53 mM Ca(OH)₂, 50 mM KCl, 250 mM HEPES (pH 7.35) as *trans* solution. The concentration of free Ca²⁺ in the *cis* chamber was calculated with WinMaxC program. Proteoliposomal RYR1 was added to the *cis* side. Then 400–500 mM KCl was added to generate the driving force and induce lipid bilayer fusion. The *cis* chamber was perfused with *cis* solution to remove KCl after RYR1 incorporation. For all recordings, 1 µM free Ca²⁺ was first added to the *cis* side for the 3 min baseline recordings. Then, for the NADP group, 100 nM deMASTER-NADP and 10 µM purified HN1L/JPT2 was added together and recorded for at least 3 min. For the NAADP group, 100 nM deMASTER-NAADP and 10 µM purified HN1L/JPT2 were incubated sequentially and recorded for at least 3 min for each step.

Single-channel currents were recorded at 0 mV using a Bilayer Clamp BC-525C (Warner Instruments), filtered at 1 kHz using a Low-Pass Bessel Filter 8 Pole (Warner Instruments), and digitized at 4 kHz. All experiments were performed at room temperature (23 °C). Data acquisition was performed using Digidata 1322 A and Axoscope 10.1 software (Axon Instruments). The recordings were analyzed using Clampfit 10.1 (Molecular Devices) and imaged by Graphpad Prism software. Each datapoint was normalized based on the baseline open probability (*P_o* of 1 µM Ca).

Preparation of *Hn1l/Jpt2*^{-/-} Neuro2A cells

Hn1l/Jpt2^{-/-} cell lines were developed by generating frameshift mutations by targeting exon2 (sgRNA#2 TTCAAGCAAGCCTAATAGGA) of the *Hn1l/Jpt2* gene using the CRISPR-Cas9 technology. A detailed, sequential step-by-step protocol is described in ref. 72. In brief, the sgRNA was cloned into pSpCas9(BB)-2A-GFP (PX458). Neuro2a (N2a) cells (obtained from the DSMZ German Collection of Microorganisms and Cell Cultures GmbH) were transfected, and GFP-expressing cells were isolated by FACS sorting. In the obtained individual cell clones, putative null alleles were identified by Sanger sequencing with the aid of the designated algorithm “Interference of CRISPR Edits” (ICE)⁷³. PCR amplicons covering the mutated site of high potential knockout clones (according to ICE scores) were amplified and NGS sequenced (> 33,143 reads, Amplicon-EZ service at GeneWiz, Azenta Life Sciences). In clones 1G4 and 1F3 we identified frameshift mutations on all alleles. Clone 1G4: Δ+1 allele c.G203Δ + 1 AfsX214 with insertion of Δ+1(A) at position 203 of the transcript (ENSMUST00000024981.9) identified in 49.57% of 33,143 reads leading to a preliminary stop codon at position 46 of protein sequence (p.R42KfsX46), and a Δ+2 allele c.G203Δ + 2TAfsX331 with insertion of Δ+2(TA) at position 203 of the transcript in 49.40% of all reads leading to a preliminary stop codon at position 85 of protein sequence, (p.R42IfsX85). Clone 1F3: a Δ+1 allele c.G203Δ + 1AfsX214 with insertion of Δ+1A at position 203 of the transcript was identified in 98.72% of 130,074 reads leading to a preliminary stop codon at position 46 of protein sequence (p.R42KfsX46). Lack of HN1L/JPT2 protein expression was confirmed by SDS-PAGE/western blot (detail see below). *Hn1l/Jpt2*^{-/-} clones 1G4 and 1F3 were then used for Ca²⁺ imaging experiments with respect to analysis of MASTER-NAADP mediated Ca²⁺ signaling.

SDS-PAGE and western blot of HN1L/JPT2

For the preparation of the S10 cytosolic fraction, a suspension of 10 to 30 million WT Jurkat, WT KHYG-1, or Neuro2a (WT and *Hn1l/Jpt2*^{-/-}) cells were centrifuged (518 *g*, 5 min, room temperature), followed by resuspension of the cell pellet in Dulbecco's phosphate-buffered saline without Ca²⁺ and Mg²⁺ (pH 7.4) with protease inhibitor (cOmplete protease inhibitor cocktail EDTA free, Roche). Next, using Sonopuls GM 70 (Bandelin Electronic) the suspension was ultrasonicated for 30 s on ice to lyse the cells. This procedure was repeated 3 times. Cell lysate was then centrifuged (518 *g*, 5 min, 4 °C) to pellet cell debris. The supernatant was removed and subjected to another centrifugation step (6000 *g*, 10 min, 4 °C), and the resulting supernatant was collected for further centrifugation (10,000 *g*, 30 min, 4 °C), which finally yielded the S10 fraction. Bradford assay and Nanodrop was used to measure the protein concentration. Until use, the S10 fraction was stored at -20 °C with protease inhibitor. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Therefore, samples were pre-mixed with 4× Laemmli buffer [73.8% (v/v) 6× SDS-PAGE loading buffer stock solution, 21.1% (v/v) 20% SDS solution, and 5.1% (v/v) β-mercaptoethanol], then samples were boiled at 95 °C for 5 min to ensure denaturation of the proteins and either 12.5% precast gels (Bio-Rad Laboratories) or 15% self-cast gels were used for separation. SDS-PAGE was run for 10 min at 90 V to ensure proper loading of protein samples, continued by separating for 40–45 min at 180 V. Next, western blotting experiments were

performed. For this purpose, the separated proteins were transferred from the gel onto a 0.45 µm pore-sized polyvinylidene difluoride (PVDF) membrane (Merck Millipore Ltd.) by either tank electroblotting (at constant current of 200 mA for 90 min) or semi-dry blotting (constant current of 200 mA for 20 min). For detection of HN1L/JPT2, PVDF-membranes were incubated overnight at 4 °C with a primary anti-HN1L antibody (1:1000, orb1412, Biorbyt or 1:2000, HPA041888, Atlas Antibodies). PVDF-membranes were then rinsed, following incubation for 1 h at RT with an anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:5000, ab6721, Abcam) or peroxidase AffiniPure goat anti-rabbit immunoglobulin G (1:20,000, 111-035-045, Jackson ImmunoResearch Europe LTD.). Incubation of PVDF-membranes with the chemiluminescent substrate Super Signal West Pico (Thermo Fisher Scientific, Waltham, Massachusetts, USA) resulted in chemiluminescence catalyzed by HRP. Chemiluminescence of the blot was detected using the fusion FX edge system (Vilber). After the detection of HN1L/JPT2, the PVDF-membranes were stripped by 30 min incubation with 30% (v/v) H₂O₂ at RT with constant shaking, followed by 6 times washing with TBS-T (Tris-buffered saline with 0.05% (v/v) Tween20). As loading control, actin was stained with anti-α-actin (1:500, MAB1501, Merck) or anti-β-actin (1:500, SC-47778, Santa Cruz).

Statistical analysis

Prism 10 (GraphPad Software, USA) and Excel (Microsoft, USA) were used for data analysis. All data are shown as mean ± SEM of at least three independent experiments. Datasets were first tested for normal distribution using the Kolmogorov-Smirnov test. The none-normally distributed data sets were further tested using a non-parametric Mann-Whitney U test or a Kruskal-Wallis test (one-way ANOVA) with subsequent Dunn's correction for multiple testing. Normally distributed data sets were tested using ordinary one-way ANOVA with Tukey's multiple comparisons test or ANOVA mixed effects test with Holm-Šidák correction for multiple testing as indicated. Linear regression was performed using the "simple linear regression" function in Prism 10 and slope deviation from zero was analyzed using a F test. Differences with a *P* value of <0.05 were considered statistically significant: **P* < 0.05; ***P* < 0.005; ****P* < 0.001; *****P* < 0.0001.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The data supporting the findings of this study are available within the paper and its Supplementary Information and Source Data files. Large high-resolution imaging raw data files that were generated and used in this study are available from the corresponding author upon request. Source data are provided with this paper.

Code availability

A Python implementation of the Ca²⁺ image postprocessing and microdomain analysis routines is publicly available (license: Apache 2.0) at Kovacevic D, Woelk L-M, Hussein H, Förster F, Werner R. IPMI-ICNS-UKE/DARTS: DARTS FIMMU 2024 release (Version 1.0.0). Zenodo entry [10459242](https://doi.org/10.5281/zenodo.10459242). The corresponding documentation can be found in ref. 51.

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Author contributions

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3 DISCUSSION

3.1 Summary of implemented results

The aim of this thesis was to understand the Ca^{2+} dynamics in adenine nucleotide-dependent signaling. On the one hand this includes NAADP-dependent Ca^{2+} signaling dynamics which was studied by using a new molecular probe mimicking NAADP. The novel asset to the NAADP toolset - MASTER-NAADP and its control - was characterised here in its deprotected form in lipid-planar bilayer measurements and in a permeabilised cell assay. Further, MASTER-NAADP allows to reliably evoke Ca^{2+} microdomains as well as global Ca^{2+} signals, thus being a *bona fide* NAADP mimic. Initial Ca^{2+} microdomains are defined by localised, highly dynamic and hard to detect short Ca^{2+} elevations. Therefore, the existing live cell high-resolution Ca^{2+} imaging method was extended, by implementing the established post-processing pipeline as an open-source Python module. This allows for sharing the post-processing steps for Ca^{2+} microdomain detection with the broader scientific community. In addition, a general user interface (GUI) allows for easy handling of the module without prior programming knowledge. The implementation of a shape normalisation module, as well as the selection of different deconvolution algorithms enhances the possibilities of post-processing of the acquired imaging data. Furthermore, the role of eATP-dependent Ca^{2+} signaling via P2X4 and P2X7 in CD8^+ T cells was assessed across timescales from (milli-)seconds to days. Here, P2X4 and P2X7 shape the initial Ca^{2+} microdomain formation after TCR stimulation in primary CD8^+ T cells. Globally, these purinergic receptors play a minor role in shaping the sustained Ca^{2+} elevation needed for full T cell activation. Of note, NFAT1 translocation was impaired in CD8^+ T cells from $P2rx4^{-/-}$ or $P2rx7^{-/-}$ mice, and cytokine and activation marker expression (e.g. $\text{IFN}\gamma$, Nur77, granzyme B) were downregulated in these cells. Consequently, proliferation was reduced in $P2rx4^{-/-}$ or $P2rx7^{-/-}$ CD8^+ T cells compared to WT cells.

3.2 Ca^{2+} microdomains: tools for hotspot detection

Local and short-lived Ca^{2+} signaling events can be differentiated into different categories such as:

- Ca^{2+} quarks or sparks, involving one or clusters of RyR (Cheng et al. 1993; Lipp and Niggli 1998)
- Ca^{2+} blips or puffs, with single IP_3R or clusters of IP_3R openings (Bootman et al. 1997)
- Ca^{2+} sparklet, due to short opening of PM Ca^{2+} channels (e.g. Transient receptor potential channel vanilloid subfamily 4 (TRPV4)) (Sonkusare et al. 2012)
- Ca^{2+} microdomains (reviewed in: Diercks 2024)

(reviewed in: Barak and Parekh 2020; Guse et al. 2021; Montoya et al. 2023).

