Acidic Ca^{2+} stores and immune-cell function

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Abstract/Summary

Acidic organelles act as intracellular Ca^{2+} stores; they actively sequester Ca^{2+} in their lumina and release it to the cytosol upon activation of endo-lysosomal Ca^{2+} channels. Recent data suggest important roles of endo-lysosomal Ca^{2+} channels, the Two-Pore Channels (TPCs) and the TRPML channels (mucolipins), in different aspects of immune-cell function, particularly impacting membrane trafficking, vesicle fusion/fission and secretion. Remarkably, different channels on the same acidic vesicles can couple to different downstream physiology. Endo-lysosomal Ca^{2+} stores can act under different modalities, be they acting alone (via local Ca^{2+} nanodomains around TPCs/TRPMLs) or in conjunction with the ER Ca^{2+} store (to either promote or suppress global ER Ca^{2+} release). These different modalities impinge upon functions as broad as phagocytosis, cell-killing, anaphylaxis, immune memory, thrombostasis, and chemotaxis.

Keywords

Calcium, endosomes, lysosomes, TPC, TRPML, immune

1 Acidic Ca^{2+} stores

Ca^{2+} mobilization from intracellular stores is an ancient and conserved signal transduction pathway from plants to mammals. Whilst the study of the endoplasmic reticulum (ER) Ca^{2+} store has led Ca^{2+} signalling studies for decades, another organelle Ca^{2+}-store family – the acidic Ca^{2+} stores – is assuming a prominence in its own right (**Figure 1**). Acidic Ca^{2+} stores encompass a continuum of organelles with an acidic lumen (pH 4.5-6.0) such as lysosomes, endosomes, secretory vesicles, lysosome-related organelles (that include vacuoles, secretory lysosomes and secretory granules), phagolysosomes and autolysosomes, that contain Ca^{2+}-uptake and –release mechanisms (**Figure 1**). We now recognise acidic Ca^{2+} stores as an assortment of highly dynamic, heterogeneous and functionally distinct organelles that differ in their subcellular position, morphology, and function.

What distinguishes acidic Ca^{2+} stores from most other organelles is, of course, their characteristic H^+ content, but other luminal ions (e.g. Ca^{2+}, Cl^-) have emerged as crucial contributors to acidic-vesicle physiology [1]. That these organelles are key for diverse cellular pathways is without doubt; that they also store and release Ca^{2+} may be less familiar but arguably just as essential. Moreover, dysfunction in endo-lysosomal Ca^{2+} homeostasis underlies (or exacerbates) various mammalian diseases [1-7]. Consequently the endo-lysosomal system is much more than the cellular 'stomach' but a pleiotropic 'hub' that organises many biochemical pathways [5]. Such coordination requires communication with other parts of the cell, and endo-lysosomes can send and receive signals via the messenger, Ca^{2+}.

How Ca^{2+} is sequestered into these acidic stores is unclear in mammalian cells but, by comparison with the better understood plants and yeast vesicles (i.e. vacuoles), it is likely to comprise some form of Ca^{2+}/H^+ exchange (**Figure 1**). Luminal Ca^{2+} must be predominantly stored in a complexed, bound form (to avoid precipitation at high concentrations of free Ca^{2+}), but again, we are uncertain as to the Ca^{2+}-binding matrix (it could be a polyanion *e.g.* polyphosphate, or Ca^{2+}-binding proteins). By contrast, in the last decade we have discovered much more about the potential Ca^{2+}-release channels (Section 1.2). The acidic-store Ca^{2+} can be swiftly released via resident Ca^{2+} channels and evoke downstream responses that are often unique from those recruited by Ca^{2+} released from the ER. In other words, this is another Ca^{2+} source that the cell can select to drive specific but diverse processes.

How do acidic Ca^{2+} stores play a role in diverse cellular pathways? First, acidic vesicles can act as *targets* for Ca^{2+}: *e.g.* Ca^{2+} stimulates exocytosis (secretory vesicles), Ca^{2+} is essential for trafficking and fusion (endo-lysosomes) or autophagy (autophagic vacuoles). Alternatively, acidic vesicles act as *sources* of Ca^{2+} to regulate unique downstream processes: *e.g.* endo-lysosomal Ca^{2+}

regulates plasma membrane excitability, cell differentiation, and triggers global Ca^{2+} signals by recruiting the ER Ca^{2+} store [2, 3, 8-10]. There are even occasions when acidic vesicles are both *source* and *target*: in some cell types, the Ca^{2+} for exocytosis can be provided by the secretory vesicle itself in an example of 'paracrine' organelle signalling. These different roles will be highlighted in the immune-cell context in this review.

1.1 Specialized immune-cell acidic Ca^{2+} stores

Many immune cell types have combined features of lysosomes with secretory granules – a dual function secretory-lysosomal organelle – termed a 'secretory lysosome' [11]. The selective use of secretory lysosomes by cells of the immune system means that the mechanisms which control the secretory aspects of the lysosome play a central role in maintaining immunity to infection according to the proteins that each cell type synthesises, stores and secretes. For example, cytotoxic T-lymphocytes (CTLs) and natural killer (NK) cells recognize and destroy tumorigenic and virus-infected cells by the exocytosis of perforin and granzymes from cytolytic granules (**Figure *6*B**). Macrophages, dendritic cells and B-cells (antigen-presenting cells) degrade and process endocytosed material in order to load antigen onto MHC class II molecules (**Figure *6*A,C**). Mast cells and basophils release histamine stored in their dense-core granules upon recognition of IgE to initiate an inflammatory response (**Figure *6*D**). We are now beginning to understand the role of resident Ca^{2+} channels on these secretory lysosomes and their role in immune-cell function; this will be discussed further, below.

1.2 Acidic Ca^{2+}-store channels

Just as the ER/SR possesses multiple channel families stimulated by second messengers (IP_3 or cyclic ADP-ribose), so too is acidic-store Ca^{2+} release stimulated by ligands: the hydrophilic, cytosolic messenger, nicotinic acid adenine dinucleotide phosphate (NAADP) or the membrane-restricted, endo-lysosome-specific lipid, phosphatidylinositol-3,5-biphosphate ($PI(3,5)P_2$). One set of messenger/channel couples is NAADP/TPCs and another is $PI(3,5)P_2$/TRPMLs. However, the fidelity of these couples has been questioned and the reported ability of the messengers to trans-activate multiple channel types has led to notions of messenger promiscuity [6, 12-14]. Whilst some of these effects are very clear under broken-cell experimental conditions, whether a messenger activates multiple channels physiologically requires deeper investigation in some systems.

1.2.1 Two-Pore Channels (TPC)

The TPC family is a member of the superfamily of voltage-gated ion channels that function as dimers [15] and it is comprised of three homologs (TPC1-3), with TPC3 being absent in humans and

mice and only present in a few species including sea urchins [16, 17]. Whilst mammalian TPCs are restricted to the endo-lysosomal system, they exhibit an isoform-selective vesicular distribution; TPC1 shows a broader localization ranging from early to late endosomes and including recycling endosomes, whereas TPC2 is more restricted to late endosomes and lysosomes. Based on gene expression data TPC1 and TPC2 are ubiquitously expressed in cells of the immune system, including B-cells, T-cells, dendritic cells, NK cells and mast cells (***Figure 2***). Of special note is the particularly high expression of endo-lysosomal channels in macrophages, including both TPC1 and TPC2 [18] (***Figure 2***).

As cation-selective channels, and similar to other intracellular Ca^{2+}-release channels, TPCs mediate both monovalent (Na^+, K^+) and divalent (Ca^{2+}) ion fluxes [14]. TPC1/2 can be stimulated by NAADP [19-23], $PI(3,5)P_2$ [24-28] and by voltage [29]. Co-regulation of TPCs by these different stimuli have been reported [30-32]. However, the Ca^{2+}/Na^+ selectivity of TPCs depends upon the stimulus: NAADP activates a higher Ca^{2+}/Na^+ permeability ratio than does the lipid $PI(3,5)P_2$, these are unusual ligand-induced ion-flux changes [13, 31]. That is, TPC2 conducts different ions in response to different activating ligands. This raises the possibility that TPCs could signal under different modalities (Ca^{2+} vs Na^+) simply by recruiting the appropriate 'messenger' or combinations thereof.

1.2.2 Transient receptor potential cation channel, mucolipin subfamily (TRPML)

TRPMLs are non-selective cation channels that conduct Ca^{2+} and Na^+ currents from the lumen of vesicles (spanning the endocytosis and exocytosis pathways) to the cytoplasm [33]. There are three TRPML subtypes – TRPML1, TRPML2, TRPML3 – sharing ~40% amino acid sequence identity [33] and all are activated by $PI(3,5)P_2$ [33]. Additionally, TRPML1 has an intraluminal loop whose protonation also promotes channel activation [34]. More recently, TRPML2 emerged as being the only member of the family that is osmo/mechanosensitive, being activated by hypotonic vesicular swelling [35]. TRPML1 is inhibited by sphingomyelins, phosphatidylinositol-4,5-biphosphate [$PI(4,5)P_2$], and lysosomal adenosine [36, 37]. Na^+ removal or less acidic/neutral pH activate TRPML3 and TRPML2, respectively [33].

TRPML1 is widely expressed in many tissues, including adrenal gland, lung, bladder and placenta as well as in thymus, spleen and immune cells [38]. TRPML3 is also expressed in all major organs, albeit at different levels, with higher levels of expression in the brain, thymus, kidney, lung and spleen [39]. At the single-cell level, TRPML3 is enriched in melanocytes [38, 40] and displays differential levels across different types of macrophage e.g. very low levels or absent in peritoneal macrophages (under basal conditions)[41] but higher in specific-tissue macrophages (CD11b+ lung and alveolar) [41, 42]. Germane to this review, TRPML2 has a more limited expression pattern,

where it is mainly detected in immune cells (***Figure 2***), suggesting a specialized role for TRPML2 in cellular immunity [43]. Interestingly, TRPML2 expression correlates with the presence and/or activity of TRPML1 [44], and so the transcriptional regulation of TRPML2 in immune cells may be under the control of TRPML1. In macrophages, TRPML2 upregulation occurs, not only in response to purified Toll-like Receptor (TLR) ligands, but also to infection with live bacteria (*Mycobacterium smegmatis)* [45].

1.2.3 $P2X_4$ receptor

$P2X_4$ receptor belongs to the family of purinoceptors that are better known as plasma membrane cation channels. However, mammalian $P2X_4$ is also localised intracellularly in lysosomes where it functions as a Ca^{2+}-permeable channel [46]. $P2X_4$ is trafficked to the plasma membrane upon lysosome exocytosis [46]. $P2X_4$ channel is activated by luminal ATP and during alkalization of the lysosomal lumen (remaining inactive in the acidic lysosomal milieu) [47, 48].

1.2.4 Transient receptor potential melastatin-2 (TRPM2)

The transient receptor potential melastatin-2 (TRPM2), is a redox-sensitive Ca^{2+}- and Na^{+}-permeable cation channel that contains a C-terminal enzymatic domain with pyrophosphate activity, which specifically binds ADP-ribose (ADPR) [49]. Whilst the principal channel-gating mechanism is mediated by ADPR [49], in T-lymphocytes and neutrophils, NAADP can synergize with ADPR to enhance TRPM2 activation [50, 51].

