Abstract

Local Ca2+ elevations boosting phagocytosis are generated in part by the store-operated Ca2+ entry (SOCE) sensor STIM1, which recruits ER cisternae to phagosomes and opens phagosomal Ca2+ channels at ER-phagosome junctions. However, residual ER-phagosome contacts and periphagosomal Ca2+ hotspots remain in Stim1-/- cells. Here, we tested whether junctate, a molecule that targets STIM1 to ER-plasma membrane contacts upon Ca2+-store depletion, could cooperate with STIM1 to recruit ER cisternae to phagosomes. Junctate expression in Stim1-/- and Stim1-/-/ Stim2-/- phagocytic fibroblasts increased phagocytosis and promoted periphagosomal Ca2+ elevations, yet without completely restoring global SOCE. These periphagosomal Ca2+ hotspots were abrogated by the InsP3R specific blocker xestospongin C, revealing that the junctate-mediated Ca2+ ions originate predominantly from Ca2+ stores. Accordingly, junctate elongated ER-phagosome junctions in Stim1-/- cells. Thus, junctate mediates an alternative mechanism for generating localized Ca2+ elevations within cells, promoting Ca2+ release from internal stores recruited to phagosomes, [...]
The role of junctate in the regulation of phagocytosis

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# CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>II</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>VI</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>1</td>
</tr>
<tr>
<td>RÉSUMÉ</td>
<td>2</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>4</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td>4</td>
</tr>
<tr>
<td>Phagosome Formation</td>
<td>5</td>
</tr>
<tr>
<td>The role of Actin</td>
<td>9</td>
</tr>
<tr>
<td>Phagosome Maturation</td>
<td>11</td>
</tr>
<tr>
<td>How microbes can escape phagocytic killing</td>
<td>18</td>
</tr>
<tr>
<td>Lipid signalling during phagocytosis</td>
<td>19</td>
</tr>
<tr>
<td>Phosphoinositides</td>
<td>19</td>
</tr>
<tr>
<td>Glycerophospholipids</td>
<td>21</td>
</tr>
<tr>
<td>Cholesterol and Sphingolipids</td>
<td>22</td>
</tr>
<tr>
<td>Lipid diffusion limiting barrier</td>
<td>25</td>
</tr>
<tr>
<td>The role of calcium during phagocytosis</td>
<td>26</td>
</tr>
<tr>
<td>FcγR-mediated calcium signals</td>
<td>26</td>
</tr>
<tr>
<td>Complement-mediated Ca^{2+} signals</td>
<td>28</td>
</tr>
<tr>
<td>Ca^{2+} signals during phagosome formation</td>
<td>28</td>
</tr>
<tr>
<td>Ca^{2+} signals during phagosome maturation</td>
<td>29</td>
</tr>
<tr>
<td>Store operated Ca^{2+} entry</td>
<td>31</td>
</tr>
<tr>
<td>Junctate</td>
<td>36</td>
</tr>
<tr>
<td>PURPOSE OF THE PROJECT</td>
<td>38</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>48</td>
</tr>
<tr>
<td>CONCLUSION</td>
<td>56</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>58</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>60</td>
</tr>
</tbody>
</table>
List of abbreviations