Ca^{2+} microdomains can be defined as subcellular, highly localised and short-lived Ca^{2+} signaling events (Barak and Parekh 2020), with rises in Ca^{2+} of a few hundred nanomolar and extending on 1-3 microns in each Ca^{2+} microdomain. Amplitude, frequency and duration of a Ca^{2+} microdomain determine which underlying signaling pathway will be triggered in different cell types, hence leading to specific global Ca^{2+} signaling patterns. Detection of these Ca^{2+} microdomains is achieved by live-cell fluorescence microscopy and is limited by the spatio-temporal resolution of the imaging technique. To be able to detect and characterise Ca^{2+} microdomains further limitations arise. One, high phototoxicity and photobleaching only allows for low photon doses, as well as out-of-focus light, lead to low SNR in the acquired images (Ettinger and Wittmann 2014). Further, the transient nature of localised Ca^{2+} signals relies on high imaging speed. Hence, the analysis of Ca^{2+} microdomains could be improved by computational restoration of the acquired image sequences to improve SNR. Schetelig et al. (2015) provided a novel semi-automated framework for postprocessing to identify local subcellular Ca^{2+} microdomains (Fig.6; left side), which is now an established tool in our group to analyse Ca^{2+} microdomains, e.g. identifying ORAI1/STIM1&2 and RyR1 as important players for initial Ca^{2+} microdomains in T cells upon TCR/CD3 stimulation (Diercks et al. 2018). One limitation of this postprocessing workflow however is the implementation in MATLAB, as usage, adaption and distribution of the code requires extensive coding skills and an expensive license. Therefore, implementation in an open-source object-oriented programming environment as Python 3 allows for sharing our modular Ca^{2+} microdomain detection pipeline with the broader scientific community (Publication 2.1; Fig.6). Adaption of the postprocessing pipeline by setting up the ratio images first before further postprocessing allows for minimising the timeframe needed to analyse whole data sets for the user. Using the MATLAB pipeline 5-10 min postprocessing time was needed per cell, leading up to 3 days of postprocessing for 1 measurement day, whereas when using DARTS the user sets all bead contacts or stimulus time directly, therefore allowing an 'over-night' analysis of the data without further supervision.

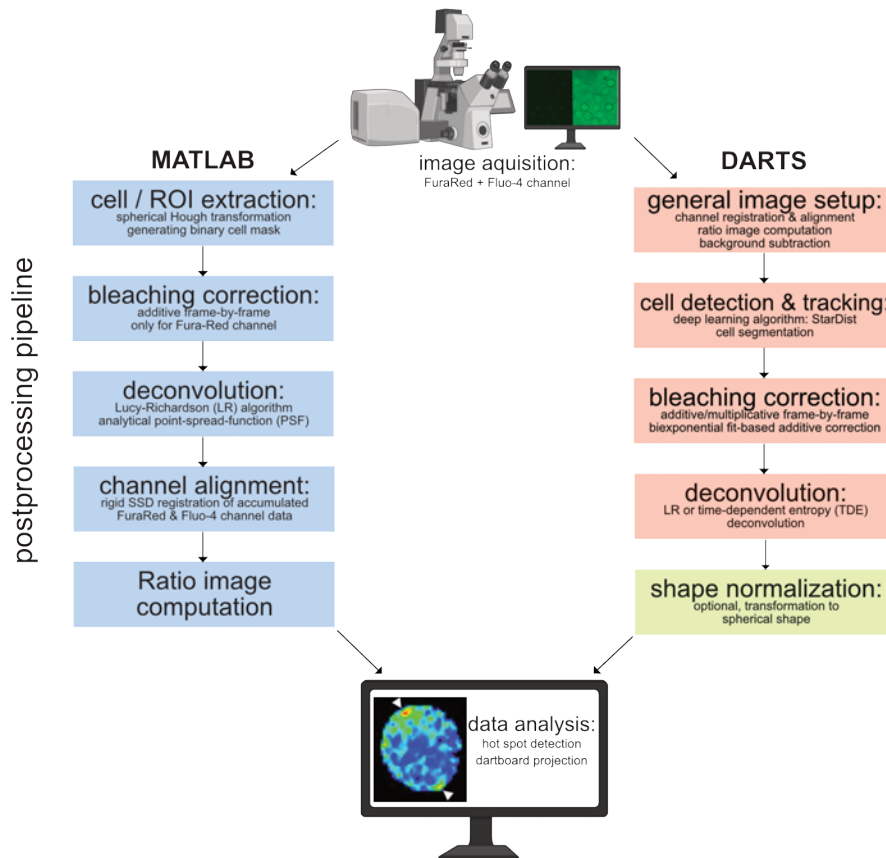


Figure 6: Comparison of image acquisition and analysis workflow for Ca^{2+} microdomains. Modified after Schetelig et al. (2015), Diercks et al. (2019), Woelk et al. (2024)(Publication 2.1)

Further, the postprocessing pipeline is altered (Fig. 6), starting with a general image setup, followed by state-of-the-art implementation of a deep-learning algorithm (StarDist, Schmidt et al. (2018)) for cell detection and cell segmentation. An optional novel step for cell shape normalisation (based on work by Schmidt et al. (2018)) is implemented, allowing to produce the so-called dartboard projection to also non-circularly shaped cells. This is advantageous as Ca^{2+} microdomain dynamics in different populations of cells can be compared, e.g. in Jurkat T cells (Publication 2.1, Fig. 3D,E), which was not possible previously, due to their initial non-round shape and changes in shape of Jurkat T cells upon (antibody-coated bead) stimulation. One limitation of the shape normalization is that the cells to be transformed need some form of convexity/circularity. Despite changes in the quantitative outcome in detection of hotspots for the presented use cases (Publication 2.1, Fig. 2,3) in comparison to the previously published results (Wolf et al. 2015; Diercks et al. 2018; Roggenkamp et al. 2021), the qualitative analysis revealed the same effect as previously published. Of note, comparing the localisation of Ca^{2+} microdomains in Jurkat T cells in the dartboards of shape normalized cells stimulated by either a bead (Publication 2.1, Fig. 3E) or MASTER-NAADP (Publication 2.3, Fig. S5A,B) reveal, albeit differences in the first seconds (due to bead contact), a similar spreading of the signaling pattern in the inner rings of the dartboard.

3.2.1 Deconvolution: a necessary tool to improve SNR to achieve super resolution

Live-cell imaging using a fluorescence microscope captures cellular changes induced by stimuli, but the generated images do not provide the ground truth, as the imaging process itself distorts the object that is imaged, also termed convolution. Consequently, the quantitative analysis of specimens is challenging. Deconvolution algorithms attempt to characterise this distortion and try to restore the convoluted image (McNally et al. 1999). By using DARTS (Publication 2.1) one can choose between two deconvolution algorithms. One is the classical LR (Richardson 1972; Lucy 1974) deconvolution algorithm, which has been extensively used to improve SNR to detect Ca^{2+} microdomains (Wolf et al. 2015; Diercks et al. 2018; Gu et al. 2021; Roggenkamp et al. 2021; Brock et al. 2022; Weiß et al. 2023), (Publications 2.2; 2.3). Especially for low SNR images, the LR algorithm has its challenges to deconvolve the image sequences to a satisfying quality for hotspot detection. The entropy-like deconvolution algorithm by Arigovindan et al. (2013) is an iterative approach using a frame-to-frame deconvolution algorithm, which was extended by integrating the time dependency (time-dependent entropy-like deconvolution, TD ER) ensuring temporal consistency over time (Woelk et al. 2021). Applying TD ER to low SNR images results in a reduction of noise and an enhancement of SNR compared to LR (Woelk et al. 2021) and is provided as an option in DARTS (Publication 2.1). A limiting factor is the high computation time needed for TD ER, as well as a tendency of entropy based approaches to blur high $[\text{Ca}^{2+}]$ spots visually (Woelk et al. 2021). Hence, to analyse Ca^{2+} microdomains from high(er) SNR images, like acquired here (Publications 2.1; 2.2; 2.3), the LR algorithm is sufficient.

To further disentangle the spatio-temporal dynamics of Ca^{2+} microdomains, especially in the spatial dimension, requires improvement of i) the technical setup (e.g. confocal spinning disk super resolution (SoRa)), ii) Ca^{2+} fluophores used and iii) deployment of denoising and deconvolution models. Recent advances in computational modelling and machine learning (ML) using neural networks (deep learning, DL) lead to the development of multiple tools deploying DL methods to analyse live-cell fluorescent imaging data and improve SNR to achieve (or improve) super resolution (SR). Zeroshot deconvolution network (ZS-DeconvNet) is an unsupervised model that can be adapted to different imaging modalities (e.g. 3D widefield microscopy, confocal microscopy, two-photon microscopy), as well as fixed probes and live-cell imaging, enhancing resolution while needing lower fluorescence levels than previously necessary (Qiao et al. 2024). Further, an advantage of such zero-shot models is that no ground truth data is necessary, as well as low as only one training data set (= 1 image) ought to be sufficient to train the DL SR network, being of advantage as state of the art supervised DL SR networks need good quality and large quantity of training data (Qiao et al. 2024). Figure 7 compares the original noisy image of lysosomes with LR deconvolution, which is used as the standard deconvolution method in our established post processing pipeline (Schetelig et al. 2015; Wolf et al. 2015; Diercks et al. 2019), with Sparse deconvolution (Wang et al. 2021) and ZS-DeconvNet (Qiao et al. 2024), showing better spatial resolution of single lysosomes / structures in Sparse

or ZS-DeconvNet deconvoluted images. Of note, the smoothing of signals, especially in the ZS-DeconvNet deconvolved image, could result in hallucinations of signals if trained and used improperly.

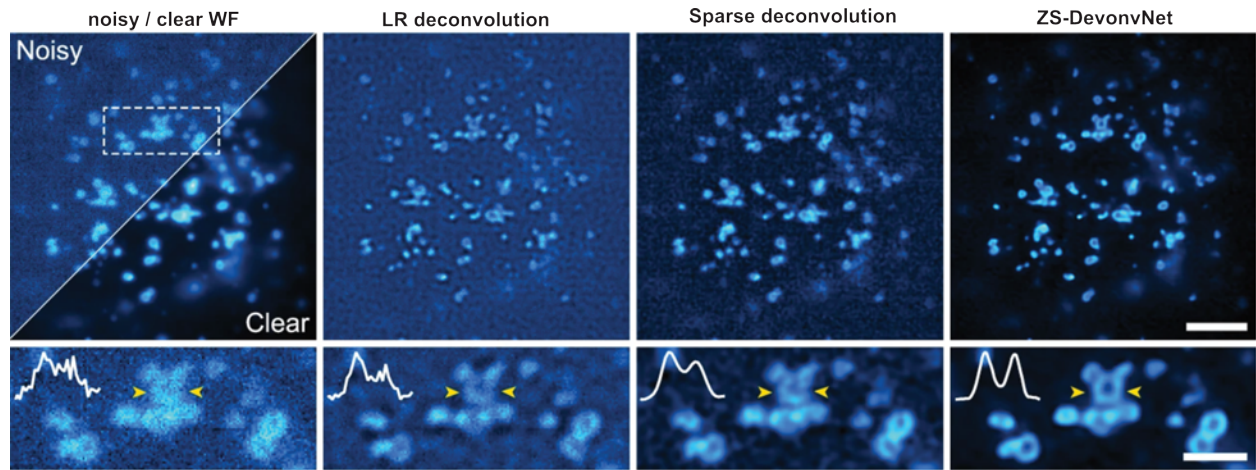


Figure 7: Different deconvolution models applied to live-cell imaging data adapted from Qiao et al. (2024).