TRPM2 is detected in immune cells (neutrophils, megakaryocytes, monocytes, macrophages, B lymphoblast cells, T lymphocytes, and mast cells) [52]. Although TRPM2 channels are primarily located in the plasma membrane where, when activated, they effect intracellular Ca^{2+} signalling, TRPM2 has been reported to function as a lysosomal Ca^{2+} release channel in mature dendritic cells [53]. By contrast, in macrophages, TRPM2 was absent from lysosomes, but present in phagosomes (presumably engulfed from the plasma membrane) [54].

1.2.5 NAADP signalling in immune cells

The best-defined signal transduction pathway coupled to acidic-store Ca^{2+}-release is the second messenger, NAADP, activating two-pore channels (TPCs) via accessory proteins [55, 56], the lysosomal equivalent of the ER's IP_3/IP_3R axis. There is an ever-growing list of cell stimuli that couple to NAADP/TPCs in diverse cell contexts [57-61] and this axis can act in parallel and/or interact with

the canonical ER signalling pathways (IP_3 and cADPR). Immuno-physiologically, a role for NAADP has been steadily increasing, with NAADP implicated in processes including T-cell exocytosis [62], T-cell motility and recruitment [63], effector and naïve T-cell functions cytokine release [64], and in macrophage FcR-mediated phagocytosis [65], which we shall explore in greater depth in this review.

NAADP levels increase in response to either T-cell receptor (TcR) activation (T-cells) [66] or to interleukin-8 (in lymphokine-activated Killer cells) [67, 68]. However, the signal transduction pathway(s) that couples stimuli to NAADP synthesis is frustratingly unclear and several transduction pathways have been implicated in driving NAADP increases in various cell types and include Ca^{2+} itself or cAMP [67, 69-71]. Two different multi-functional enzymes can synthesise NAADP, the ADP-ribosyl cyclase, CD38 [72], and a NAD(P)ase, SARM1 [73]. There is even evidence for Ca^{2+}-mobilizing nucleotide messengers being synthesised within the lumen of acidic vesicles themselves and transported out to act in the cytosol [74, 75]. Although the physiological role of CD38 in NAADP signalling in immune cells remains clouded, CD38 has been linked in leukaemia [72], FcR-mediated phagocytosis [76] and in inflammation and autoimmunity [77, 78]. In B-lymphocytes and macrophages, ligation of plasma membrane CD38 resulted in the internalization of a CD38-LRRK2 (leucine-rich repeat kinase 2) complex and its targeting to the endolysosomal system [79]. This resulted in an NAADP-dependent Ca^{2+} signal and the activation of the transcription factor TFEB [79].

The identity of the Ca^{2+}-release channel targeted by NAADP had long been debated, with several candidate channels proposed over the years including ryanodine receptors (RyRs), TRPML1, TRPM2 and TPCs [80-82]. NAADP may not bind directly to its target channels but rather to an intermediary accessory protein: photoaffinity labelling of various cell extracts (including Jurkat T-lymphocytes) revealed a set of polypeptides (20–45 kDa) to which NAADP binds [83]. Very recently, Jupiter microtubule-associated homolog 2 (JPT2), also known as Haematological and Neurological expressed 1-like protein (HN1L), was identified as an NAADP-binding protein from Jurkat T-lymphocytes [84] and erythrocytes [55]. In the same year, another NAADP-binding protein was discovered, an Sm-like protein, LSm12 [56]. RNA sequencing data show ubiquitous expression of both JPT2 and LSm12 in immune cells, with LSm12 RNA levels consistently greater than TPCs' (***Figure 2***).

LSm12 has the ability to chaperone RNA-RNA and RNA/protein interactions, having important roles in RNA processing and degradation [85, 86], but a role in immune cells is currently unknown. On the other hand, JPT2 has a role in viral infectivity; supporting SARS-CoV-2 translocation through the endolysosomal system [55] (a role ascribed to the TPC complex [87, 88]), and inhibiting apoptosis in response to viral infection [89]. JPT2 also participates in cancer invasion and metastasis;

JPT2 overexpression stimulates cell proliferation, tumor growth, and metastasis, whereas JPT2 knockdown suppresses cell growth and migration [90-93]. TPCs have also recently been implicated in angiogenesis, tumour growth and invasion [94-97], so an NAADP-JPT2-acidic Ca^{2+} store signalling axis might be anticipated. Since macrophages phagocytose and kill tumour cells, and TPC/TRPML and endo-lysosome Ca^{2+} release regulate these processes (Section 3.1.2), a role for JPT2 in macrophage immune responses can also be envisioned.

2 Ca^{2+} Signalling Modalities

As a framework for discussion, we shall group immune cells by the Ca^{2+}-signalling modality that their acidic Ca^{2+} stores have adopted, and we arbitrarily define three modalities: (a) endo-lysosomes acting alone; (b,c) endo-lysosomes interacting with the ER in either positive (b) or negative (c) feedback loops to promote or inhibit ER Ca^{2+} release respectively. These modalities need not be mutually exclusive, and in some cells lysosomes can act both in isolation as well as in concert with the ER, as shall be touched upon.

2.1 Lysosomes Fly Solo

It's worth re-emphasising that the total volume of acidic vesicles in a single cell is small — typically a tenth of the ER's — and therefore they can only maximally release a small total amount of Ca^{2+} compared with the ER and Ca^{2+} influx across the plasma membrane. Indeed, when measuring global cytosolic Ca^{2+} signals, the pure endo-lysosomal Ca^{2+} release component is often too small to even be detected, and this is the net product of a small Ca^{2+} store, a low-conductance and -density of endo-lysosomal Ca^{2+} channels and the limited distance of Ca^{2+} diffusion. By definition, this will constrain endo-lysosomal Ca^{2+} release to small Ca^{2+} nanodomains around the mouth of the channel that are higher than the surrounding bulk cytosol (**Figure 3**). In spite of the apparently diminutive Ca^{2+} signals, this 'solo' modality nonetheless has a profound impact on immune-cell physiology, as we will outline below.

These local endo-lysosomal Ca^{2+} signals are utterly unique in time and space and cannot be substituted by other Ca^{2+} sources, so there is an obligate fidelity between the local Ca^{2+} domain and its Ca^{2+}-decoding downstream pathway, a 'privileged conversation' that no one else can break into. For this premise to hold, the Ca^{2+} decoder should be located intimately with the channel (perhaps in a complex) to sense exclusively the locally high $[Ca^{2+}]$ (**Figure 3**), as has been observed for non-endo-lysosomal Ca^{2+}-channel/decoder couples [98].

2.2 Endo-lysosome/ER Coupling

The dynamic and intimate interaction of endo-lysosomes and the ER at membrane contact sites (MCSs) is a subject of intense investigation across multiple fields of biology [99, 100]. Be it metabolism, organelle dynamics or signalling, a bidirectional flow of information occurs between acidic vesicles and the ER to mutually affect their respective activities. For the purposes of this article, we place emphasis upon anterograde Ca^{2+} signalling from the endo-lysosomes to the ER (while acknowledging that retrograde ER to endo-lysosome signalling may also be of importance [101]). We shall consider first positive and then negative feedback between endo-lysosomes and the ER.

2.2.1 Positive Feedback

The limited concentration and diffusion of Ca^{2+} released from the endo-lysosomes cannot *per se* give rise to substantial cytosolic Ca^{2+} signals and yet endo-lysosomal stimuli patently evoke global Ca^{2+} signals. How can this be? The conundrum is resolved by invoking the 'two-store' or 'trigger model' of Ca^{2+} release whereby a small Ca^{2+} store (endo-lysosomes) secondarily recruits a large Ca^{2+} store (ER) whose Ca^{2+} signals are the ones readily detected. In such a scheme (**Figure 4**), a small amount of Ca^{2+} is released from the endo-lysosomes and this, in turn, activates Ca^{2+} channels on the ER (IP_3Rs or RyRs) via Ca^{2+}-induced Ca^{2+} release (CICR); the ER is therefore the 'amplifier' to the endo-lysosomal 'trigger'. In this way, Ca^{2+} signals that ostensibly emanate from endo-lysosomes can still generate global Ca^{2+} responses via the ER, and often explains why agents that target acidic Ca^{2+} stores (e.g. LLOMe, GPN, ML-SA1, NAADP, nigericin) evoke large global Ca^{2+} signals [102-105].

2.2.2 Negative Feedback

An emerging modality is the inhibition of ER-mediated Ca^{2+} release by endo-lysosomes. Mechanistically, this is somewhat ill-defined but conceivably could be due to any of several possibilities. One way is that Ca^{2+} released by IP_3Rs is avidly sequestered by endo-lysosomes at MCSs to repress the global Ca^{2+} signal [106]. It is difficult to envision this as a simple 'sponge' effect when the limited endo-lysosomal buffering capacity is unlikely to be sufficient to buffer the vast ER Ca^{2+} store, and so it might be instead that endo-lysosomes shape the essential Ca^{2+} feedback at the IP_3R in much the same way as mitochondria do [107]. Alternatively, endo-lysosomes may reduce ER Ca^{2+}-filling, and there are several potential mechanisms; although speculative, perhaps a low level of chronic basal endo-lysosomal Ca^{2+} release raises the IP_3R/RyR open probability via CICR to leak Ca^{2+} and lower the ER steady-state Ca^{2+} concentration. Another possibility is that the ER Ca^{2+} refilling mediated by the SERCA pump is somehow inhibited by endo-lysosome recruitment; there is certainly

a precedent in the heart for Ca^{2+} pumps (and SR store filling) to be regulated by endo-lysosomal Ca^{2+} release [108-110]. Regardless of details, these illustrate that different cell types evince opposing net results of acidic Ca^{2+} store-ER conversations.

3 Acidic Ca^{2+} stores in immune cell function

3.1 Modality I: acidic vesicles acting alone

The most conceptually straightforward modus operandi of endo-lysosomes is that of their acting in isolation to generate unique local Ca^{2+} nanodomains which, in turn, evoke their own downstream physiological correlates (**Figure 3**). Other parallel Ca^{2+} sources (e.g. IP_3Rs, SOCE) which are often activated at the same time cannot substitute and do not drive the same downstream processes. We highlight several examples which explicitly (or implicitly) conform to this mode.

3.1.1 Lymphocyte Secretion

Target-cells can be killed by cytolytic factors such as granzyme B (GrB) and perforin that are rapidly secreted from CTLs and NK cells upon exocytosis (**Figure *6*B**). TCR activation is renowned for its strong Ca^{2+} signalling via the IP_3/SOCE pathways [111, 112] and this is accompanied by the vectorial exocytosis of cytolytic granules (and the release of cytolytic factors) at the immunological synapse of CTLs [113]. However, in our hands, this ER/plasma membrane Ca^{2+} signalling was shown to be largely irrelevant for driving Granzyme B secretion because manipulating IP_3Rs or SOCE failed to impact exocytosis [62]. Something else was needed during TCR activation, and that, we argued, was NAADP/TPC Ca^{2+} signalling [62]: TCR-dependent Granzyme B release was inhibited by the NAADP antagonist, Ned-19, or bafilomycin A1 (an inhibitor of endo-lysosomal Ca^{2+}-store loading in these cells) [62]. Conversely, a cell-permeant form of NAADP (NAADP/AM) efficiently drove Granzyme B secretion (whereas cell-permeant IP_3 did not). *i.e.*, there was a *selectivity* for the acidic Ca^{2+} store pathway and NAADP, and siRNA revealed that this was reliant upon TPC1 and TPC2 [62].