- adenosine triphosphate-binding cassette transporter 1 (ABC1)
- arachidonic acid (AA)
- arachidonic acid-activated channel (ARC)
- arginine-containing SNARE (R-SNARE)
- aspartyl-β-hydroxylase (AβH)
- calcium binding (EF)
- calcium release-activated calcium (CRAC)
- cationic antimicrobial peptides (CAP)
- chronic granulomatous disease (CGD)
- circulating angiogenic cells (CACs)
- class I phosphatidylinositol 3-kinase (PI3K)
- coiled-coil domains 1 and 3 (CC-1-CC-3)
- colony forming unit-endothelial cells (CFU-ECs)
- complement receptor (CR)
- Complement receptor 1, 3 and 4 (CR1, CR3 and CR4)
- CRAC activation domain (CAD)
- dendritic cells (DCs)
- detergent-resistant membranes (DRMs)
- diacylglycerol (DAG)
- early endosomal antigen 1 (EEA1)
- endoplasmic reticulum – plasma membrane (ER-PM)
- endoplasmic reticulum (ER)
- endothelial colony forming cells (ECFCs)
- endothelial progenitor cells (EPCs)
- ER-Phagosome (ER-Ph)
- fetal bovine serum (FBS)
- formin-binding protein 17 (FBP17)
- GFP-exange factor (GEF)
glutamine-containing SNAP (Q-SNAP)
GTPase-binding domain (GBD)
homotypic fusion and protein sorting (HOPS)
hypoxia-inducible factor-1α (HIF-1α)
immunoreceptor tyrosine-based activation motif (ITAM)
immunoreceptor tyrosine-based inhibition motif (ITIM)
inducible NOS (iNOS)
inositol-1,4,5-trisphosphate (IP3)
inositol-1,4,5-trisphosphate receptors (InsP3Rs)
intracellular adhesion molecules (ICAM)
Janus-activated-kinase–signal transducer and activator of transcription–interferon regulatory factor 1 (JAK–STAT–IRF1)
linker of activated T cells (LAT)
lipopolysaccharide (LPS)
lyso-bisphosphatidic acid (LBPA)
Lysosome-associated membrane protein 1 and 2 (LAMP-1 and LAMP-2)
membrane contact sites (MCSs)
metastatic renal cellular carcinoma (mRCC)
mononuclear cells (MNCs)
mouse embryonic fibroblasts (MEFs)
natural killer cells (NK)
natural resistance-associated macrophages protein 1 (NRAMP)
neuronal WASP (N-WASP)
nicotinamide adenine dinucleotide phosphate (NADPH)
nitrogen intermediates (RNI)
nitrous oxide (NO)
nucleation-promoting factors (NPFs)
overall survival (OS)
oxysterol-binding protein-related protein 1 (ORPL1)
pathogen-associated molecular patterns (PAMPs)
pattern recognition receptors (PRRs)
phosphatidic acid (PA)
phosphatidylcholine (PC)
phosphatidylinositol 3-phosphate [PI(3)P]
phosphatidylinositol 4-phosphate 5-kinase (PI4P-5K)
phosphatidylinositol-3,4,5-trisphosphate PI(3,4,5)P$_3$
phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P$_3$]
phosphatidylinositol-3,4-bisphosphate PI(3,4)P$_2$
phosphatidylinositol-4,5-bisphosphate [PI(4,5)P$_2$]
phosphatidylserine (PS)
phosphatidylserine synthase 1 (PSS1)
phosphatidylserine synthase 2 (PSS2)
phospholipase A$_2$ (PLA$_2$)
phospholipase C$_{\gamma}$ (PLC$_{\gamma}$)
phospholipase D (PLD)
pleckstrin homology (PH)
polybasic domain (PBD)
protein kinase C (PKC)
Rab7-interacting lysosomal protein (RILP)
reactive oxygen species (ROS)
sarco/ER calcium-adenosine trisphosphatase (SERCA)
Scr family kinases (SFKs)
Scr homology domains (SH2)
secretory acid phosphatase (SapM)
serum-opsonized erythrocytes (RBCs)
Severe Combined Immunodeficiency (SCID)
signal regulatory protein $\alpha$ (SIRP$\alpha$)
soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)
sorting nexin (SNX)
sphingomyelin (SM)
sphingosine kinase 1 (SK1)
Sphingosine-1-phosphate (S1P)
spleen tyrosine kinase (Syk)
Src homology 2 domain-containing inositol 5’-phosphatase (SHIP)
Src homology 2 domain-containing protein tyrosine phosphatase (SHP)
sterile alpha motif (SAM)
STIM-Orai activating region (SOAR)
stromal derived factor-1α (SDF-1α)
stromal interaction molecule 1 (STIM1)
T cell immunoglobulin mucin (TIM)
the so-called store operated calcium entry (SOCE)
the trans-Golgi network (TGN)
transmembrane (TM)
vacuolar protein sorting-associated protein 26 (Vps26)
vacuolar-ATPase (V-ATPase)
vascular endothelial grow factor (VEGF)
VEGF receptor-2 (VEGFR-2)
voltage-gated proton channel (Hv1)
WASP family verprolin-homologous
WASP homology domain (WH1)
WAVE homology domain (WH2)
Wiskott-Aldrich syndrome protein (WASP)
List of figures

Figure 1. Signalling cascade during Fcγ-mediated phagosome formation.

Figure 2. Representative scheme of phagosome maturation in macrophages.

Figure 3. Cell type-dependent phagosomal pH and ROS production.