Of high interest is the achieved spatio-temporal resolution in a knock-in breast cancer cell line (SUM-159) of EGFP-Rab11 and mCherry-LAMP1, allowing to track recycling of endo-lysosomes for a prolonged and higher resolution than previously possible (Guo et al. 2018) (1500 frames, 3 fps, ~ 150 nm) using ZS-DeconvNet (Qiao et al. 2024). Albeit the temporal resolution of the imaging setup used for Ca^{2+} microdomain detection (Publications 2.1, 2.2, 2.3; e.g. Wolf et al. (2015); Diercks et al. (2018); Gu et al. (2021); Brock et al. (2022); Weiß et al. (2023)) is higher (~ 40 fps), the spatial resolution after LR deconvolution is ~ 2 fold lower. Therefore it would be of interest to test either Sparse deconvolution or ZS-DeconvNet on the imaging data acquired here to possibly further enhance spatial resolution. Others developed a DL approach to analyse local Ca^{2+} signals, being able to robustly differentiate between Ca^{2+} sparks/puffs/waves in cardiomyocytes, which were confirmed by manual analysis of these local Ca^{2+} signals and classification by experts (Dotti et al. 2024). Deploying DL approaches to our Ca^{2+} microdomain data might help to reduce human error and bias, as well as serve to reach computational super resolution.

3.3 Ca^{2+} signaling dynamics in T cells: a complex network

Initial activation of T cells *in vivo* requires a complex interplay of various molecules and second messengers to shape their fate and proper immune response (Hwang et al. 2020). Their cytokine profile and transcription factor expression regulates the division into CD4^{+} T cells, with proinflammatory subsets, e.g. $\text{T}_{\text{H}1}$, $\text{T}_{\text{H}2}$, $\text{T}_{\text{H}17}$ and anti-inflammatory subsets (T_{reg}), as well as cytotoxic CD8^{+} memory and effector subsets (Golubovskaya and Wu 2016). Whether the fate of a T cell can be steered by initial Ca^{2+} microdomains and thus shaping the immune response

is unclear. So far, it is known for CD4^+ T cells that adhesion to extracellular matrix (ECM) proteins, such as laminin-1 and collagen IV, locally activates focal adhesion kinase, PLC- γ and subsequent local store depletion via the IP_3 receptor (Weiß et al. 2023). In addition, preformed clusters of ORA1/STIM1 are already present in this state and allow for local Ca^{2+} influx (Diercks et al. 2018), as well as autocrine P2X4 stimulation further facilitates local Ca^{2+} entry (Brock et al. 2022). These mechanisms allow for low sensitising of the T cell, resulting in lower amplitude Ca^{2+} microdomains (Weiß et al. 2023; reviewed in: Diercks 2024). For full T cell activation both antigen recognition and co-stimulation are necessary, resulting in rapid NAADP formation by DUOX2 (Gasser et al. 2006a; Gu et al. 2021). NAADP-dependent Ca^{2+} microdomains are evoked by NAADP binding to HN1L/JPT2 and subsequent Ca^{2+} release from the ER through RyR1 (Wolf et al. 2015; Diercks et al. 2018; Roggenkamp et al. 2021). The local decrease of ER $[\text{Ca}^{2+}]$ stimulates SOCE through ORA1/STIM1/2, as well as mitochondrial trafficking to the IS, ATP release and autocrine stimulation of P2X4 and P2X7, further elevating $[\text{Ca}^{2+}]_i$ leading to translocation of NFAT-1 to the nucleus and promoting secretion of inflammatory cytokines (e.g. IL-17, IFN- γ) (Lee et al. 2018; Diercks et al. 2018; Gu et al. 2021; Brock et al. 2022; Weiß et al. 2023).

Here, we tried to understand the role of purinergic receptors P2X4 and P2X7 in initial CD8^+ T cell activation and downstream responses (Publication: 2.2). Both, $P2rx4^{-/-}$, $P2rx7^{-/-}$ and depletion of eATP by recombinant apyrase resulted in significantly reduced Ca^{2+} microdomains in the first 15 seconds after bead contact in CD8^+ T cells (Publication 2.2, Fig.1 & 2). This effect is already observable in the first second after bead contact (Publication 2.2., Fig.1C), which is in line with previous findings by Brock et al. (2022) in CD4^+ T cells. Interestingly, the global Ca^{2+} response in both $P2rx4^{-/-}$ and $P2rx7^{-/-}$ CD8^+ T cells is only affected to a minor extent (Publication 2.2, Fig.3), whereas knockout of these purinergic channels significantly decreased global Ca^{2+} signals in CD4^+ T cells (Brock et al. 2022). The signal onset velocity was significantly lowered in $P2rx4^{-/-}$ CD8^+ T cells, and both $P2rx4^{-/-}$ and $P2rx7^{-/-}$ needed significantly longer time to reach 200 nM Ca^{2+} compared to WT CD8^+ T cells (Publication 2.2, Fig.3). Different Ca^{2+} response patterns, e.g. sustained plateaus or oscillations in CD4^+ and CD8^+ human T cells have been attributed to an increased $[\text{Ca}^{2+}]_i$ threshold for activation of CD8^+ T cells due to their cytotoxic properties (Lyubchenko et al. 2001; Arrol et al. 2008), as well as the duration and strength of Ca^{2+} plateaus or oscillations shaping transcription profiles driving T cell subset differentiation and proliferation (Trebak and Kinet 2019). One transcription factor that is activated upon Ca^{2+} microdomain formation at the PM is NFAT-1 (Macian 2005; Kar et al. 2021). Previously, eATP and P2X7 receptors have been associated with NFAT translocation in microglial cells, rat pheochromocytoma neuronal cells (PC12) and Jurkat T cells (Ferrari et al. 1999; Yip et al. 2009; Prasai et al. 2011). NFAT translocation has been linked to INF- γ and TNF- α expression, driving CD8^+ T cell subset differentiation (Teixeira et al. 2005; Xu et al. 2019). In CD8^+ T cells, NFAT-1 translocation is regulated by functional P2X4 and P2X7 upon TCR stimulation (Publication: 2.2, Fig. 4). In both, $P2rx4^{-/-}$ and $P2rx7^{-/-}$ CD8^+ T cells, no or only to a minor extent NFAT-1

translocation could be observed after 7.5 or 15 minutes of bead stimulation compared to the WT (Publication 2.2, Fig. 4B). Further, in WT CD8^+ T cells $\text{TNF-}\alpha$ was significantly increased after 4h of stimulation and $\text{INF-}\gamma$ after 18h (Publication 2.2, Fig. 5A, B). $P2rx4^{-/-}$ resulted in significantly reduced $\text{TNF-}\alpha$ expression, whereas both $P2rx4^{-/-}$ and $P2rx7^{-/-}$ CD8^+ cells showed significantly lowered $\text{INF-}\gamma$ expression, with stronger effect in $P2rx7^{-/-}$. Further, Nur77, a transcription marker associated with TCR signal strength (Ashouri and Weiss 2017), is downregulated in $P2rx4^{-/-}$ and $P2rx7^{-/-}$ from both CD4^+ (Brock et al. 2022) and CD8^+ T cells (Publication 2.2 Fig. 5C). Granzyme B expression and release is an important factor for effective killing of target cells by cytotoxic lymphocytes (Voskoboinik et al. 2015). Here, significant reduction in granzyme B expression from $P2rx4^{-/-}$ and $P2rx7^{-/-}$ cells in comparison to the WT were observed (Publication 2.2, Fig. 5D). Furthermore, the proliferation of $P2rx4^{-/-}$ and $P2rx7^{-/-}$ cells were also negatively affected in comparison to the WT (Publication 2.2, Fig. 5E). These data collectively suggest that both P2X4 and P2X7 play a role in not only shaping the initial T cell activation, but also affecting further downstream effector functions in CD8^+ T cells. ATP affinities of P2X receptors differ, from high-affinity (0.05 - 1 μM) for P2X1, to intermediate concentrations (1 - 10 μM) for P2X4 and low affinity for P2X7 (above 100 μM) (Junger 2011). Albeit both knockout models generated a similar profile, $P2rx4^{-/-}$ resulted in a more pronounced effect in initial Ca^{2+} microdomains and NFAT-1 translocation, whereas the effect of $P2rx7^{-/-}$ was more striking in later timepoints (Publication: 2.2, Fig. 6). At the IS, local ATP secretion via PANX1 and autocrine P2X4 activation is directly induced upon TCR stimulation (Woehrle et al. 2010; Brock et al. 2022), hence signaling via P2X4 is mostly affected in the milliseconds to seconds after TCR stimulation. $P2rx7^{-/-}$ effects later stages, as eATP concentration rises due to release by (other) immune cells or cell death (Junger 2011; Cekic and Linden 2016). It is generally accepted that P2X4, PANX1 and mitochondria must accumulate at the IS directly after TCR stimulation for proper T cell activation, where mitochondria produce ATP and act as a Ca^{2+} buffer, release ATP into the synaptic cleft via PANX1 and triggering autocrine stimulation of P2X4, resulting in Ca^{2+} influx necessary for mitochondrial ATP production by oxidative phosphorylation (Quintana et al. 2011; Trebak and Kinet 2019; Ledderose and Junger 2020). Recently, Ledderose et al. (2021) identified P2Y11, which has similar ATP affinities as P2X4, as responsible for mitochondrial trafficking along microtubuli to the IS. Further, redistribution of P2Y11 to the back of a polarised human T cells induces cAMP/PKA signaling to stabilise the polarised state (Ledderose et al. 2021). In rodents, such as mice, P2Y11 is not expressed (Kennedy 2017), hence studying its implications in Ca^{2+} microdomain formation in our model renders difficult. Proposedly, adenosine receptors such as A2A or A2B receptors could take over P2Y11s role in rodents, but further investigation is needed (Ledderose et al. 2021). Very recently, Winterberg et al. (2026) identified activation of DUOX2 in a TCR independent pathway by autocrine A2A receptor stimulation and subsequent DUOX2 phosphorylation through a PKA isoform. Further, NAADP-dependent Ca^{2+} microdomain formation could be stimulated by A2A activation and subsequent cAMP production through extracellular addition of adenosine (Winterberg et al. 2026), linking purinergic signaling and

NAADP-dependent Ca^{2+} signaling directly.

3.4 The NAADP toolbox: NAADP-AM under the magnifying lens

The development and synthesis of NAADP-AM by Parkesh et al. (2008), using the same synthesis route as described for cAMP-AM, cGMP-AM (Schultz et al. 1993) and IP_3 -AM (Li et al. 1997), contributed a novel tool to simplify studying NAADP-dependent Ca^{2+} signaling in intact cells. Albeit reporting stability issues in the primary publication (Parkesh et al. 2008), NAADP-AM was used in a wide range of publications to study NAADP-dependent Ca^{2+} signaling, highlighting the need for a user-friendly tool in the NAADP community. Attempts to synthesise NAADP-AM by our collaboration partners failed under testing various conditions (Ruthenbeck 2018; Dekiert 2021). Furthermore, HPLC analysis of purchased NAADP-AM lots presented in this study revealed a mixture of compounds and only negligible traces of NAADP after digestion with pig liver esterase (Publication 2.3, Fig. S9, 10) and in one lot small trace amounts of substrate NAADP were detected as an impurity (Publication 2.3, Fig. S10C). This compound mixture probably derives due to the non-selective reaction sites at the NAADP molecule and the low stability of AM esters (Srivastva and Farquhar 1984; Iyer et al. 1989; Ruthenbeck 2018). Parkesh et al. (2008) state in their original publication four possible AM-masking sites for NAADP, one at the pyrophosphate backbone, two at the 2'-phosphate of the adenosine part and one masking the carboxylic acid, resulting in a reduction of the highly negative charge of the NAADP molecule and thus making it membrane permeant. After the synthesis reaction, purification of NAADP-AM was attempted by flash-column chromatography, but resulted in multiple inseparable bands due to rapid hydrolysis; hence, the compound was used without further purification in their verification process (Parkesh et al. 2008). Acetoxymethylation of pyrophosphate moieties in small molecules like dinucleotides has been reported to result in unstable products (Bonnaffé et al. 1996; Jessen et al. 2008), further emphasising the challenge to use AM groups as a masking concept for NAADP.