A further twist in the tale was that TPCs were on the acidic lysosome related organelles (the cytolytic granules) themselves, and TPCs accordingly translocated towards the immunological synapse. The elegance of the CTL system is that the secretory vesicles themselves (as lysosome related organelles) act as the acidic Ca^{2+} store that drive their own Ca^{2+}-dependent exocytosis in an 'autocrine' manner by virtue of the TPCs resident on the secretory vesicle membrane [62]. We postulated that privileged local Ca^{2+} nanodomains around TPCs on the acidic Ca^{2+} stores are detected by the neighbouring exocytotic machinery and this locally high Ca^{2+} is what distinguishes NAADP from the other stimuli. As already mentioned, another potential lysosomal Ca^{2+}-channel activator, is the lysosomal lipid, $PI(3,5)P_2$, and this too may drive exocytosis in other contexts. In NK cells it is the $PI(3,5)P_2$/TRPML1 axis that appears to promote GrB release via Ca^{2+} release from endo-lysosomes [114].

Whether the local Ca^{2+} domain is a function of each *individual* channel (**Figure 3**) or a *collective* summation of multiple channels is unclear. For example, the translocation to and polarization of TPCs at the immunological synapse could concentrate Ca^{2+} release at a restricted locus; because the single-channel conductance of TPCs is quite modest (Human TPC1 and TPC2 exhibit a Ca^{2+} conductance of 15-40 pS [31, 115, 116]) the clustering of TPCs at the immunological synapse, could cooperatively generate domains of high [Ca^{2+}] sufficient to activate exocytosis.

TPCs join a list of other ion channels that also cluster at the immunological synapse, including the Orai1/STIM1 complex, and different types of K^+ channels [117-120]. In addition to channels, Ca^{2+}-buffering systems are also recruited to the immunological synapse, including mitochondria [121] and the plasma membrane Ca^{2+}-ATPase [122]. It is tempting to speculate that NAADP provides local Ca^{2+} nanodomains around the cytolytic granules that are isolated from these Ca^{2+} buffering systems (contrasting with ER Ca^{2+} release/Ca^{2+} entry that are sensitive to them), potentially another reason why NAADP is a unique stimulus of exocytosis.

How does our model compare with the existing literature? The sources of Ca^{2+} for vesicular secretion in CTLs has, of course, a long history and it has been known for decades that a Ca^{2+} ionophore by itself cannot evoke exocytosis, in spite of the sizeable Ca^{2+} response [62, 123]. Only when PKC is artificially co-stimulated by phorbol ester [62, 123] or PKC over-expression [124] will degranulation occur in response to ionophore. Mechanistically, Ca^{2+} ionophores elevate cytosolic Ca^{2+} by mobilizing the ER and recruiting SOCE [65, 103, 125], but not the acidic Ca^{2+} stores [126]. This further reinforces that ER/SOCE Ca^{2+} sources are insufficient and require an additional pathway (PKC or NAADP). This is not to say that these energetically expensive global Ca^{2+} excursions are unimportant, rather that they must be driving other downstream roles in these cells (e.g. stimulating mitochondrial ATP production, or longer term responses such as gene expression or cytokine production [127] or proliferation [127]). Then there is the issue of kinetics; studies commonly report outputs after hours of stimulation, e.g. cell killing. Certainly, human patient CTL degranulation and cytolysis appears to be Orai-dependent over these longer time courses [128]. Likewise different CTL types may exhibit differential dependence on global signals (e.g. GrB secretion is decreased in Orai-KO cells [129]).

Interestingly, canonical blockers of voltage-gated Ca^{2+} channels (VGCCs) inhibit T-cell responses such as proliferation [130, 131] and, of relevance here, granule exocytosis [123] and its correlate, cell-killing [132]. Indeed, Ca^{2+} release from T-cell intracellular stores can also be inhibited by these agents, independent of Ca^{2+} influx [131]. Initially, the effect of these blockers was attributed to Ca^{2+}-channel inhibition, but the concentrations that affect T-cells are too high to match effects on

VGCCs and, more damning, it transpires that T-cells (and T-cell lines) do not possess functional VGCCs [133] (but see [134]). Logically, off-site drug targets were invoked as an explanation, and these included K^+ channels [133] and SOCE [135]. However, in view of the more recent realization that these 'Ca^{2+} influx' agents inhibit NAADP-dependent Ca^{2+} release [69, 87, 136], and endo-lysosomal TPCs [25, 137, 138], these old results may be consistent with the NAADP/TPC hypothesis and are worthy of reassessment.

Acidic stores are a primary source of Ca^{2+} for NAADP-mediated Ca^{2+} release in conventional naïve and effector T-cells [64]. Interleukin-2 and interferon γ are synthesised and secreted after T-cell activation; the inhibition of acidic store Ca^{2+} release by the NAADP antagonist Ned-19 reduced the nuclear translocation of NFAT, and consequently expression of NFAT-dependent Interleukin-2 and interferon γ genes [64].

The role of TRPML1 in lymphocytes is somewhat underexplored. In B-lymphocytes, TRPMLs modulate lysosomal morphology [139] and the abnormal accumulation of adenosine within the lysosomes of B-cells is linked to SCID (severe combined immunodeficiency disease) via its inhibition of TRPML1 and cell death [140]; although a demonstrable decrease in TRPML1-dependent Ca^{2+} signalling was evident in these cells, how aberrant TRPML1 signalling resulted in pathogenesis was unclear. The fact that it was specific for TRPML1 channels implies that this is a highly localized lysosome-specific effect.

Clearly, different cell types have adopted different pathways and cell architectures to drive secretion. If T-cells exploit the NAADP/TPC axis, then macrophages potentially utilize $PI(3,5)P_2$ in conjunction with TRPML1 [141] or TPC2 [13], whereas mast cells and NK cells require ER Ca^{2+} release (see Sections **Error! Reference source not found.** and **Error! Reference source not found.**). Why NAADP/TPC2 stimulates exocytosis in T-cells [13, 62] is unclear, but could conceivably depend upon cell-type-dependent levels of expression of channels and/or locale of the Ca^{2+}-decoders.

3.1.2 Phagocytosis

Another complex pathway that is increasingly associated with localized endo-lysosomal Ca^{2+} signalling is phagocytosis. Even more remarkably, different points throughout the entire lifecycle of the phagosome are subject to regulation by endo-lysosomal Ca^{2+} channels, likely using local Ca^{2+} signalling (with one major exception — see below) [6]. Phagocytosis is a specialized form of endocytosis by which a wide range of particles are engulfed and destroyed by cells of the innate immune system (**Figure *6*A**). Particles varying in size from viruses to effete host cells are internalized

into phagosomes which, in turn, fuse with the endo-lysosomes that both acidify and deliver degradative hydrolases to the phagosomal lumen. A raft of professional phagocytes including macrophages, microglia, B-cells, and neutrophils express cell-surface receptors that recognize particles tagged for engulfment by a coating of the opsins such as antibodies or complement i.e. Fc receptors (FcRs) and complement receptors (CRs) respectively [142-144]. Engagement of these receptors by their cognate ligands initiates multifarious cell-signalling pathways that evoke the substantial cytoskeletal and membrane rearrangement and trafficking events required to drive particle uptake.

Ca^{2+} signalling is one such pathway recruited by FcRs and CRs, although its physiological relevance has proven to be polemical [145]. This confusion may, in part, be due to the fact that different phagocytes (and receptors) may rely on different intracellular signals, but another is that, endo-lysosomal Ca^{2+} channels have a more prominent role than was originally appreciated. Invariably recruited in parallel with canonical IP_3 and SOCE pathways, endo-lysosomal Ca^{2+} signals independently drive both early and later phagocytic events. As discussed above (Section 2.1), endo-lysosomes are tailor-made for local Ca^{2+} nanodomain signalling and, as such, couple to unique downstream responses that other Ca^{2+} sources cannot i.e. the physiology obligatorily requires endo-lysosomal Ca^{2+}.

3.1.2.1 Early Phase

In macrophages, phagocytosis has been linked to several endo-lysosomal Ca^{2+} channels including TPC1, TPC2 and TRPML1 [65, 141, 146]. However, selection from this channel palette is nuanced to allow differential channel use, depending upon the cell type, opsin receptor, or even particle size. Even within the same macrophage and FcR, small and large particles recruit different endo-lysosomal channels: TPC1 and TPC2 are universally recruited by all sizes (and shapes) tested (≤ 3-µm diameter), whereas TRPML1 is only activated by large particles (6-µm diameter) [65, 141, 146]. These conclusions were supported by the differential effects of channel knockouts as well as by monitoring Ca^{2+}-channel activation with channel-GECI fusions [65, 141, 146]. This pattern of activation may make more sense when considering the downstream pathways; TPCs appear to be important for dynamin activation (via calcineurin-mediated dephosphorylation), itself a universal phagocytic requirement for actin rearrangement, and scission of the neck of the incoming phagosome [147, 148]. In contrast, TRPML1 selectively couples to lysosomal exocytosis (probably via synaptotagmin-7) which provides additional membrane that is essential for coating larger particles [141, 149]. The TRPML1 pathway is therefore fuelling a specialist emergency supply. This is a remarkable form of extreme signal compartmentation whereby different channels on the same

small vesicle drive different physiological responses. CR-mediated phagocytosis similarly manifests a reliance on TPCs since TPC-blockers or -knockout likewise reduced uptake of iC3b-coated red blood cells [150].

These data collectively raise issues of how coupling fidelity is generated and preserved i.e. how are channels uniquely activated and uniquely decoded? The working hypothesis is that different channels are activated by different second messengers, and that each stimulus recruits its appropriate synthetic pathway(s). In the case of FcR-mediated phagocytosis, we envision that NAADP activates TPCs whereas $PI(3,5)P_2$ stimulates TRPML1. This is not necessarily a 'given' considering the concerns surrounding messenger/channel promiscuity and/or synergy (Section 1.2) with reports of NAADP activating TRPML1 [151-153] and $PI(3,5)P_2$ opening TPCs [24-28]. Indeed, CR-mediated phagocytosis is inhibited by an inhibitor of $PI(3,5)P_2$ synthesis [150]. As for the downstream signalling, it is implicit that the Ca^{2+} nanodomains of one channel family are not only insulated from those of another, but strictly couple to their own Ca^{2+}-sensitive decoder (e.g. Calcineurin, Synaptotagmin-7). The latter implies an intimate proximity (complex) between the Ca^{2+} channel and decoder in 'private' conversation. These frameworks await formal confirmation.

ATP-sensitive $P2X_4$ or $P2X_7$ receptors are unusual purinoceptors localized to the endo-lysosomal system, with the ATP-binding domain facing the lumen [154]. The naturally acidic luminal pH prevents ATP activation, but the channel is stimulated upon a relative vesicle alkalinization [47, 48]. Whilst TRPML1 is thought to couple to lysosomal fission, the $P2X_4$ receptor is believed to couple to lysosomal fusion [48]. There is increasing evidence that $P2X_4$ receptors are important in macrophage-like cells. In macrophages, phagocytosis results in the insertion of lysosomal $P2X_4$ receptor into the plasma membrane, which may lead to increased Ca^{2+} flux through $P2X_4$ receptor channels and the enhancement of pro-inflammatory molecule release [155]. In a murine model of the autoimmune disease, multiple sclerosis, $P2X_4$ receptors drive myelin phagocytosis in microglia, and this improves the clinical outcome [156]. Macrophage $P2X_4$ or $P2X_7$ receptors also enhance the killing of phagocytosed bacteria [157, 158], although there is discrepancy whether this is via promoting phagosome-lysosome fusion [158], or not [157].