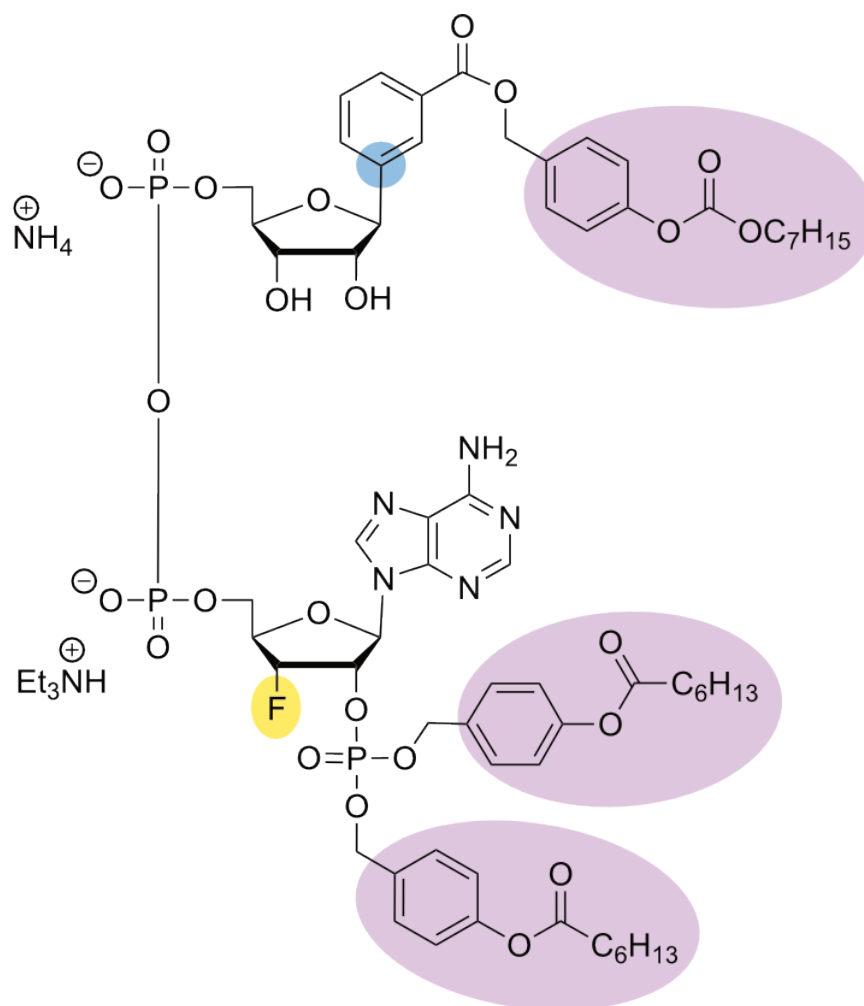
Reported NAADP-AM concentrations in the literature highly differ, spanning from low nanomolar to high micromolar concentrations, hence a variety of NAADP-AM concentrations (100 nM to 100 μM) and multiple (purchased) lots were used here (Publication 2.3, Fig. 5, S8). Interestingly, even at the highest concentration, no differences between the DMSO control and NAADP-AM could be found in the Ca^{2+} mobilizing activity in Jurkat T cells, but these cells still reacted to the stimulation of the TCR/CD3 complex (Publication: 2.3, Fig. S8). In contrast, low nanomolar doses of NAADP-AM induced Ca^{2+} flux in murine memory CD4^+ T cells which could be antagonised by Ned-19 (Chakraborty et al. 2024). Also, NAADP-AM induced Ca^{+2} release in cytotoxic T cells, showing the characteristic NAADP bell-shaped concentration-response curve (Davis et al. 2012).

Off-target effects of remaining substrate NAADP in NAADP-AM lots should be considered. A study in naive CD4^+ T cells examined Ca^{2+} inducing dynamics of exogenous NAADP addition

(Ali et al. 2016), which surprisingly resulted in a similar bell-shaped concentration-response curve as NAADP microinjection (Gasser et al. 2006a). In contrast, addition of exogenous NAADP in cytotoxic T cells did not result in Ca^{2+} responses (Davis et al. 2012). Early reports in rat astrocytes correlate addition of NAADP in extracellular solution with Ca^{2+} release via an IP_3 -dependent pathway, which ought to be activated via P2Y receptors (Singaravelu and Deitmer 2006). Furthermore, in P2Y11 overexpressing astrocytes, exogenous NAADP resulted in Ca^{2+} release mediated by P2Y11 with a transient Ca^{2+} release dependent on IP_3 and sustained Ca^{2+} release by cAMP/cADPR cyclase signaling cascade and induced Ca^{2+} influx (Moreschi et al. 2008). In addition, exogenous NAADP activated the endogenous P2Y11 in human granulocytes, highlighting NAADP as a potential proinflammatory cytokine (Moreschi et al. 2008). In cardiomyocytes, extracellular NAADP or a P2Y11 agonist resulted in elevated contractility and intracellular elevation of NAADP, cADPR and cAMP, which could be antagonised by NF157 (P2Y11 antagonist) (Djerada and Millart 2013), further providing evidence that exogenous NAADP could activate P2Y11 receptors on the cell surface resulting in elevated $[\text{Ca}^{2+}]_i$. As stability issues for NAADP-AM have been reported by Parkesh et al. (2008) and Zhang et al. (2013) and small traces of NAADP before digestion were found in our hands (Publication 2.3, Fig. S10C), the data acquired using NAADP-AM should be treated with reasonable doubt. Furthermore, it emphasises the need in the community for a reliable membrane-permeant NAADP-derivative to study intracellular signaling cascades induced by intracellular NAADP.

3.5 MASTER-NAADP: a novel member of the NAADP toolbox

As discussed in the previous section, synthesis of NAADP-AM failed in the hands of our collaboration partners and purchased NAADP-AM lots failed to reliably induce $[\text{Ca}^{2+}]_i$ responses. Thus, the need for an easily applicable tool to directly study the NAADP induced signaling pathways remains. In the past, our collaboration partners have introduced a new way to protect nucleoside di-/tri-phosphates by using acyloxybenzyl (AB) and alkoxycarbonyloxybenzyl (ACB) moieties, which can be cleaved by cytosolic esterases or lipases, to introduce membrane permeability for e.g. HIV prodrugs (Jessen et al. 2008; Gollnest et al. 2015, 2016). This masking strategy was applied to develop the **membrane-permeable, stabilised, bio-reversibly protected precursor of NAADP** (MASTER-NAADP; Fig. 8) described here (Publication 2.3).



MASTER-NAADP

Figure 8: Chemical structure of MASTER-NAADP. AB/ACB masks are highlighted in lilac. Substitution of N- to a C-nucleosidic bond is highlighted in blue. The 3'-fluorination is highlighted in yellow. Created with ChemDraw 23.1.2.

Previous work by Dekiert (2021) provided important insights in developing such a novel tool; highlighting the incompatibility of the nicotinic acid with the required reaction conditions and the need to prevent phosphate migration from the 2' to the 3'-OH group in the adenosine part. Hence, the N-glycosidic bond in the nicotinic acid moiety was replaced by a C-nucleosidic bond (Fig. 8: blue; 'northern-part') and the 3'-OH group was replaced by a fluorine atom to prevent 2'-3'-phosphate migration (Fig. 8: yellow; 'southern-part'). Furthermore, three AB/ACB masks were introduced, one at the carboxyl group of the benzoic acid (ACB) and two at the 2'-phosphate of the adenosine (AB) (Fig. 8: lilac), masking the negative charge to allow for membrane-permeability. The synthesis of MASTER-NAADP was achieved by synthesising the 'northern' and 'southern' part separately and finally coupling of the two molecules (Publication 2.3, Fig.1; 2). Additionally, an inactive control compound - MASTER-NADP - was synthesised, lacking the 3'-fluorination (Publication 2.3, Fig. S1). As NADP (or the deprotected mimetic of NADP) should not be able to release Ca^{2+} , 2'-3'-phospho-migration is tolerated. The exact

synthesis route for both compounds is elaborated in the thesis of Krukenberg (2025), thus will not be discussed here.

3.5.1 Design strategy for a new stable membrane-permeant NAADP derivative

The design of a compound mimicking adenine nucleotides needs careful consideration which parts of the molecules can be modified to not lose their activity, but achieve membrane permeability. For NAADP, three important structural parts for its Ca^{2+} releasing ability have been identified by Lee and Aarhus (1997) using NAADP-analogues in sea urchin egg homogenates:

- negative charge at the 3' position of the pyridine ring (Fig. 9, highlighted in red)
- amino group at the 6. position of the adenine ring (Fig. 9, highlighted in blue)
- 2'-phosphate of the adenosine (Fig. 9, highlighted in green)

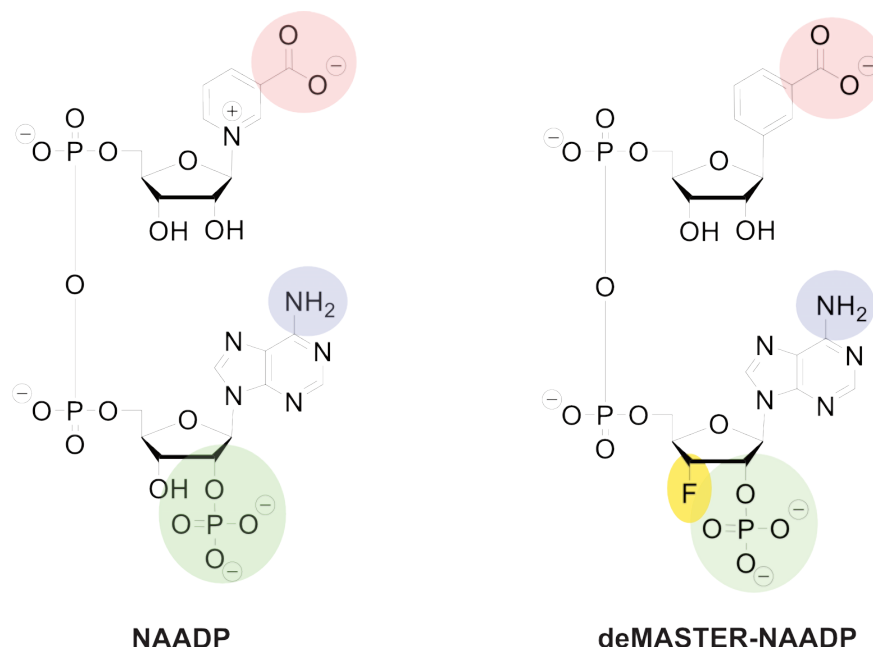


Figure 9: Structural comparison of endogenous NAADP and deprotected MASTER-NAADP (deMASTER-NAADP). Structural similarities are highlighted in red, violet and green, difference in yellow. Created with ChemDraw 23.1.2.

Modification of the 3'-carboxyl group of the nicotinic acid moiety of NAADP by introducing an uncharged carbinol or movement of the carboxyl-group to the 4'-position eliminated Ca^{2+} releasing activity of the molecules (Lee and Aarhus 1997). Changing the 3'-carboxyl group to other negatively charged groups resulted in a 100 to 200 fold EC_{50} shift and modifying the 6'-amino group of the adenine to a hydroxyl group drastically reduced the Ca^{2+} releasing activity of the NAADP-analogue in sea urchin egg homogenates (Lee and Aarhus 1997). The modification of the 2'-phosphate to a 2'-3'-cyclic-phosphate or 3'-phosphate also lead to a 5 to 20 fold higher EC_{50} respectively (Lee and Aarhus 1997). Others reported a complete loss of the agonistic effect by modifying the 4'- position of the nicotinic acid moiety, but a good agonistic effect by modifying the 5'-position, in sea urchin egg homogenates (Jain et

al. 2010). In SKBR3 cells, caged forms of the 4'-/5'-modified NAADP analogues both resulted in concentration dependent Ca^{2+} mobilizing activity with a reduced effectiveness compared to NAADP (Ali et al. 2014), highlighting possible differences between species in the NAADP receptor and agonist recognition.

Taking into account the structural dependencies for NAADPs capacity to release Ca^{2+} revealed by Lee and Aarhus (1997) and others (Jain et al. 2010; Ali et al. 2014), the deprotected form of MASTER-NAADP (deMASTER-NAADP), *benzoic acid C-nucleoside, 2'-phospho-3'-F-adenosine-diphosphate* (Fig. 9: right side), possesses all the necessary parts of the NAADP molecule to act as an agonist (Fig. 9: highlighted in red, violet and green). As described above, the changes introduced were necessary to increase stability of the protected molecule. Furthermore, using molecular modeling, an overlay of the endogenous NAADP molecule and the deMASTER-NAADP revealed high structural similarity (Publication 2.3, Fig. 2C(b)). Thus, deMASTER-NAADP should result in similar Ca^{2+} releasing activity as endogenous NAADP.

3.5.2 Ca^{2+} mobilizing activity of (MASTER-) NAADP

NAADP has long been established as a Ca^{2+} mobilizing adenine nucleotide acting as a second messenger in the field. How and in which timely fashion NAADP is produced and acts upon its binding proteins and receptors is still under debate. Our group has established the role of NAADP in Ca^{2+} signaling in T cells over the last decade. Microinjection of low nanomolar concentrations of NAADP resulted in oscillating Ca^{2+} responses in Jurkat T cells (Berg et al. 2000; Langhorst et al. 2004) and Dammermann and Guse (2005) could identify subcellular Ca^{2+} signals upon NAADP microinjection for the first time. Furthermore, Gasser et al. (2006a) showed that NAADP is produced already after 10 seconds upon OKT3 stimulation in Jurkat T cells. Deciphering the NAADP-producing enzyme DUOX2 (Gu et al. 2021), as well as identifying the NAADP-binding protein in T cells as HN1L/JPT2 (Roggenkamp et al. 2021), indirectly linked NAADP to be crucial for TCR/CD3-dependent Ca^{2+} microdomain formation by using knockout models of either DUOX or HN1L/JPT2. In addition, knockdown or knockout of RyR1 in T cells resulted in lower TDCM (Wolf et al. 2015; Diercks et al. 2018). Hence, it is of interest to study pure NAADP evoked Ca^{2+} microdomains to further elucidate NAADP's role in these highly dynamic Ca^{2+} release events.

Visualisation of such subcellular Ca^{2+} signals on the one hand requires the differentiation between different subcellular signal types and on the other hand tools to identify them. Intracellular Ca^{2+} signals can be classified into two larger subgroups - global and local Ca^{2+} signals. Increase of $[\text{Ca}^{2+}]_i$ throughout the cytosol, extending into the nucleus, describes a global Ca^{2+} signal (Tsien et al. 1985). Using fluorescent Ca^{2+} indicators, such as Fura2-AM, NAADPs role in inducing global Ca^{2+} signaling events was established, e.g. in SKBR3 (Brailoiu et al. 2009) and Jurkat T cells (Steen et al. 2007; Dammermann et al. 2009). MASTER-NAADP was able to evoke global Ca^{2+} signals in different cell types; with working concentrations

ranging between 100 nM in T cells and 100 μ M in KHYG-1 NK or Neuro2a neuronal cell lines and are dependent on HN1L/JPT2 (Publication 2.3; Fig. 5, 6). Delta Ca^{2+} peaks induced by 100 nM NAADP microinjection were in the range of 500 - 750 nM in Jurkat T cells (Steen et al. 2007; Dammermann et al. 2009), whereas here the peak amplitude of the oscillations for acute addition of 100 nM MASTER-NAADP in Jurkat T cells is two to three-fold lower ($\sim$ 200 nM) (Publication 2.3; Fig. 5). On the one hand, the lower amplitude here (Publication 2.3; Fig. 5) might be due to the direct inhibition of SOCE via ORAI channels using Synta66. On the other hand, one can only speculate on the intracellular concentration of deMASTER-NAADP released by endogenous esterases. Generally, the observed signals were higher in amplitude and frequency while performed at 37 degree Celsius. The different working concentrations established here for the different cell types might be due to differences in esterase activity, but also the metabolic stability of deMASTER-NAADP might differ depending on the cell type. Furthermore, the diffusion rate of the compounds needs to be established, as well as if and at what rate the cleaved compound is pumped out again into the extracellular space by xenobiotic efflux pumps. Nevertheless, MASTER-NAADP is a novel tool to study 'pure' NAADP-evoked Ca^{2+} signal, not only globally but also locally.

MASTER-NAADP induces Ca^{2+} microdomains across cell types

NAADP, as a main driver for Ca^{2+} microdomain formation in T cells, has been characterised so far by either microinjection of NAADP (Dammermann and Guse 2005) or indirectly by knockout models of the players involved in the NAADP-dependent signaling cascade in T cells (Wolf et al. 2015; Diercks et al. 2018; Gu et al. 2021; Roggenkamp et al. 2021). Studying MASTER-NAADPs ability to induce Ca^{2+} microdomains (Publication 2.3, Fig. 4A-E) revealed comparable numbers in hotspot detection to TCR/CD3 and CD28 induced Ca^{2+} microdomains in Jurkat and primary murine WT T cells [Gu et al. (2021); Roggenkamp et al. (2021); Wolf et al. (2015); Diercks et al. (2018); Brock et al. (2022); Publication 2.2]. Moreover, acute stimulation of KHYG-1 NK cells with MASTER-NAADP resulted in local Ca^{2+} signals (Publication 2.3, Fig. 4F,G). NAADP production via CD38 in the endo-lysosomes has been linked to Ca^{2+} signaling in IL8 stimulated lymphokine activated killer (LAK) cells (Rah et al. 2010; Nam et al. 2020), as well as an observed reduction in number of signals in KHYG-1 NK cells after BZ194 treatment (unpublished data), provides evidence for a NAADP-dependent Ca^{2+} signaling cascade in these cells. Further, MASTER-NAADP evoked Ca^{2+} microdomains in Neuro2A cells, whereas the control did not evoke any responses (Publication 2.3, Fig. 4 H,I). In mouse hypothalamic neurons, NAADP-dependent Ca^{2+} release from lysosomes via TPCs was crucial for oxytocin secretion (Martucci and Cancela 2022). Glutamate evoked Ca^{2+} response is modulated in a NAADP-dependent manner in mouse hippocampal neurons (Hermann et al. 2020), strengthening the role of NAADP-dependent Ca^{2+} signaling in the brain.

Additionally, MASTER-NAADP was able to induce Ca^{2+} microdomains in human CD4+ T cells derived from healthy donors, whereas the control compound only to a minor extend evoked

Ca^{2+} hotspots in these cells (Fig. 10). Further, 57% of hCD4⁺ T cells responded upon MASTER-NAADP addition, whereas only 25% of cells responded to the control compound, but the $[\text{Ca}^{2+}]_i$ amplitude of responding cells was not significantly altered. Thus, MASTER-NAADP can be established as a novel addition to the NAADP-toolbox to study NAADP-dependent Ca^{2+} microdomains in several cell types.

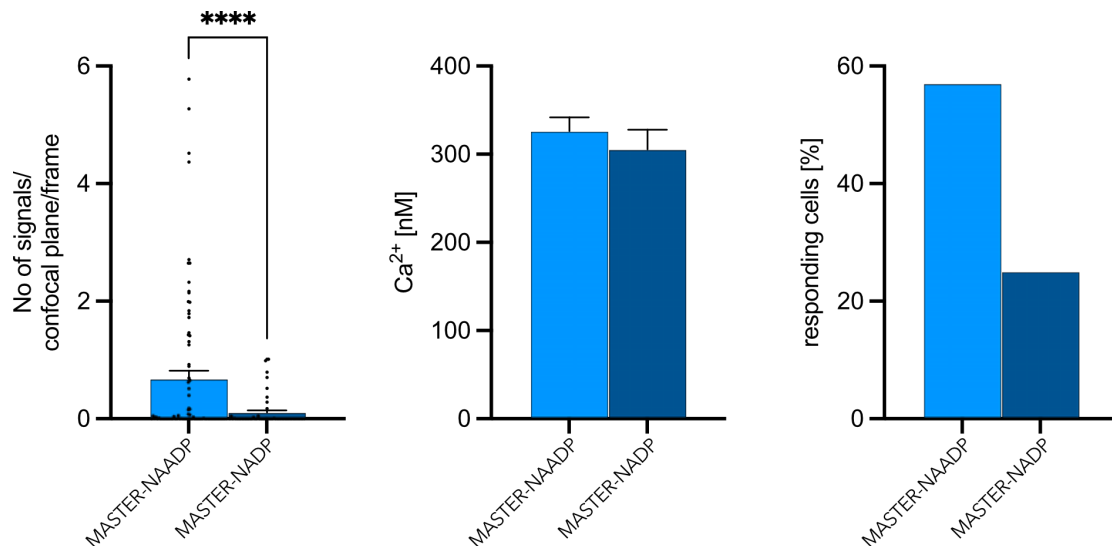


Figure 10: Initial Ca^{2+} microdomains evoked by MASTER-NAADP in human CD4⁺ T cells. Primary hCD4⁺ T cells were isolated from buffy coats by negative selection. The cells were loaded with a combination of Fluo4-AM and FuraRed-AM and Ca^{2+} imaging was performed as described in Material and Methods section of 2.1, 2.2, 2.3. Cells were stimulated with 100 nM MASTER-NAADP or control and the characteristics of initial Ca^{2+} microdomains were analysed using MATLAB post-processing workflow. In every frame of the first 15 seconds Ca^{2+} signals with an amplitude ≥ 112.5 nM and size ≥ 368 nm were detected and the starting point was determined by an exponential fit. Data are presented as means $\pm$ SEM (MASTER-NAADP: n=83 and MASTER-NADP: n=57 cells). Statistical testing was performed using Mann-Whitney U test, **** $p < 0.0001$.

NAADP-dependent signaling axis in T cells revisited

Recent discovery of NAADP-binding proteins HN1L/JPT2 (Roggenkamp et al. 2021; Gunaratne et al. 2021) and LSM12 (Zhang et al. 2021) emphasized the necessity of accessory proteins for NAADP-dependent Ca^{2+} release from internal stores. In primary rat and Jurkat T cells knockout of HN1L/JPT2 resulted in diminished TCR/CD3-evoked Ca^{2+} microdomains and subsequent reduced signal onset velocity, Ca^{2+} peak and plateau phase (Roggenkamp et al. 2021). To directly study the impact of NAADP and its binding proteins on the formation of Ca^{2+} microdomains, addition of MASTER-NAADP, rather than TCR/CD3 stimulation resulting in NAADP production, should be addressed. Accordingly, in *Hn1l/jpt2*-deficient Jurkat T cells (Publication 2.3, Fig. 4C), as well as *Hn1l/jpt2* deletion mutants in Neuro2a cells (Publication 2.3, Fig. 4I), direct stimulation using MASTER-NAADP resulted in diminished Ca^{2+} microdomain formation compared to WT cells, emphasizing deMASTER-NAADP as a potent mimic of endogenous NAADP. Further evidence for the necessity of NAADP-binding proteins was acquired using deMASTER-NAADP in i) a permeabilised cell assay (Publication 2.3, Fig. 3 A-D) and ii) lipid-planar bilayer measurements (Publication 2.3, Fig. 3 E,F), where HN1L/JPT2 addition was necessary to see Ca^{2+} flux in

permeabilised T cells or RyR1 channel opening by deMASTER-NAADP. Further, the de-esterified control compound did neither evoke Ca^{2+} responses, nor RyR1 channel opening in presence of HN1L/JPT2. Of interest, deMASTER-NAADP lacks the observed NAADP-specific bell-shaped concentration-response curve in the presence of HN1L/JPT2, where high levels of NAADP are suppressing Ca^{2+} responses (Berg et al. 2000). This finding is in line with an early study in artificial planar lipid bilayer experiments using partially purified RyR1 preparations (Hohenegger et al. 2002). There, physiological NAADP concentrations were sufficient for channel opening, but the typical bell-shaped concentration-response of NAADP was not observed (Hohenegger et al. 2002), coinciding with the loss of necessary binding proteins in the purification process (Guse 2015). Wagner et al. (2014) reported no effect of NAADP on the open probability of RyR1, which can be explained by the lack of binding proteins in their experimental setup.