3.1.2.2 Later Phase

Following its internalization, the phagosome subsequently undergoes maturation and, finally, resolution; maturation is the development of a luminal environment that kills and/or degrades the ingested particle, whereas resolution is the subsequent 'dissolution' of the phagosome after the job is done. Remarkably, these too are reliant on lysosomal Ca^{2+} channels. Maturation

involves fusion between the phagosome and the lysosome, the latter equipping the hybrid vesicle with an acidifying and hydrolytic capacity to clear the ingested particle. As might be expected, phagosome-lysosome fusion is Ca^{2+}-dependent and is, in this instance, promoted by the lysosomal Ca^{2+} channel, TRPML1 activated by $PI(3,5)P_2$ in macrophages and neutrophils [146, 159]. As already mentioned, $P2X_4$ receptors do not appear to contribute to fusion [157], and it remains to be seen whether TPCs have a role. Certainly, TPCs can promote endo-lysosomal fusion in other systems as well as enhance the lysosomal motility that might favour the interaction [150, 160-162], so this is a testable possibility. Furthermore, TPC1 and TPC2 protein interactomes contain membrane-fusion machinery (syntaxins and VAMPs), including the very VAMP3 that traffics to phagosomes [23, 163, 164]. Certainly, the reduced lysosomal Ca^{2+} content during *Mycobacterium tuberculosis* infection of macrophages [165] would be predicted to alter TPC-induced Ca^{2+} release (and phagosome-lysosome fusion).

The role of endo-lysosomal Ca^{2+} channels in the resolution of the phagosome has only been more recently investigated. Resolution is a relatively under-explored, complex process that coordinates both ionic/fluid movements (osmotic shrinkage) and membrane retrieval (tubulation, scission). The scope for Ca^{2+} channel involvement should therefore be self-evident. To date, workers have studied the resolution of phagosomes or other, related internalized vesicles (entotic vacuoles and macropinosomes). In mammalian or worm macrophages, the TRPML1/$PI(3,5)P_2$ axis was implicated in the resolution of phagosomes, macropinosomes and entotic vacuoles [166]. The system may be more complex, though, if we accept at face value the observation that the NAADP antagonist, Ned-19, also abrogated vesicle resolution [166]. On the other hand, TPCs were definitively invoked as mediators of macropinosome resolution in murine macrophages [150]. Mechanistically, the latter study proffered a novel mechanism whereby Na^+ fluxes through TPCs were the osmotic driving force for vesicle shrinkage [150], rather than via Ca^{2+} nanodomains, but this seems to be, thus far, a unique example of endo-lysosomal Na^+ signalling.

3.1.2.3 Long Term

In addition to these relatively acute roles of channels, long-term phagocytic competency is sustained by recycling lysosomal membrane from the phagosome [167] and in particular is modulated by endo-lysosomal channels, especially by the TRPMLs. By priming macrophages with an initial phagocytosis substrate, subsequent rounds of FcR-mediated phagocytosis and bacterial killing are enhanced by lysosomal expansion [168]; this required TRPML1-dependent stimulation of the transcription factor TFEB [168]. TRPML1 is also important for sustaining an adequate level of phagocytosis during continual exposure to substrates: bacterial degradation in the phagolysosome

releases peptidoglycans that maintain phagocytic capacity by stimulating NFκB [169]; as already noted, TRPML1 is important for phagosome maturation and pathogen killing, and is therefore key to this process.

3.1.2.4 Other Roles

Since macrophages lack typical secretory granules, exocytosis is mediated by other vesicle populations including recycling endosomes and late endosomes/lysosomes [141, 170]. Secretion of the chemokine, CCL2, from macrophages is enhanced by LPS in a TRPML2-dependent pathway [45]. In accordance, activation of TRPML2 with a selective agonist alone was sufficient to induce this CCL2 secretion, and could be traced to this mucolipin isoform that is located in less acidic compartments (early/recycling endosomes) [170].

Finally, endo-lysosomal tubulation is implicated in several immune-cell processes such as antigen processing, immune synapse formation and phagosome maturation (reviewed [171]). The endo-lysosomal morphological change from spheroids to tubules in macrophages was recently shown to be accompanied by luminal gradients of proteolysis, Ca^{2+} and pH along the tubule [172]. Endo-lysosomal tubulation is a dramatic consequence of protein kinase C activation or LPS-stimulation of macrophage and dendritic cells [173, 174]. The advantages conferred by this morphological change are debated and proposals include lysosomal expansion and increased fusogenic potential and motility but, again, TRPML1 has been implicated in contributing to this process [171]. Factors that potentially alter endo-lysosomal tubulation are several e.g. [174-178], but one factor of relevance is endo-lysosomal Ca^{2+} channel activity: TPCs and TRPMLs are pro-tubulation factors and may impact immune cell function [35, 150, 161, 176].

3.2 Modality II: acidic vesicles promote global Ca^{2+} signals

The other modality for which endo-lysosomes are renown is that of secondarily recruiting the ER Ca^{2+}-release systems by CICR (the 'two-pool model'), be they the IP_3R [104] or RyR [179] (**Figure *4***; Section 2.2.1). In cells with a normal cohort of endo-lysosomes, this is the only way in which a globalized Ca^{2+} response can be entrained by vesicular trigger-Ca^{2+} release. Without the endo-lysosomal input, the global Ca^{2+} responses will be smaller, and it transpires that some immune cells exhibit this behaviour.

3.2.1 T-cell Ca^{2+} globalization

Above, we highlighted the importance of the NAADP/TPC axis for Granzyme B secretion, likely via local Ca^{2+} nanodomains, but the same CTLs also provide an example of the typical mechanisms underpinning lysosome-ER coupling and Ca^{2+} globalization. First, cell-permeant NAADP/AM primarily evoked Ca^{2+} release from acidic Ca^{2+} stores and yet it also produced repetitive global Ca^{2+} oscillations [62]. Ca^{2+} oscillations are a hallmark of the pulsatile opening of ER Ca^{2+} channels [180] and these NAADP-stimulated spikes in CTLs were shown to be additionally dependent upon the IP_3Rs on the ER (but not RyRs in our hands, and in contrast to others [181]). Importantly, when the ER was Ca^{2+}-depleted, the pure acidic Ca^{2+} store component evoked by NAADP was undetectable in global signals. This is consistent with the pure endo-lysosomal signals being too small to readily record.

Using cell-permeant messengers, a synergy could be demonstrated between NAADP and IP_3 in CTLs: sub-threshold concentrations of each messenger alone failed to elicit global Ca^{2+} release from the ER, but their co-application was supra-additive to give repetitive spiking [62]. That is, co-activation of both systems was required to give a full-blown response. More physiologically, activation of the TCR likewise required both systems for a full response. In spite of the fact that the pure NAADP component is too small to be detected globally, inhibition of the NAADP pathway (pharmacologically or genetically) significantly reduced the cytosolic TCR Ca^{2+} response. This disproportionate reduction of the global Ca^{2+} signal can only be explained by the synergism between the NAADP and IP_3 pathways during TCR engagement.

3.2.2 Macrophage Ca^{2+} globalization

Similar to CTLs, macrophages also utilize concurrently the two modalities of local and global endo-lysosomal Ca^{2+} signalling. In addition to the above section (Section 3.2.1), endo-lysosomal Ca^{2+} release also contributed to FcR-stimulated global Ca^{2+} signals even though the ‘pure’ endo-lysosomal signals were invisible and only detectable by channel-GECI fusions [65]. In these cells, abrogating endo-lysosomal NAADP/TPC signalling in various ways compromised the global cytosolic Ca^{2+} signals by a disproportionate amount (up to 50%) [65]. Both endosomal TPC1 and lysosomal TPC2 were associated with ER-coupling and globalization in macrophages [65], a duality shared by CTLs [62] and SKBR3 cells [20, 116], but not by HEK293 cells [182].

The interplay between lysosomes and ER has an additional ramification, which relies on a two-way (bidirectional) Ca^{2+} dialogue between the two organelles [69]. Upon FcR activation, TPC2 is activated and generates Ca^{2+} nanodomains that are only detected by the TPC2-G-GECO1.2 [65]. During cell stimulation, such local TPC2 signals are oscillatory, with multiple Ca^{2+} spikes being

entrained by FcR engagement i.e. pulsatile, repetitive TPC2 openings. Perhaps surprisingly, the ER Ca^{2+}-release appears to drive the continuance of these TPC2 openings because when the ER component is removed (by ER store emptying or cytosolic Ca^{2+} buffering) only a single TPC2 Ca^{2+} spike is observed. That is, the lysosome-ER dialogue is essential for maintaining a train of multiple lysosomal Ca^{2+} spikes. The mechanism is currently unknown but, in other systems, the ER has certainly been implicated in maintaining the Ca^{2+} filling state of lysosomal stores [183-185] and promoting NAADP synthesis [69].

Given that there was no role whatever for global Ca^{2+} in the early phases of particle phagocytic uptake, it raises the question of what this energetically expensive Ca^{2+} signal is actually doing. We do not yet know, and can only suggest that there might be other early Ca^{2+}-dependent responses that do not immediately impact particle uptake and/or that longer-term responses (such as gene expression) require prolonged Ca^{2+} signals. Whether other endo-lysosomal channels of the TRPML family globalize Ca^{2+} responses in immune cells has not been explicitly investigated to the best of our knowledge, although TRPML1 activation in fibroblasts and HeLa cells does couple to ER Ca^{2+} release, so there are precedents [186].

3.2.3 Dendritic-cell Ca^{2+} globalization

As shall be described in more detail below (Section 3.3.3.2), dendritic cells are part of the immune surveillance system that mature and migrate to the lymph nodes upon encountering foreign antigens (**Figure *6*C**). Dendritic-cell maturation and chemotaxis is regulated by transient receptor potential melastatin-2 (TRPM2)-mediated lysosomal Ca^{2+} release [53]. TRPM2 is a Ca^{2+}-permeable nonselective cation channel [187], sometimes expressed upon acidic Ca^{2+} stores of melanocytes and pancreatic β-cells [188], and was found to be preferentially expressed on lysosomes in dendritic cells (cf. a plasmalemmal localization in polymorphonuclear leukocytes) [53]. The authors present evidence that lysosomal TRPM2 is gated by another nucleotide second-messenger, ADP-ribose (ADPR), in a pathway stimulated by the receptor for the chemotactic chemokine, CXCL12 [53]. Not only TRPM2 but IP_3Rs are also required for Ca^{2+} release which is at least suggestive of positive feedback between acidic Ca^{2+} stores and the ER in this system [53].

3.3 Modality III: acidic vesicles suppress global Ca^{2+} signals

The final classification embraces immune cells in which the acidic Ca^{2+} stores are inhibitory to global Ca^{2+} signalling (**Figure *5***; Section 2.2.2). Since intracellular global Ca^{2+} signals emanate from the large ER Ca^{2+} store, acidic vesicles appear to modify ER Ca^{2+} release (independent of their ability to shape Ca^{2+} influx [189]). As outlined below, it is the global ER Ca^{2+} signals (and not endo-lysosomal

Ca^{2+} nanodomains) that drive exocytosis in both mast cells and NK cells. Acidic Ca^{2+} stores ultimately repress secretion.