RyRs as potential NAADP targets in T cells

In T cells, NAADP action via DUOX2-NAADP-HN1L/JPT2-RyR1 axis was established in a long chain of evidence. *In vitro* studies revealed NOX/DUOX as NAADP-producing enzymes and HN1L/JPT2 as the NAADP-binding protein in T cells (Gu et al. 2021; Roggenkamp et al. 2021). Further, TCR/CD3-mediated Ca^{2+} microdomains were diminished in *Hn1l/jpt2* and *Duox2*-deficient T cells (Gu et al. 2021; Roggenkamp et al. 2021), underlining NAADP as the trigger for initial Ca^{2+} microdomains. Early studies revealed RyR as the NAADP-target receptor in NAADP-mediated global Ca^{2+} signaling (Langhorst et al. 2004) along with initial local Ca^{2+} signals being dependent on RyR, specifically RyR1, expression (Dammermann and Guse 2005; Dammermann et al. 2009). Further, RyR1 proved to be necessary for the initial Ca^{2+} microdomain formation upon TCR/CD3 stimulation and further downstream global Ca^{2+} elevations (Wolf et al. 2015; Diercks et al. 2018). Accordingly, RyR knockdown in Jurkat T cells resulted in diminished Ca^{2+} microdomain formation in the first 15 seconds after stimulation with MASTER-NAADP compared to the WT (Fig. 11).

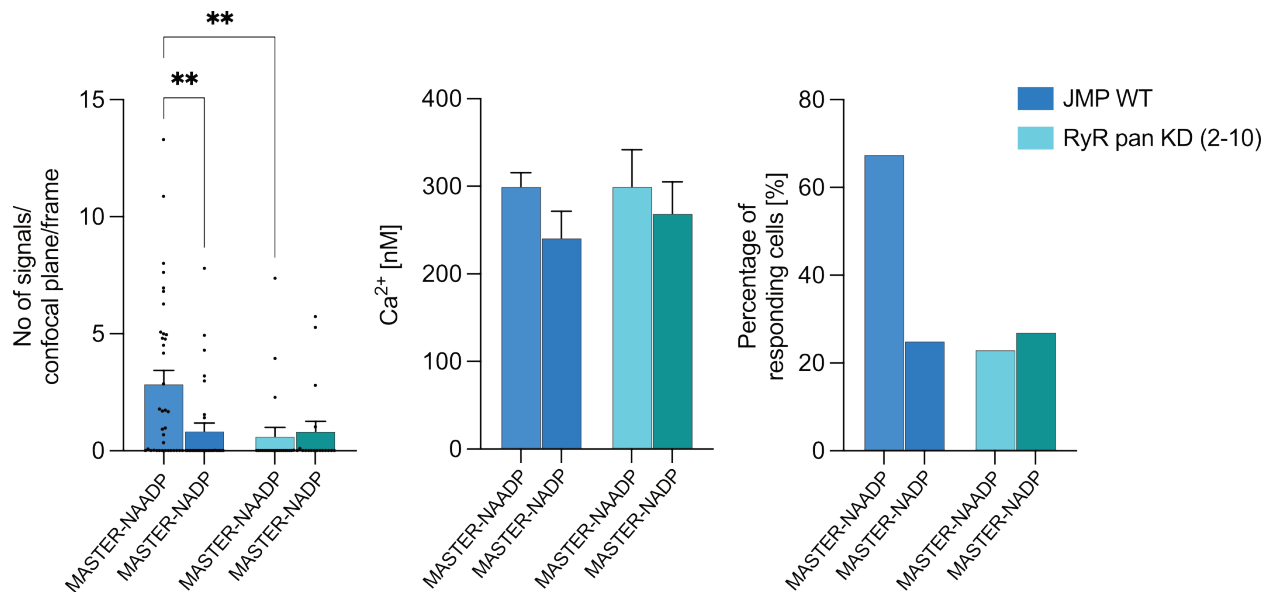


Figure 11: Decreased Ca²⁺ microdomains by MASTER-NAADP in RyR pan knockdown T cells. Jurkat WT (blue) or RyR deficient (turquoise) T cells were loaded with Fluo4-AM and FuraRed-AM and high resolution Ca²⁺ imaging was performed as described in Material and Methods section of 2.1, 2.2, 2.3. Cells were stimulated with 100 nM MASTER-NAADP or control and the characteristics of initial Ca²⁺ microdomains were analysed using MATLAB post-processing workflow. In every frame of the first 15 seconds Ca²⁺ signals with an amplitude ≥ 72 nM and size ≥ 368 nm were detected and the starting point was determined by an exponential fit. Data are presented as means $\pm$ SEM (WT: MASTER-NAADP: n=37, MASTER-NADP: n=31 cells; RyR pan kd: MASTER-NAADP: n=22, MASTER-NADP: n=18). Statistical testing was performed using non-parametric Kruskal-Wallis test and Dunn's correction for multiple testing, ** p < 0.01.

The number of responding cells was threefold reduced in the *RyR* deficient T cells compared to the WT, showing a similar number of responding cells as the WT stimulated with the control compound. In the cells that responded, no differences in the Ca²⁺ amplitude was observed. Of note, the mean responsiveness, a parameter derived by multiplication of number of signals and Ca²⁺ amplitude, allows for rapid comparison of different stimuli among cell types (Wolf et al. 2015).

In addition, comparison of WT T cells stimulated with either α CD3/ α CD28 coated beads (Wolf et al. 2015) or MASTER-NAADP revealed similar mean responsiveness, with a moderate elevation for MASTER-NAADP (Table 2). Knockdown of *RyR* in Jurkat cells resulted in significantly lower mean responsiveness compared to WT T cells independent of the stimuli. Collectively, these data further promote the NAADP-HN1L/JPT2-RyR1 axis as the main driver for initial Ca²⁺ microdomain formation in T cells. Additionally, comparison of MASTER-NAADPs ability to evoke Ca²⁺ microdomains with α CD3/ α CD28 bead stimulation reveals MASTER-NAADP as a valuable tool to study NAADP-dependent Ca²⁺ microdomains directly.

Table 2: Comparison of mean responsiveness of initial Ca^{2+} microdomains in Jurkat T cells upon activation by different stimuli.

<i>Cells</i>	<i>Stimulation</i>	<i>Mean responsiveness</i>
Jurkat WT (Wolf et al. 2015)	$\alpha\text{CD3}/\alpha\text{CD28}$ coated beads	685 ± 231
Jurkat RyR knockdown (Wolf et al. 2015)	$\alpha\text{CD3}/\alpha\text{CD28}$ coated beads	251 ± 151
Jurkat WT (Publication: 2.3; Fig. 11)	100 nM MASTER-NAADP	931 ± 195
	100 nM MASTER-NADP	211 ± 87
Jurkat RyR knockdown (Fig. 11)	100 nM MASTER-NAADP	201 ± 132
	100 nM MASTER-NADP	262 ± 150

TPCs as potential NAADP-mediated endolysosomal Ca^{2+} channels in T cells

TPCs, located primarily in membranes of acidic organelles, such as endosomes and lysosomes, have been proposed to regulate Ca^{2+} homeostasis mediated by NAADP (Steiner et al. 2023). In immune cells, NAADP-mediated Ca^{2+} signaling through TPCs is poorly understood. In cytotoxic T lymphocytes (CTL), TPCs were present in the immunological synapse and NAADP-evoked activation of TPCs triggered the release of perforin and granzyme B (Davis et al. 2012). Others reported activation of the NAADP-TPC pathway as essential for phagocytosis in macrophages (Davis et al. 2020). In contrast, evidence for NAADP-mediated Ca^{2+} release from endolysosomal stores could not be obtained in Jurkat T cells (Dammermann and Guse 2005; Steen et al. 2007). Furthermore, in *tpcn1* and *tpcn2*-deficient CD4^{+} murine T cells initial Ca^{2+} microdomain formation was not altered (Diercks et al. 2018). Hence, to decipher the role of NAADP-mediated local Ca^{2+} signaling in T cells, MASTER-NAADP was used in *tpcn*-deficient CD4^{+} T cells (Fig. 12).

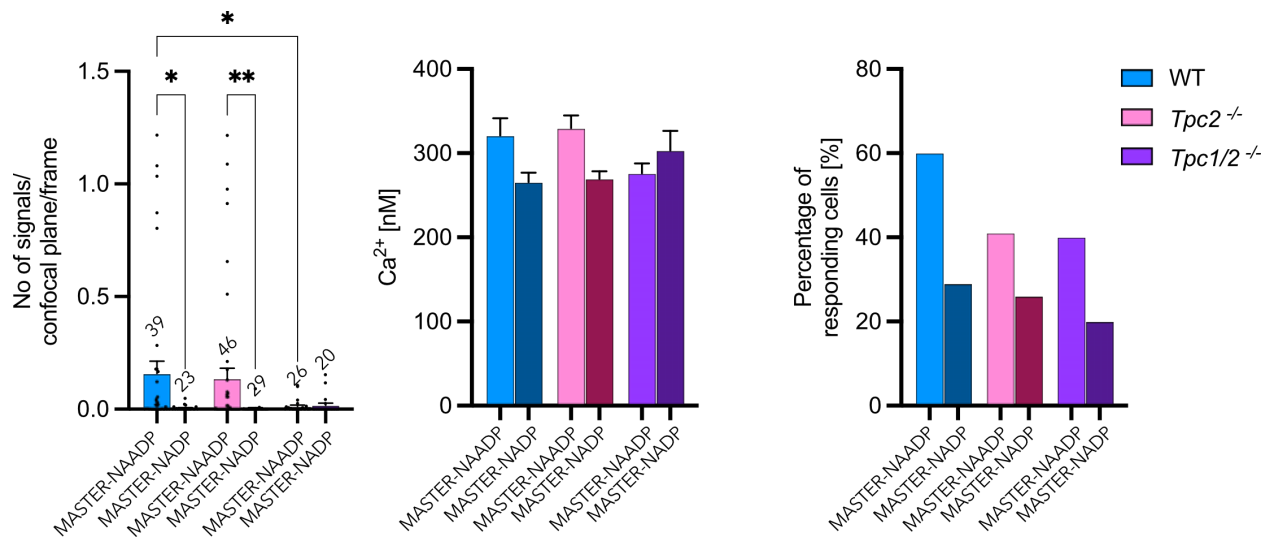


Figure 12: Effect of TPC deletion on Ca^{2+} microdomains evoked by MASTER-NAADP. Primary murine CD4^{+} T cells from WT, *Tpcn2*^{-/-} and *Tpcn1/2*^{-/-} were isolated from spleens and lymph nodes by negative selection. The cells were loaded with a combination of Fluo4-AM and FuraRed-AM and Ca^{2+} imaging was performed as described in Material and Methods section of 2.1, 2.2, 2.3. Cells were stimulated with 100 nM MASTER-NAADP or control and the characteristics of initial Ca^{2+} microdomains were analysed using MATLAB post-processing workflow. In every frame of the first 15 seconds Ca^{2+} signals with an amplitude ≥ 112.5 nM and size ≥ 368 nm were detected and the starting point was determined by an exponential fit. Data are presented as means $\pm$ SEM (WT: MASTER-NAADP: n=39, MASTER-NADP: n=23 cells; *Tpcn2*^{-/-}: MASTER-NAADP: n=46, MASTER-NADP: n=29 cells; *Tpcn1/2*^{-/-}: MASTER-NAADP: n=26, MASTER-NADP: n=20 cells). Statistical testing was performed using non-parametric Kruskal-Wallis test and Dunn's correction for multiple testing, * p < 0.05, ** p < 0.01.

Here, WT as well as TPC2 and TPC1/2 knockout CD4^{+} T cells were stimulated with 100 nM MASTER-NAADP. Both the WT and TPC2 knockout elicited Ca^{2+} microdomains upon MASTER-NAADP addition, whereas the response to the control compound was significantly decreased. Surprisingly, TPC1/2 double knockout resulted in a significant reduction of initial Ca^{2+} microdomains compared to WT cells, similar to the control compound. In contrast, TCR/CD3 stimulation of *tpcn1/2* knockout did not alter initial Ca^{2+} microdomain formation (Diercks et al. 2018). As Ca^{2+} microdomains were neither completely abolished in RyR knockdown (Fig. 11) nor TPC double knockout (Fig. 12) T cells, the question arises whether both TPCs and RyR, as well as NAADP binding proteins, need to be present and potentially interact with each other. As a consequence, (endo)lysosomes must be present in the immunological synapse at PM/ER junctions. Davis et al. (2020) proposed local Ca^{2+} nanodomains formed by Ca^{2+} release from endolysosomes through TPCs independently of ER Ca^{2+} signals to drive phagocytosis. Others reported full NAADP-mediated intracellular Ca^{2+} release to be orchestrated by both RyRs and TPCs in pancreatic acinar cells (Gerasimenko et al. 2015). Further, TPC1 has been postulated as an important regulator for intracellular Ca^{2+} homeostasis in murine mast cells through endolysosomal-ER contact sites (Arlt et al. 2020). However, to date an exact identification of these contact sites remains elusive.

3.6 Outlook

NAADP is rapidly formed upon TCR/CD3 engagement and its binding proteins as well as their respective targets are of utmost importance for the initial Ca^{2+} microdomain formation and subsequent global $[\text{Ca}^{2+}]_i$ elevation. Here, a novel extension to the NAADP toolbox was synthesised and characterised in its Ca^{2+} releasing ability from intracellular stores. MASTER-NAADP was able to evoke local Ca^{2+} microdomains and global Ca^{2+} signals in immune and neuronal cells. Furthermore, the introduction of a shape normalisation tool into the analysis pipeline enables for better comparison of the obtained data regarding the intracellular origin of Ca^{2+} hotspots. Of note, the dartboard projection only segments the cell, hence the real subcellular distribution of these Ca^{2+} hotspots can not be determined exclusively based on the provided data. Another limitation is the definition of the startpoint for the detection of Ca^{2+} microdomains. By using magnetic beads, one can estimate the starting point by eye when the bead first contacts the cell. Using a soluble compound as MASTER-NAADP the determination of the startpoint is more complex. We used an exponential fit approach, where the startpoint was determined in the linear phase before an exponential global signal. Although this procedure has less user bias, we could not detect a starting point for each cell; consequently, we used the average starting point for cells without a starting point. Further optimisation of the DARTS analysis pipeline is needed, implementing a model that can detect a startpoint above a certain local threshold to further reduce user bias.

The established working concentrations of MASTER-NAADP differed quite drastically between cell types, but differences in HN1L/JPT2 protein expression were ruled out (2.3, Fig. S8). This raises the question of how fast the compound is taken up and liberated in the cell and at what rate it is pumped out by xenobiotic efflux pumps. Detection and quantification of adenine nucleotides, in general and NAADP in particular, in cellular lysates or supernatants is challenging due to their hydrophilicity. Hiefner et al. (2023) developed a hydrophilic interaction high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) based method to detect adenine nucleotides in various settings, e.g. derived from plasma, cell culture supernatants and cell lysates. This HPLC-MS/MS-based method should be tested and applied to cells treated with MASTER compounds, as well as the supernatant of these cells, to determine the liberated compound concentration inside the cell. One challenge is the detection limit in the low micromolar to nanomolar range (Hiefner et al. 2023), as low nanomolar concentrations of deMASTER-NAADP, which would be sufficient to mediate Ca^{2+} release, might not be detectable.

Moreover, as mentioned in a recent comment by Mu et al. (2024), MASTER-NADP is not the perfect control as it lacks the 3'-fluorination. Additionally, the synthesis of both MASTER compounds is quite complex and results in rather low yields. Therefore, a second generation of MASTER compounds ought to be synthesised and characterised in their ability to evoke Ca^{2+} signaling events in a similar manner. Possible modifications of MASTER-NAADP could include introducing other protection groups to prevent phosphate migration from the 2' to 3' position,

re-introducing the nicotinic acid moiety in the 'northern part,' as well as changing the lengths and positions of the lipophilic masks. Furthermore, structural dynamics of NAADP bound to HN1L/JPT2 or LSM12 would provide further insights into the moieties necessary for NAADP binding and subsequent action.

Unravelling the detailed formation and dynamics of NAADP-mediated Ca^{2+} microdomains and nanodomains requires live-cell imaging at the highest spatial and temporal resolutions, along with specific, case-based processing and analysis tools. Recent advances in microscopy techniques, e.g. SoRa, allow for improved spatial resolution of up to ~ 150 nm. To release NAADP in a controlled manner, caged-NAADP was synthesised and used in sea urchin egg homogenates resulting in Ca^{2+} release upon photolysis at nanomolar concentrations (Lee et al. 1997). Photocaged probes of NAADP are of advantage, as NAADP can be released in a controlled manner but still need to be microinjected. Therefore, one could combine the MASTER compounds with a nitrobenzyl-based (Gaur et al. 2019) or coumarin-based fluorescent photocage (Kaufmann et al. 2023). This, in combination with the FRAP (fluorescence recovery after photobleaching / photo-activation) module included in our SoRa system, should allow for controlled release of deMASTER-NAADP not only globally but also at localised areas in the cell. Further, transferring the established widefield imaging protocol for Ca^{2+} microdomains to super-resolution Ca^{2+} imaging at the SoRa system should allow for detection of NAADP-mediated Ca^{2+} nanodomains with an anticipated spatial resolution of ~ 120 nm (after deconvolution) and temporal resolution of 30 ms. This, in combination with DL models for deconvolution, might allow for distinguishing between intracellular structures during live-cell imaging, allowing for differentiation between PM/ER or endolysosomal-derived Ca^{2+} release. As discussed above, local Ca^{2+} nanodomains via lysosomes were essential in phagocytosis in macrophages and not dependent on IP_3 -mediated Ca^{2+} release from the ER (Arlt et al. 2020). The data acquired here (Fig. 12) contradicts findings by Diercks et al. (2018), implying a role for TPCs in pure NAADP-evoked Ca^{2+} microdomains in T cells. To further disentangle the NAADP signaling cascade in T cells, a combination of Ca^{2+} nanodomain imaging and the photocleavable MASTER compounds should be applied to WT as well as *ryr1*, *tpcn1*, *tpcn2* and *tpcn1/2* deficient CD4^+ T cells. Additionally, to visualize Ca^{2+} signals directly at the point of origin, low-affinity TPC1/TPC2-GECI constructs are available and ought to be used in combination with the photocleavable MASTER compounds to visualize potential (endo)lysosomal-derived Ca^{2+} nanodomains.

Additionally, not only Ca^{2+} microdomains, but also ATP microdomains have been proposed. So far, identifying eATP concentrations proved to be challenging, due to their rapid diffusion and degradation and low sensitivity of sensors. Kitajima et al. (2020) developed an ATP sensor (ATPOS) with a high ATP affinity in the nanomolar range, which could further be modified using a fluorophore (Alexa488) coupled by the streptavidin-biotin system allowing the sensor to be used ratiometrically. Hence, targeting the ATPOS sensor specifically to the PM of T cells would allow determination of the ATP concentrations in ATP-halos and microdomains upon TCR stimulation.

Furthermore, it would be interesting to visualize PM-mitochondrial contact sites and the Ca^{2+} concentrations produced in these subcellular compartments.

In summary, this work highlights the need for novel tools to study the initial events following TCR stimulation, as well as the complexity of the initial Ca^{2+} mobilisation network in T cells.

4 Publications, awards, conferences and other contributions

ISI publications in chronological order

1. Winterberg KJ, Schwentner V, Gu F, **Möckl F**, Li G, Bauche A, Etzold S, Rosche A, Weiß M, Thuille N, Förster F, Woelk L, Werner R, Kovacevic D, Fehse B, Kurelic R, Nawrocki M, Huber S, Mittrücker HW, Meier C, Müller CE, Baier G, Skålhegg BS, de Deken X, Wahl-Schott C, Mair T, Siebels B, Cugota Canals R, Odoardi F, Lodygin D, Flügel A, Nikolaev VO, Diercks BP, Guse AH. Multiple signaling events are required for NAADP synthesis by DUOX2 and formation of Ca²⁺ microdomains to initiate T cell activation. *Sci Signal*. 2026 Jan 13;19(920):eadp4326. doi: 10.1126/scisignal.adp4326. Epub 2026 Jan 13. PMID: 41529097.
2. **Möckl F**, Kovacevic D, Werner R, Diercks BP, Weiß M. Visualization and Quantification of NFAT1 Translocation in T Cells. *Methods Mol Biol*. 2025;2904:11-19. doi: 10.1007/978-1-0716-4414-0_2. PMID: 40220223.
3. Gerlach F[#], **Möckl F[#]**, Kovacevic D, Brock VJ, Winzer R, Meyer L, Lohr D, Woelk LM, Tolosa E, Werner R, Diercks BP. Imaging Initial Ca²⁺ Microdomains in Primary T Cells. *J Vis Exp*. 2024 Oct 4;(212). doi: 10.3791/67075. PMID: 39431792. [#]equal contribution
4. Krukenberg S[#], **Möckl F[#]**, Weiß M, Dekiert P, Hofmann M, Gerlach F, Winterberg KJ, Kovacevic D, Khansahib I, Troost B, Hinrichs M, Granato V, Nawrocki M, Hub T, Tsvilovskyy V, Medert R, Woelk LM, Förster F, Li H, Werner R, Altfeld M, Huber S, Clarke OB, Freichel M, Diercks BP, Meier C, Guse AH. MASTER-NAADP: a membrane permeable precursor of the Ca²⁺ mobilizing second messenger NAADP. *Nat Commun*. 2024 Sep 13;15(1):8008. doi: 10.1038/s41467-024-52024-y. PMID: 39271671; PMCID: PMC11399135. [#]equal contribution
5. Brock VJ, Lory NC, **Möckl F**, Birus M, Stähler T, Woelk LM, Jaeckstein M, Heeren J, Koch-Nolte F, Rissiek B, Mittrücker HW, Guse AH, Werner R, Diercks BP. Time-resolved role of P2X4 and P2X7 during CD8⁺ T cell activation. *Front Immunol*. 2024 Feb 15;15:1258119. doi: 10.3389/fimmu.2024.1258119. PMID: 38426095; PMCID: PMC10902106.
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 11. Gattkowski E, Johnsen A, Bauche A, **Möckl F**, Kulow F, Garcia Alai M, Rutherford TJ, Fliegert R, Tidow H. Novel CaM-binding motif in its NudT9H domain contributes to temperature sensitivity of TRPM2. *Biochim Biophys Acta Mol Cell Res.* 2019 Jul;1866(7):1162-1170. doi: 10.1016/j.bbamcr.2018.12.010. Epub 2018 Dec 22. PMID: 30584900; PMCID: PMC6646794.