3.3.1 Mast Cells

Endo-lysosomal channels were recently found to impact anaphylaxis at the level of the mast cell [190] in the inhibitory configuration of **Figure *5***. Upon engagement of the $F_c\varepsilon$ receptor by IgE, the release of pro-inflammatory histamine from mast-cell secretory granules is primarily driven by Ca^{2+} released from the ER [191] (**Figure *6*D**). Perhaps unexpectedly, mast cells in TPC1-knockout mice exhibited an enhanced release of histamine per cell, even though mast-cell numbers were down [190]. Secretory granule size and number were unchanged in TPC1-KO cells, but the histamine content was threefold higher. However, the histamine content alone could not fully explain the TPC1-KO phenotype because vesicular fusion with the plasma membrane (as measured by capacitance) was also increased. Together, the data indicate that TPC1 is inhibitory to degranulation at the level of the exocytotic pathway.

A key point is that the suppression of histamine release by TPC1 is not merely a chronic adaptation to TPC1 gene deletion because acute application (over minutes) of pharmacological modulators of TPC1 function in wild-type cells had a similar effect: the TPC channel-blocker, tetrandrine, enhances secretion whereas the TPC activators, NAADP or $PI(3,5)P_2$, reduce it. Considering that ER-dependent Ca^{2+} release is the stimulus for exocytosis, could TPC1 be suppressing this essential signal? This indeed appeared to be the case because IgE-induced Ca^{2+} release from intracellular stores was enhanced by either TPC1 deletion or inhibition (including by the NAADP antagonist, Ned-19) [190]. Ultimately, the authors demonstrate directly with luminal ER Ca^{2+} recordings that, in wild-type cells, TPC1 acutely reduces the Ca^{2+} content of the ER Ca^{2+} store (hence the inhibition of the cytosolic Ca^{2+} signals). This ER-Ca^{2+} reduction is the primary reason for a suppression of histamine release.

The perplexing mast-cell circuitry that underlies this inhibitory interaction between endo-lysosomes and the ER is yet to be uncovered, although as already mentioned, there are precedents in the literature for mutual effects of one organelle on the Ca^{2+} loading of the other [183-185]. This inhibitory modality of endo-lysosomal Ca^{2+} signalling (**Figure 5**) may well occur in privileged MCSs between TPC1-positive endosomes and the ER, with TPC1 able to promote MCS formation in some cell types [192]. How, potentially, an increased juxtaposition might result in partial ER-depletion is less clear but might conceivably involve altered endosome-ER Ca^{2+} recycling [184] or low-level CICR at ER IP_3Rs or RyRs to induce a Ca^{2+} leak (although endosome-ER coupling might be cell-type specific for TPC1 e.g. compare [193] and [20]).

3.3.2 Natural Killer Cells

Natural killer (NK) cells are analogous to CTLs in that they respond to target-cell presentation with the formation of an immunological synapse and secretion of cytolytic factors such as Granzyme B to kill the target [194] (**Figure *6*B**). Although there are far fewer secretory lysosomes in NK cells than in mast cells or neutrophils, their exocytosis is still Ca^{2+}-dependent [194, 195] and up-regulated by lysosomal biogenesis [195]. Secretory lysosome exocytosis not only delivers luminal factors to kill the target, but also inserts unique, densely packed lipids into the NK-cell's own plasma membrane which affords self-protection against the released cytolytic factors [196].

NK cells have an advantage over T-cells in that they do not require self-markers of MHC class I and can act quickly without antibodies. Rather than expressing the T-cell receptor or B-cell receptor, NK cells express a range of receptors loosely grouped as Activating or Inhibitory. One feature of NK cells is their ability to adjust to their environment in a form of immunological memory, and so-called 'NK education' translates 'self' inhibitory signals into, paradoxically, *enhanced* Granzyme B and cell killing.

Unexpectedly, self-specific inhibitory receptors impact endo-lysosomes: Granzyme B expression is enhanced and stored in secretory lysosomes that are increased in size and relocated closer to the microtubule organizing centre (MTOC); such a phenotype correlates with enhanced exocytosis in educated NK cells when challenged with target cells [114]. The changes to the Ca^{2+} signalling that underlie the secretion are complex and only beginning to emerge, but there is an involvement of the TRPML1/$PI(3,5)P_2$ axis. This lysosomal couple is an inhibitory brake to secretion, because strategies that interfere with the channel or lipid actually *promote* secretion. This is the opposite of the situation in CTLs and is more reminiscent of the inhibitory role of TPC1 in mast-cell degranulation (see above) because NK-cell degranulation is likewise dependent upon ER Ca^{2+} release, be it via PLCγ/IP_3Rs [194] or via TRPM2/ADP-ribose [197]. Is it possible that TRPML1/$PI(3,5)P_2$ suppresses ER-induced Ca^{2+} release and this is a reason why secretion is down? Although not explicitly tested, aspects of the existing data fit such a model in that inhibitors of $PI(3,5)P_2$ synthesis promote larger (probably ER-dependent) Ca^{2+} signals in NK cells activated via the ligation of the receptors FcγRIII or DNAM-1 [114]. Again, an endo-lysosomal Ca^{2+} channel suppresses processes driven by ER-dependent Ca^{2+} release.

Conversely, in a study touching on the Ca^{2+} signals driving NK-cell degranulation (measured by LAMP1/CD107 insertion into the plasma membrane), it was proposed that Ca^{2+} release from acidic Ca^{2+} stores actually *enhanced* secretion [198]. In part, this was supported by the fact that reduced lysosomal Ca^{2+} content/release is believed to be a primary defect in the lysosomal storage

disease, Niemann-Pick C1 (NPC1) [199] and, NK-cell degranulation was reduced in NPC1 (consistent with a stimulatory role for endo-lysosomal Ca^{2+}). Whilst the negative impact of NPC1 on degranulation was quite clear, the conclusions about which Ca^{2+} stores are the primary drive of exocytosis were more equivocal and requires further work.

Taken together, endo-lysosomal Ca^{2+} channels can elicit inhibitory signals, and somehow suppress Ca^{2+} release from the ER and its downstream corollary, exocytosis, in mast and NK cells.

3.3.3 Undefined modes of operation

There are other examples in the literature implicating endo-lysosomal Ca^{2+} channels in immune-cell functions, but detailed Ca^{2+}-signalling analyses were either not performed or were unable to provide sufficient detail to subject them to the classification above. The following sections include these instances and are grouped by cell-type.

3.3.3.1 Antigen presentation

Antigen presentation is central in activating adaptive immunity and is mainly mediated by professional antigen-presenting cells including dendritic cells and macrophages. In mouse macrophages, TRPML1 co-localizes with the MHC-II molecules, and by heteromeric interactions with TRPML2 [200] that also contributes to MHC-II/antigen complex formation [201]. TRPML1 is required for the efficient transport of MHCII to the plasma membrane, from late endosomes/lysosomes [201].

B-lymphocytes possess specialized lysosome compartments within which antigen derived from endocytosed B-cell antigen receptors (BCRs) is processed and loaded onto class II MHC for antigen presentation to T helper cells [202]. The expression of C-terminal GFP fusions of either TRPML1 or TRPML2 in B-lymphocytes caused the development of abnormally enlarged lysosomal compartments to which internalized BCRs are transported [139]. Lysosomal adenosine accumulation impairs lysosome function by inhibiting TRPML1, resulting in lysosome enlargement, alkalinisation and dysfunction, and subsequently leads to cell death in B-lymphocytes in response to oxidative stress [140]. These studies highlight a role for TRPML1/2 in the regulation of the lysosomal compartment of B-cells, as BCR-antigen breakdown and loading onto MHC class II depend on healthy, functioning lysosomes.

3.3.3.2 Dendritic Cells

Dendritic cells (DCs) are antigen-presenting cells that connect the innate and adaptive immune systems. In their immature form, they persistently patrol and ‘sample’ their environment

for danger-associated antigens by continual macropinocytosis of extracellular material [203]. Once an exogenous antigen is encountered, say at a site of infection, the DC rapidly matures with several key outcomes: further sampling is inhibited (macropinocytosis is down-regulated) and the DCs undergo a hasty migration to lymph nodes where they 'cross-present' their precious cargo of antigen to (and thus activate) cytotoxic T-cells in order to do battle [204] (**Figure *6*C**).

Antigens are engulfed and luminally processed and fragments re-presented at their cell surface in a complex with MHC molecules. So specialized are DCs that they have adapted their endocytic and phagocytic pathways [205]. In other cells — phagocytes — phagosomes mature by fusion with lysosomes to lower the organelle pH and increase the luminal proteolytic capacity [206] (Section 3.1.2.2). Internalized antigens are processed by a similar process of lysosomal fusion in DCs, but to prevent counter-productive 'over-digestion' of the antigen, DCs limit lysosomal acidification and express lower levels of lysosomal proteases than do macrophages [207]. Similar to macrophages and neutrophils, fusion of lysosomes with the internalized compartment in DCs seems to occur via a $PI(3,5)P_2$-dependent process [208] which may well implicate TPCs or TRPMLs in antigen presentation (see below).

Activated by danger antigens, the rapid migration of DCs to lymph nodes to alert the resident T-cells is an aspect under control by endo-lysosomal Ca^{2+}. Upon recognising bacteria, the inhibition of macropinocytosis ultimately activates lysosomal Ca^{2+} release by TRPML1 (possibly via an mTORC1-dependent pathway [209]), and this has a two-fold effect. First, activation of the actin-based motor protein myosin II at the rear of the cell, stimulates a rapid migration of mature dendritic cells to the lymph nodes [210]; Rab7b interacts with TRPML1, bridging it to myosin II for the local activation at the cell rear, thereby promoting fast migration [211]. Second, TFEB translocation to the nucleus maintains TRPML1 expression (whose gene expression is controlled by TFEB [210, 212]). Upregulation of TFEB expression during DC maturation (e.g. stimulation of TLR by the bacterial product lipopolysaccharide (LPS) [213]) enhances the transcription of lysosomal genes, leading to enhanced proteolytic activity and thus a reduction in cross-presentation [214]. In summary, inhibition of macropinocytosis is sufficient to trigger Ca^{2+} signalling from lysosomes to induce TFEB nuclear translocation and the actomyosin cytoskeleton rearrangements required for fast dendritic cell migration.

In addition to migrating and cross-presenting antigens to cytotoxic T-cells, dendritic cells promote the proliferative expansion of a different T-cell class, T_{reg} cells, via the chemokine TNF. The surface of DCs is decorated with a transmembrane form of TNF (tmTNF) that activates the TNF-receptor type II (TNFR2) on T_{reg} cells which, in turn, drives their expansion. It transpires that the

levels of active tmTNF at the DC surface are a balance between upregulation by trafficking to the plasma membrane and downregulation through cleavage by the ectocellular enzyme, TACE (TNF-α-converting enzyme) which is itself trafficked to the plasma membrane. Both these processes may be regulated by TPCs [215]. Genetic or pharmacological knockdown of TPC1 or TPC2 then upregulates surface tmTNF levels on DCs and macrophages and thereby promotes T_{reg} proliferation via the TNFR2 [215]. This upregulation of tmTNF was not due to an increase in its mRNA, but was due to a downregulation of TACE upon TPC blockade (due to reduced trafficking that is TPC-dependent); ultimately, this results in a decreased proteolysis of tmTNF [215]. The pathway may be clinically relevant because the use of TPC inhibitors *in vivo* improved a mouse model of colitis via the DC-dependent expansion of T_{reg} cells [215]. Again, endo-lysosomal Ca^{2+} channels act by regulating trafficking.

Finally, we have alluded to the fact that DCs and macrophages constantly sample their environment by macropinocytosis and these internalized foreign molecules elicit their own responses within the surveillance cell (and which may go awry in autoimmune diseases). Toll-like receptors (TLRs) are located either at the plasma membrane or on intracellular organelles [216] and one, TLR7, is located on the lysosome in DCs [217]. TLRs can sense microbial nucleic acids [216] and TLR7 recognises single-stranded ribonucleic acid (ssRNA), a common feature of viral genomes that have been internalised by dendritic cells and macrophages [218]. TLR7-mediated induction of inflammatory cytokines is thus important in antiviral immune responses. The TRPML1/$PI(3,5)P_2$ axis has been reported to be involved in the TLR7 response to ssRNA, in particular by facilitating ssRNA transport to the lysosomes. Li et al. demonstrated in dendritic cells that either the inhibition of $PI(3,5)P_2$ synthesis or loss of TRPML1 function blocked the transport of ssRNA into lysosomes and a consequent reduction in TLR7 activation and cytokine release [219]. These results suggest an important role of the PIKfyve–TRPML1 axis in internalized RNA transport in dendritic cells, and reveal another trafficking process dependent upon TRPML1.

3.3.3.3 Platelets

As a part of their haemostatic role (**Figure *6*E**), platelets contain several classes of acidic vesicles including the secretory vesicles called platelet dense granules (PDGs) and the more abundant α-granules that comprise 10% of the platelet volume [220]. Both exocytotic vesicle types are laden with heterogeneous cargo integral to thrombostasis and they share their origins in acidic late endosomes/multivesicular bodies and, as such, are H^+- and Ca^{2+}-rich [221]. PDGs contain serotonin and adenine nucleotides [161], whereas α-granules are laden with fibrinogen, β-

thromboglobulin, chemokines, and von Willebrand Factor [220]. Mechanistically, the pathways regulating PDG and α-granule secretion are different, if overlapping [220].

That acidic vesicles act as Ca^{2+} stores in platelets has been known for some years and they are differentially mobilized by cell-surface receptors [222-225]. Thrombin and CRP receptors appear to couple to acidic stores via the NAADP pathway. Platelets not only contain high-affinity NAADP-binding sites [223], but NAADP levels increase in response to thrombin [225] and CRP [223]. Sensitivity to the NAADP antagonist, Ned-19, suggests that the Ca^{2+} signals evoked by thrombin and CRP are each dependent upon NAADP [223, 225]. This has downstream physiological consequences because α-granule release, platelet aggregation and cell spreading are correspondingly reduced by Ned-19 [223].

What is less clear is which acidic vesicle classes are the ultimate target of NAADP. Does NAADP mobilize Ca^{2+} from late endosomes/lysosomes or does it act on the secretory vesicles themselves (cf. the cytolytic granules of CTLs [62] – Section 3.1.1)? The V-H^+-ATPase inhibitor, bafilomycin A1, inhibited NAADP-induced Ca^{2+} release in platelet precursor megakaryocyte MEG-01 cells [226], but this doesn't distinguish between acidic vesicle populations. Do the TPC targets shed any light? MEG-01 cells are reported to express both TPC1 and TPC2, and their knockdown reduced responses to NAADP [226]. The distribution of both endogenous and heterologously expressed TPC2 was determined in MEG-01 cells and primary megakaryocytes and converged on TPC2 being located in PDGs [161]. Indeed, the authors proposed that TPC2 activity increases both the PDG luminal pH and local domains of perigranular [Ca^{2+}], both of which were inhibited by Ned-19. The physiological correlate of these ionic changes remains to be determined, but it was hypothesised that they might play a role in PDG maturation and luminal content exchange with other vesicle types [161].

Mechanistically, the picture that emerges may be a somewhat confusing one, and the downstream pathway(s) dependent on acidic-store Ca^{2+} is not immediately transparent. First, does vesicular Ca^{2+} act by itself via nanodomains (**Figure 3**) or by secondary recruitment of the ER (**Figure 4**)? Certainly Ca^{2+} can drive the exocytosis of both AGs and PDGs [227] and cytosolic Ca^{2+} chelation inhibits PDG release [228]. Indeed, there is a long history of Ca^{2+} release from the ER (dense tubular network in platelets) driving the exocytosis of α-granules and of PDGs [229-231].

In view of the fact that that ER (dense tubular system) Ca^{2+}-release may be the primary stimulus of secretion [229, 231, 232], acidic Ca^{2+} stores are more likely to act in concert with IP_3Rs on the ER in a two-pool model (**Figure 4**), but current data do not definitively address this. The identity of the vesicle class potentially interacting with the ER is also confusing: in platelets, the NAADP axis couples to the exocytosis of α-granules, and yet, in megakaryocytes, TPC2 is found upon the other

secretory vesicle family of PDGs. Does this imply that Ca^{2+} mobilized from PDGs trans-activates α-granule release? If it does, it might be via an indirect route where NAADP mobilizes the PDG Ca^{2+} store which in turn enhances IP_3R activation and the global Ca^{2+} signal that drives secretion. Indeed, global Ca^{2+} responses (measured with cytosolic Ca^{2+} reporters) are decreased by Ned-19 [223, 225] which is certainly consistent with this.

4 Conclusion

This non-exhaustive summary of how endo-lysosomal Ca^{2+} channels impinge upon immune-cell function has endeavoured to highlight what is common and what is divergent amongst different immune cells i.e. how individual cell types utilize endo-lysosomal Ca^{2+} signalling in different modalities to very unique ends. Endo-lysosomal Ca^{2+} release is usually a very local, private signalling affair but, in conjunction with the ER, can evoke global Ca^{2+} changes in some cell types. Both these modes are stimulatory, but in some cells, endo-lysosomal Ca^{2+} release has a surprisingly inhibitory impact on the physiology. One consistent and common theme is that endo-lysosomes impact the sundry trafficking and secretory processes that are so integral to immune-cell function. We suspect that there is far more waiting to be found that is attributable to endo-lysosomal Ca^{2+} signalling in immunity.

5 Figure Legends

Figure 1. Ion-transport mechanisms across the endo-lysosomal membrane. Schematic depicting Ca^{2+}-permeable endo-lysosomal channels including TPCs, TRPMLs, TRPM2 and $P2X_4$. Channel-activating second messengers include the lipid $PI(3,5)P_2$, cytosolic nucleotides (NAADP, ADPR) or luminal ATP. LSm12 and JPT2 are NAADP-binding accessory proteins for TPCs. An acidic lumen is maintained by the vacuolar V-H^+-ATPase and one potential route of Ca^{2+} uptake is via some form of Ca^{2+}/H^+ exchange (CHX).

Figure 2. Relative expression patterns of channels and accessory proteins in immune cells. (A) Gene expression of mouse TPC and TRPML endo-lysosomal channels (NCBI genes: *Tpcn1* (252972), *Tpcn2* (233979), *Mcoln1* (94178), *Mcoln2* (68279), *Mcoln3* (171166)) in immune cells, (data displayed relative to *Mcoln2* expression in bone marrow macrophages), analysed using the GeneAtlas MOE430 [233], gcrma dataset, available from biogps.org. The following Probeset was used for: *Tpcn1* 1434930_at; *Tpcn2* 1424304_at; *Mcoln1* 1416671_a_at; *Mcoln2* 1431705_a_at; *Mcoln3* 1437540_at. (B) Expression levels of human *Tpcn1*, *Tpcn2*, *JPT2* and *LSm12* in immune cells shown as normalisation of transcriptomics datasets according to The Human Protein Atlas (available from http://www.proteinatlas.org); RNA HPA blood cell gene data is based on The Human Protein Atlas [234] version 20.1 and Ensembl version 92.38.

Figure 3. Modality I — endo-lysosomes act alone. Model whereby activation of endo-lysosomal Ca^{2+} channels generates a highly localized Ca^{2+} nanodomain centred around the channel itself. Only those Ca^{2+} decoders that are proximal to (associated with) the channels are in the correct locale to detect the locally high $[Ca^{2+}]$.

Figure 4. Modality II — endo-lysosomes secondarily recruit ER Ca^{2+} release. At membrane contacts sites (MCSs), endo-lysosomes provide a small, local release of 'trigger' Ca^{2+} that, in turn, trans-activates IP_3Rs or RyRs on the juxtapose ER via Ca^{2+}-induced Ca^{2+} release (CICR). The substantial ER Ca^{2+}-release is the amplifying step that produces large global Ca^{2+} signals.

Figure 5. Modality III — endo-lysosomes repress ER Ca^{2+}-release. Speculative working hypotheses whereby Ca^{2+}-release from the ER is inhibited by closely apposed endo-lysosomes (likely at MCSs). Different possible paradigms: (1) ER Ca^{2+}-channel opening is reduced by endo-lysosomes sequestering Ca^{2+} that is released by the ER which thereby reduces the positive feedback normally provided by CICR; (2) The stored luminal ER [Ca^{2+}] is lowered by endo-lysosomal channels inducing low-level CICR and increasing the basal ER Ca^{2+} 'leak'; (3) Similarly, the luminal ER [Ca^{2+}] is lowered by endo-lysosomal Ca^{2+}-release activating a pathway that inhibits SERCA on the ER membrane e.g. via a kinase.

Figure 6. Different immune-cell functions. (A) Phagocytes, including macrophages, neutrophils and dendritic cells, engulf and destroy pathogens by phagocytosis, a series of processes: internalization into the vesicular phagosome, phagosome maturation, destruction of ingested cargo, and resolution. (B) Cytotoxic T-cells (CTL) and Natural Killer (NK) cells kill infected target cells via the vectorial exocytosis of the cytolytic proteins, Granzyme B and Perforin at the immunological synapse formed at the point of contact with the target. (C) Immature dendritic cells continuously sample their environment by macropinocytosis and upon ingesting and degrading pathogens, they migrate to the lymph nodes where they present antigens to T-cells. Dendritic cells also promote the proliferation of T_{reg}-cells via the chemokine tmTNF. (D) Mast cells release pro-inflammatory histamine upon engagement of the $F_c\varepsilon$ receptor by IgE. (E) Thrombin activates platelets to exocytose the contents of their granules, which include proteins required for platelet adhesion, aggregation and coagulation. In addition, α-granules contain proteins and peptides that recruit or activate immune cells, such as neutrophils.