Conferences: Talks, poster presentations & awards

Talks

Franziska Möckl, Sarah Krukenberg, Mariella Weiß, Patrick Dekiert, Melanie Hofmann, Fynn Gerlach, Kai J. Winterberg, Dejan Kovacevic, Imrankhan Khansahib, Berit Troost-Kind, Macarena Hinrichs, Viviana Granato, Mikolaj Nawrocki, Tobis Hub, Volodymyr Tsvilovskyy, Rebekka Medert, Lena-Marie Woelk, Fritz Förster, Li Huan, René Werner, Marcus Altfeld, Samuel Huber, Oliver Biggs Clarke, Marc Freichel, Björn-Philipp Diercks, Chris Meier, Andreas H. Guse (2025) "MASTER-NAADP – Membrane permeAble, Stabilized, biorEversibly pRotected derivative of the Ca^{2+} mobilizing second messenger NAADP," *11th European Calcium Society Symposium: Crosstalk between Ca^{2+} and adenine nucleotides*, **September 2025**, Hamburg Germany, flash talk

Sarah Krukenberg, **Franziska Möckl**, Mariella Weiß, Patrick Dekiert, Melanie Hofmann, Fynn Gerlach, Kai J. Winterberg, Dejan Kovacevic, Imrankhan Khansahib, Berit Troost-Kind, Macarena Hinrichs, Viviana Granato, Mikolaj Nawrocki, Tobis Hub, Volodymyr Tsvilovskyy, Rebekka Medert, Lena-Marie Woelk, Fritz Förster, Li Huan, René Werner, Marcus Altfeld, Samuel Huber, Oliver Biggs Clarke, Marc Freichel, Björn-Philipp Diercks, Chris Meier, Andreas H. Guse (2024) "MASTER-NAADP – Membrane permeAble, Stabilized, biorEversibly pRotected derivative of the Ca^{2+} mobilizing second messenger NAADP," *17th International Meeting of the European Calcium Society*, **September 2024**, Cambridge UK, flash talk

Franziska Möckl, Sarah Krukenberg, Mariella Weiß, Patrick Dekiert, Melanie Hofmann, Fynn Gerlach, Imrankhan Khansahib, Mikolaj Nawrocki, Volodymyr Tsvilovskyy, Tobias Hub, Rebekka Medert, Lena-Marie Woelk, Fritz Förster, René Werner, Marcus Altfeld, Samuel Huber, Marc Freichel, Björn-Philipp Diercks, Chris Meier, Andreas H. Guse (2023) "MASTER-NAADP – Membrane permeAble, Stabilized, biorEversibly pRotected derivative of the Ca^{2+} mobilizing second messenger NAADP" *10th European Calcium Society Workshop: The Ca^{2+} Signaling Toolkit in Cell Function, Health and Disease*, **November 2023**, Leuven Belgium, short talk

Franziska Möckl, Sarah Krukenberg, Mariella Weiß, Patrick Dekiert, Melanie Hofmann, Fynn Gerlach, Imrankhan Khansahib, Mikolaj Nawrocki, Volodymyr Tsvilovskyy, Tobias Hub, Rebekka Medert, Lena-Marie Woelk, Fritz Förster, René Werner, Marcus Altfeld, Samuel Huber, Marc Freichel, Björn-Philipp Diercks, Chris Meier, Andreas H. Guse (2023) "MASTER-NAADP – Membrane permeAble, Stabilized, biorEversibly pRotected derivative of the Ca^{2+} mobilizing second messenger NAADP" *2nd online junior European Calcium Society Meeting*, **October 2023**, online, flash talk

Franziska Möckl, Mariella Weiß, Björn-Philipp Diercks, Patrick Dekiert, Sarah Krukenberg, Tobias Hub, Rebekka Medert, René Werner, Melanie Hofmann, Marc Freichel, Chris Meier, Andreas H. Guse (2022) "MASTER-NAADP: Characterization of a newly developed membrane permeant NAADP derivative" *The 3rd junior European Calcium Society Meeting*, **August 2022**, Cork Ireland, short talk

Poster presentations

Franziska Möckl, Sarah Krukenberg, Mariella Weiß, Patrick Dekiert, Melanie Hofmann, Fynn Gerlach, Kai J. Winterberg, Dejan Kovacevic, Imrankhan Khansahib, Berit Troost-Kind, Macarena Hinrichs, Viviana Granato, Mikolaj Nawrocki, Tobis Hub, Volodymyr Tsvilovskyy, Rebekka Medert, Lena-Marie Woelk, Fritz Förster, Li Huan, René Werner, Marcus Altfeld, Samuel Huber, Oliver Biggs Clarke, Marc Freichel, Björn-Philipp Diercks, Chris Meier, Andreas H. Guse (2025) "MASTER-NAADP – Membrane permeAble, Stabilized, biorEversibly pRotected derivative of the Ca^{2+} mobilizing second messenger NAADP," *11th European Calcium Society Symposium: Crosstalk between Ca^{2+} and adenine nucleotides*, **September 2025**, Hamburg Germany

Sarah Krukenberg, **Franziska Möckl**, Mariella Weiß, Patrick Dekiert, Melanie Hofmann, Fynn Gerlach, Kai J. Winterberg, Dejan Kovacevic, Imrankhan Khansahib, Berit Troost-Kind, Macarena Hinrichs, Viviana Granato, Mikolaj Nawrocki, Tobis Hub, Volodymyr Tsvilovskyy, Rebekka Medert, Lena-Marie Woelk, Fritz Förster, Li Huan, René Werner, Marcus Altfeld, Samuel Huber, Oliver Biggs Clarke, Marc Freichel, Björn-Philipp Diercks, Chris Meier, Andreas H. Guse (2024) "MASTER-NAADP – Membrane permeAble, Stabilized, biorEversibly pRotected derivative of the Ca^{2+} mobilizing second messenger NAADP," *17th International Meeting of the European Calcium Society & junior European Calcium Society Meeting*, **September 2024**, Cambridge UK

Franziska Möckl, Sarah Krukenberg, Mariella Weiß, Patrick Dekiert, Melanie Hofmann, Fynn

Gerlach, Imrankhan Khansahib, Mikolaj Nawrocki, Volodymyr Tsvilovskyy, Tobias Hub, Rebekka Medert, Lena-Marie Woelk, Fritz Förster, René Werner, Marcus Altfeld, Samuel Huber, Marc Freichel, Björn-Philipp Diercks, Chris Meier, Andreas H. Guse (2023) "MASTER-NAADP – Membrane permeable, Stabilized, bioReversibly pRotected derivative of the Ca^{2+} mobilizing second messenger NAADP" *The Calcium and Cell Function Conference, FASEB, June 2023*, Dublin Ireland

Franziska Möckl, Mariella Weiß, Björn-Philipp Diercks, Patrick Dekiert, Sarah Krukenberg, Tobias Hub, Rebekka Medert, René Werner, Melanie Hofmann, Marc Freichel, Chris Meier, Andreas H. Guse (2022) "MASTER-NAADP: Characterization of a newly developed membrane permeant NAADP derivative" *The 16th International Meeting of the European Calcium Society, August 2022*, Cork Ireland

Franziska Möckl, Mariella Weiß, Björn-Philipp Diercks, Chris Meier, Andreas H. Guse (2022) "MASTER-NAADP: Characterization of a newly developed membrane permeant NAADP derivative" *The NAD⁺ Metabolism and Signaling Conference, FASEB, June 2022*, Steamboat Springs USA

Awards

Franziska Möckl, Sarah Krukenberg, Mariella Weiß, Patrick Dekiert, Melanie Hofmann, Fynn Gerlach, Imrankhan Khansahib, Mikolaj Nawrocki, Volodymyr Tsvilovskyy, Tobias Hub, Rebekka Medert, Lena-Marie Woelk, Fritz Förster, René Werner, Marcus Altfeld, Samuel Huber, Marc Freichel, Björn-Philipp Diercks, Chris Meier, Andreas H. Guse (2023) "MASTER-NAADP – Membrane permeable, Stabilized, bioReversibly pRotected derivative of the Ca^{2+} mobilizing second messenger NAADP" *10th European Calcium Society Workshop: The Ca^{2+} Signaling Toolkit in Cell Function, Health and Disease, November 2023*, Leuven Belgium: award for best short talk

Franziska Möckl, Sarah Krukenberg, Mariella Weiß, Patrick Dekiert, Melanie Hofmann, Fynn Gerlach, Imrankhan Khansahib, Mikolaj Nawrocki, Volodymyr Tsvilovskyy, Tobias Hub, Rebekka Medert, Lena-Marie Woelk, Fritz Förster, René Werner, Marcus Altfeld, Samuel Huber, Marc Freichel, Björn-Philipp Diercks, Chris Meier, Andreas H. Guse (2023) "MASTER-NAADP – Membrane permeable, Stabilized, bioReversibly pRotected derivative of the Ca^{2+} mobilizing second messenger NAADP" *2nd online junior European Calcium Society Meeting, October 2023*, online: award for best flash talk

Franziska Möckl, Mariella Weiß, Björn-Philipp Diercks, Chris Meier, Andreas H. Guse (2022) "MASTER-NAADP: Characterization of a newly developed membrane permeant NAADP derivative" *The NAD⁺ Metabolism and Signaling Conference, FASEB, June 2022*, Steamboat Springs USA: Poster award for exceptional poster presentation

Other contributions

Member of the organising committee of the *11th European Calcium Society Symposium*:

Crosstalk between Ca^{2+} and adenine nucleotides (2025)

Member of the junior European Calcium Society Board (jECS) (since 2024)

Mentor in the P4M mentoring program (Postdocs/PhD students for Medical students) of the SFB1328 (since 2022)

5 Eidesstattliche Versicherung

Hiermit versichere ich an Eides statt, die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt zu haben. Sofern im Zuge der Erstellung der vorliegenden Dissertationsschrift generative Künstliche Intelligenz (gKI) basierte elektronische Hilfsmittel verwendet wurden, versichere ich, dass meine eigene Leistung im Vordergrund stand und dass eine vollständige Dokumentation aller verwendeten Hilfsmittel gemäß der Guten wissenschaftlichen Praxis vorliegt. Ich trage die Verantwortung für eventuell durch die gKI generierte fehlerhafte oder verzerrte Inhalte, fehlerhafte Referenzen, Verstöße gegen das Datenschutz- und Urheberrecht oder Plagiate.

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Unterschrift (Franziska Möckl)

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7 Erklärung

Ich versichere, dass dieses gebundene Exemplar der Dissertation und das in elektronischer Form eingereichter Dissertationsexemplar (über den Docata-Upload) und das bei der Fakultät (zuständiges Studienbüro bzw. Promotionsbüro Biologie) zur Archivierung eingereichte gedruckte gebundene Exemplar der Dissertationsschrift identisch sind.

Hamburg, 16.01.2026

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A handwritten signature in black ink, appearing to be 'F. Möckl', written over a horizontal line.

Unterschrift (Franziska Möckl)

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