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Figure 1

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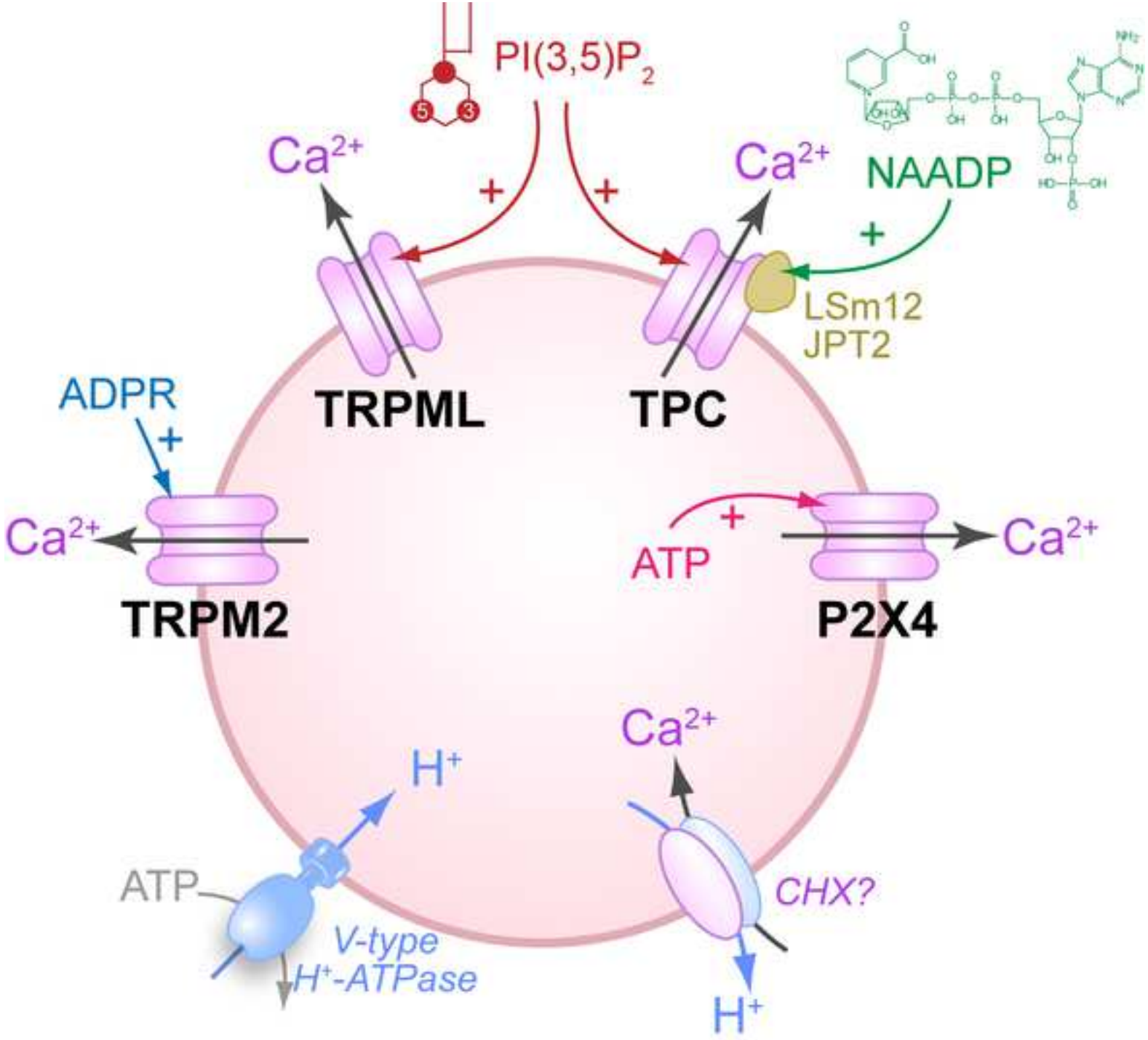


Figure 2

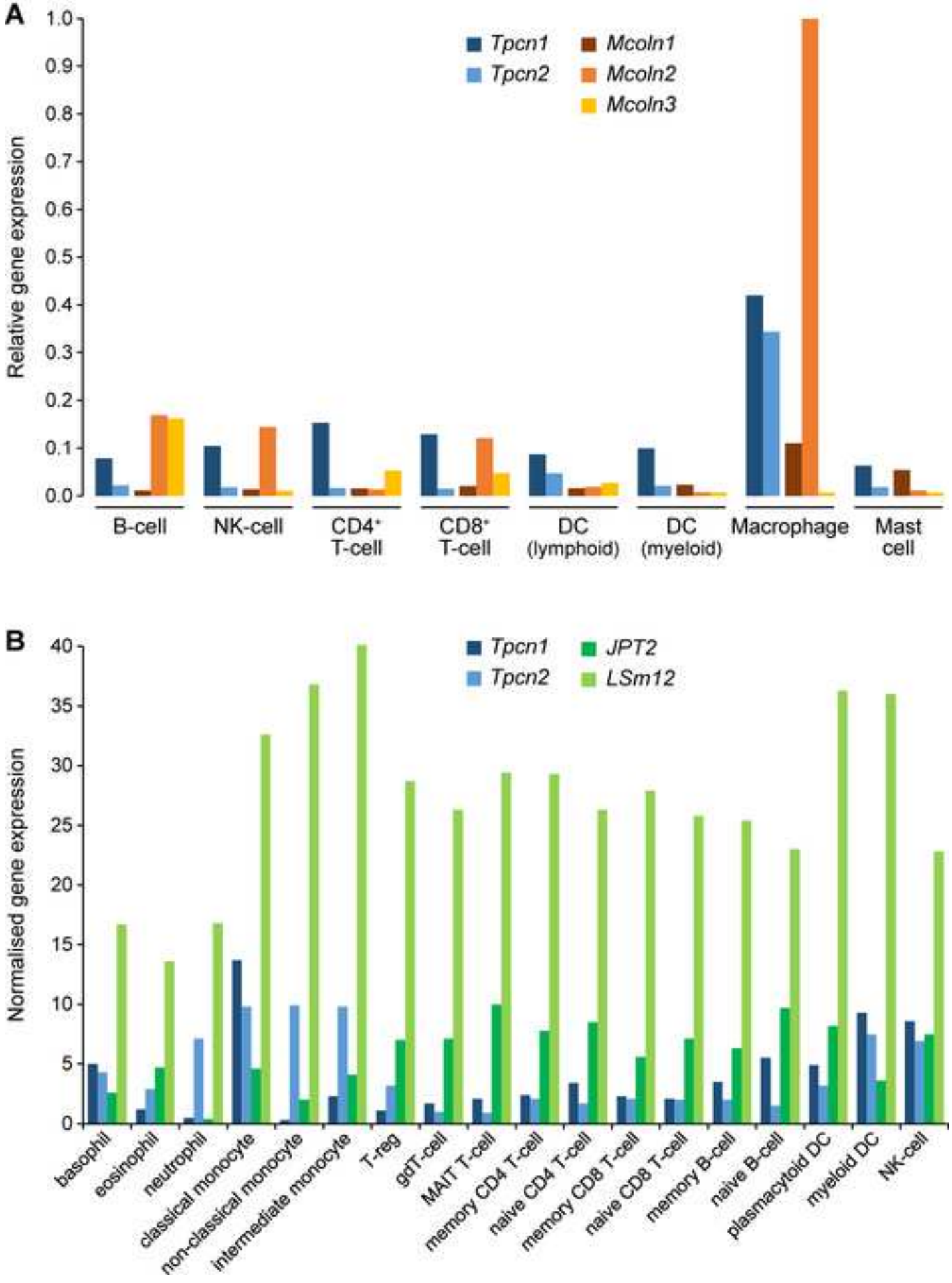


Figure 3

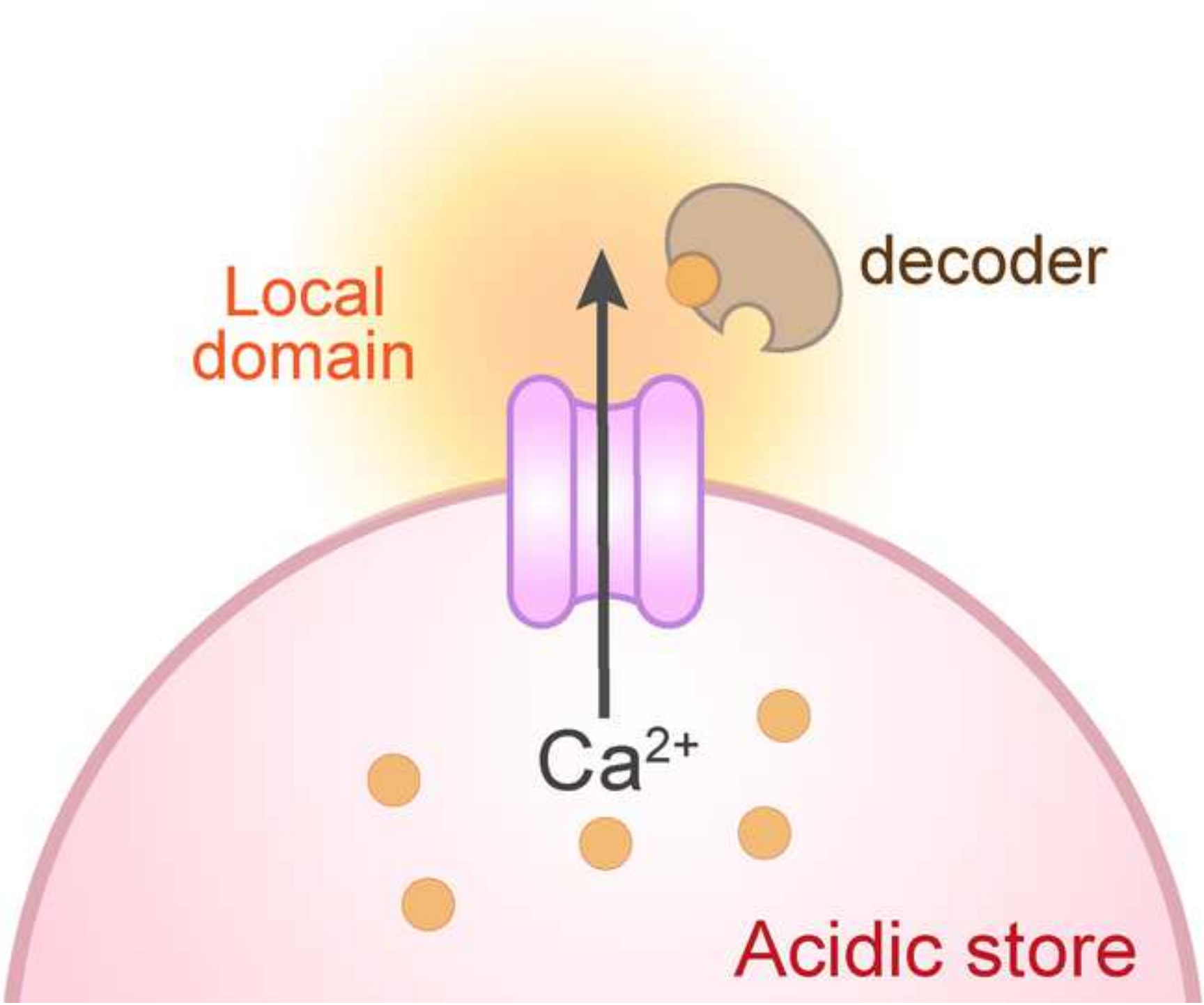


Figure 4

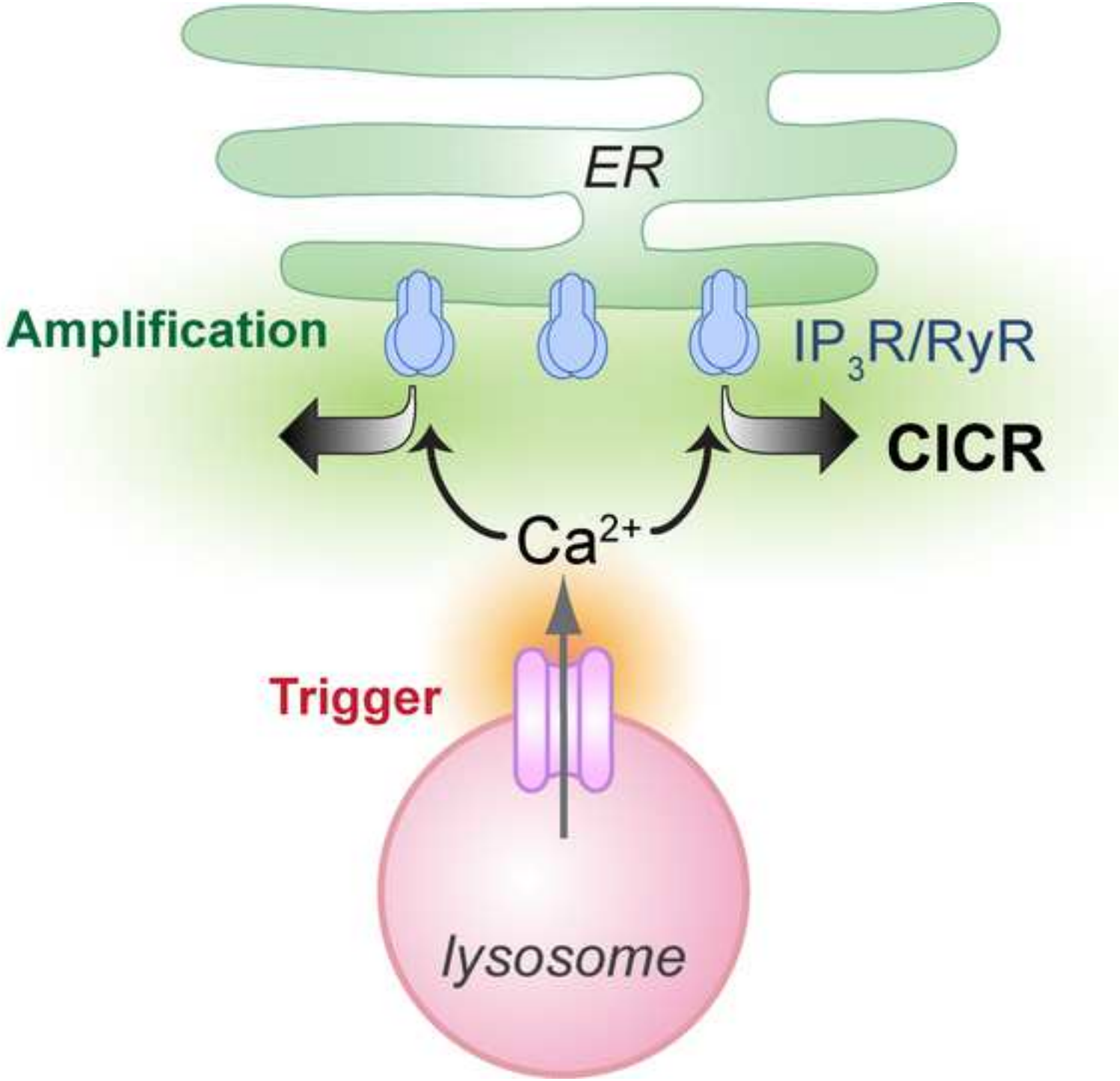


Figure 5

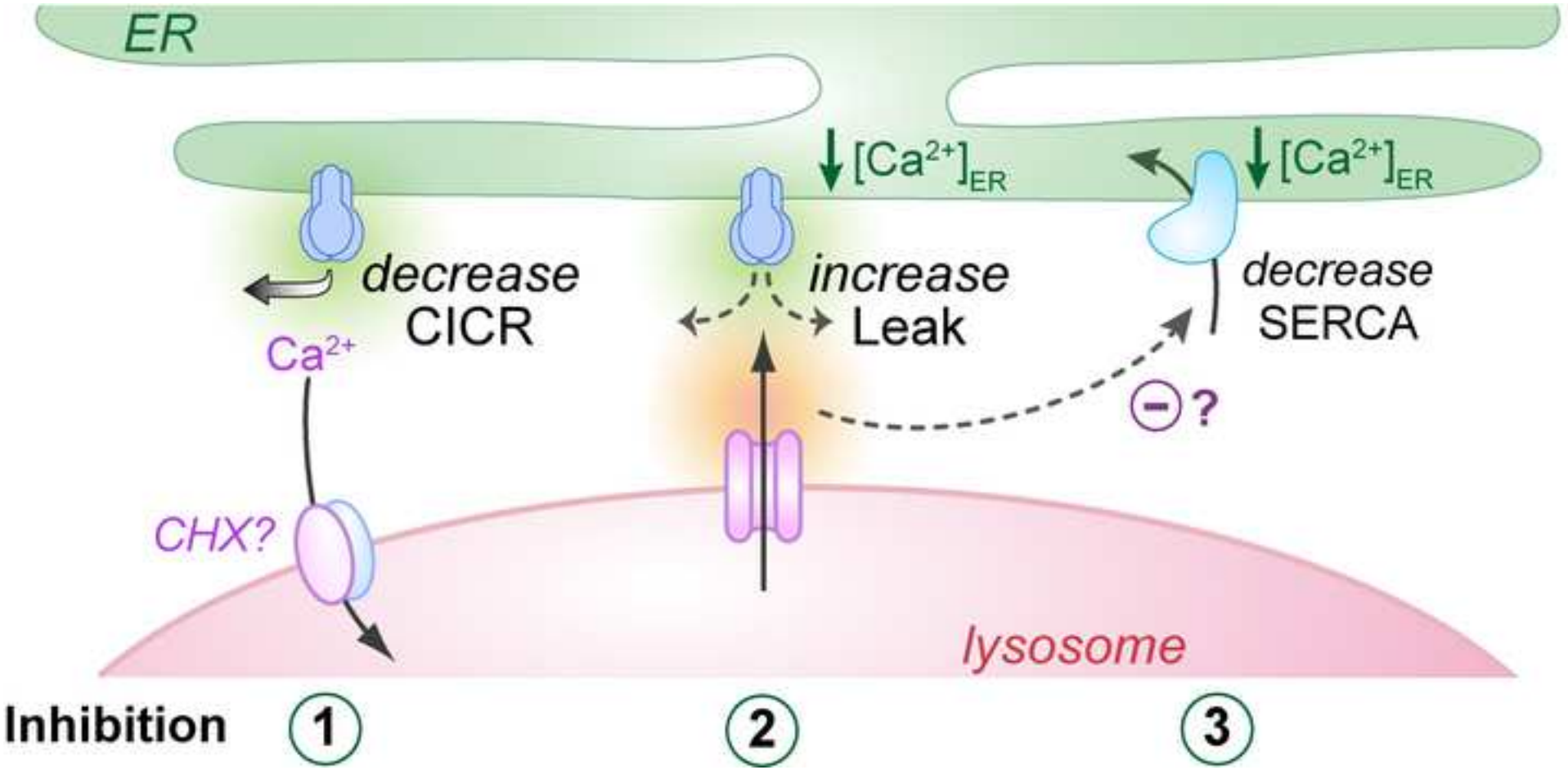
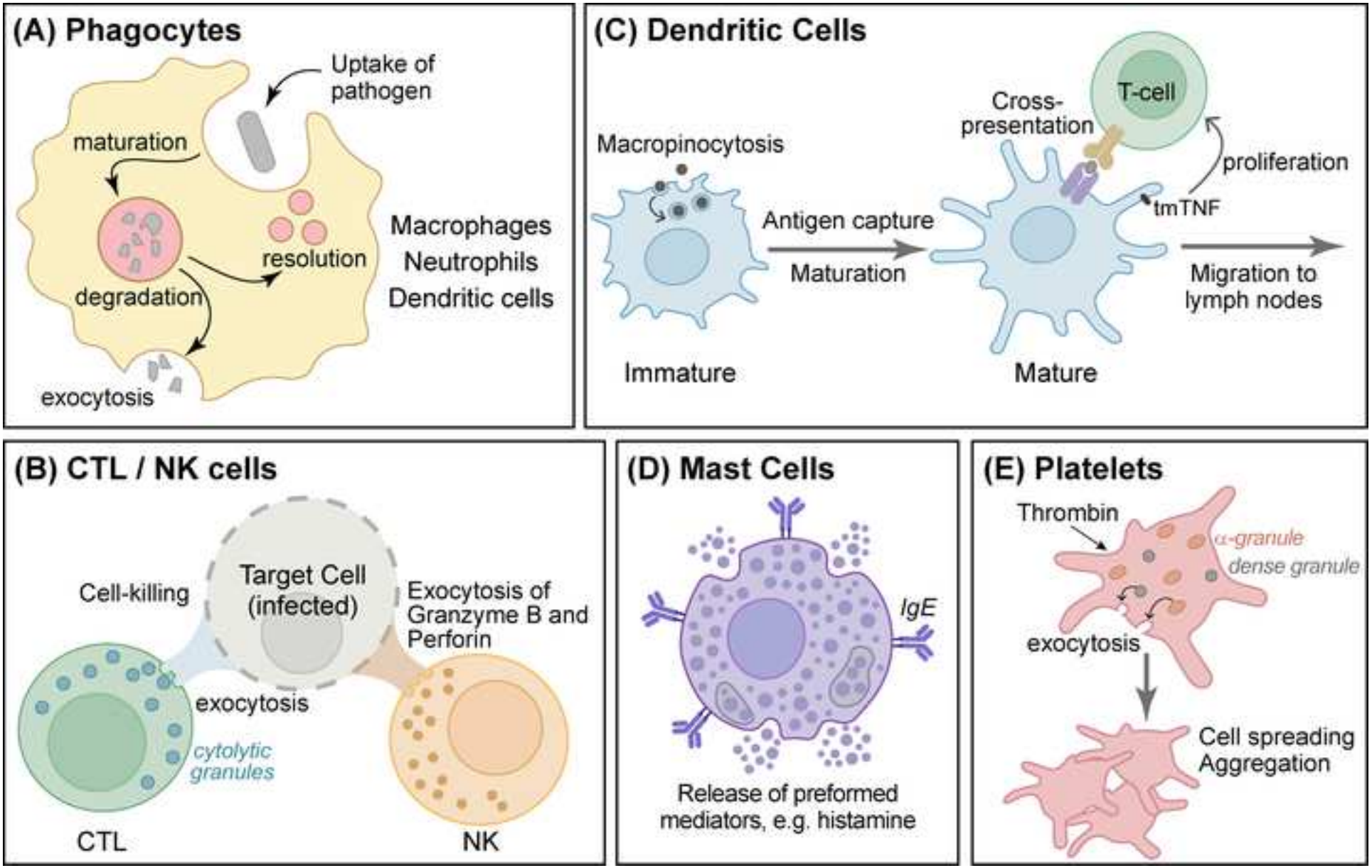


Figure 6



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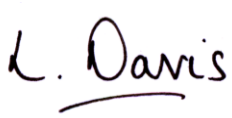
Conflict of Interest

There are no conflict of interests.



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CRediT author statement

LCD: Writing, creation of figures, reviewing and editing, AJM: Writing, reviewing and editing, AG: reviewing and editing.