Figure 4. Schematic organization of STIM proteins.

Figure 5. Representation of STIM1 activation and recruitment at ER-PM membrane contact sites.

Figure 6. SOCE at the level of ER-Ph MCS.

Figure 7. Schematic representation of junctate alternative splicing isoforms.

Figure 8. Partners of junctate at ER-PM MCS.

Figure 9. The involvement of STIM and Orai proteins in cancer.

Figure 10. Endothelial progenitor cell (EPC) subgroups.
Summary

Phagocytosis is an extremely organized mechanism used by our immune system in order to kill pathogens. Calcium plays a very important role in the regulation of phagocytosis, with both global and local calcium elevations reported to participate in this process. One of the best characterized mechanism used by cells to increase the cytosolic calcium concentration is represented by the so-called store operated calcium entry (SOCE). Two major important proteins are involved in the regulation of SOCE at the level of endoplasmic reticulum – plasma membrane (ER-PM) contact sites: stromal interaction molecule 1 (STIM1) and the calcium permeable channel Orai1, which reside in the ER membrane and plasma membrane respectively. In 2000, the group of Zorzato described a novel calcium sensor protein, called junctate. Junctate was reported to interact with inositol-1,4,5-trisphosphate receptors (InsP3Rs) and other calcium permeable channel resident in the plasma membrane, like TRPC3. Moreover, junctate can recruit STIM1 at the level of ER-PM membrane contact sites via its luminal portion and its overexpression leads to an increased SOCE in primary T cells. Recently, Nunes et al. showed that STIM1is able to recruit ER cisternae close to the phagosome membrane where it can interact with SOCE channels, thereby generating periphagosomal calcium microdomains that boost phagocytosis. Since the researchers still detected ER-Phagosome (ER-Ph) membrane contact sites (MCSs) even in the absence of STIM1, here we determine whether the novel STIM1-interactor junctate is able to modulate ER-Ph membrane contact sites formation. Junctate-YFP was expressed in STIM1−/− and STIM1−/−;STIM2−/− mouse embryonic fibroblasts (MEFs) rendered phagocytic by ectopic expression of Fcy-RIIA receptors. Confocal microscopy, Fura-2 and Fluo-8 imaging as well as electron microscopy were used to assess phagocytosis, global calcium signalling, local calcium signalling and MCS formation, respectively. Surprisingly, junctate-YFP overexpression in STIM1−/−, STIM1−/−;STIM2−/− and wild-type MEFs revealed that junctate can increase the phagocytic capability and the number of MCS around phagosomes independently of STIM proteins. The overexpression of junctate did not cause an increase in global calcium elevations in either WT or
STIM1<sup>−/−</sup> MEFs. However, Ca<sup>2+</sup> live imaging experiments showed that junctate increases the frequency of periphagosomal Ca<sup>2+</sup> microdomains both in STIM1<sup>−/−</sup> and STIM1<sup>−/−</sup>; STIM2<sup>−/−</sup> MEFs. Furthermore, the use of IP3R and Ca<sup>2+</sup> channel blockers revealed that junctate promotes the release of Ca<sup>2+</sup> from stores rather than phagosomal Ca<sup>2+</sup> release, in contrast to STIM1. Finally electron microscopy showed that the overexpression of junctate, both in STIM1<sup>−/−</sup> and WT MEFs, can increase the length of the MCS around the phagosome. These data indicate that junctate can replace the STIM1 pro-phagocytic function by creating Ca<sup>2+</sup> microdomains around phagosomes through release from calcium stores.

**Résumé**

La phagocytose est un mécanisme extrêmement sophistiqué utilisé par notre système immunitaire pour éliminer les agents pathogènes. Lors de la régulation de ce processus, la concentration en calcium joue un rôle majeur et des élévations calciques aussi bien locales que globales peuvent moduler la phagocytose. L’un des mécanismes le mieux caractérisé permettant à la cellule d’augmenter sa concentration en calcium cytosolique est appelé entrée de calcium par déplétion des stocks intracellulaires (en anglais, store operated calcium entry or SOCE). Deux protéines essentielles régulent le SOCE au niveau des sites de contact réticulum endoplasmique – membrane plasmique (RE-MP) : STIM1, pour stromal interaction molecule 1, et le canal perméable au calcium Orai1, qui résident dans le RE et la membrane plasmique respectivement. En 2000, le groupe de Zorzato a décrit un nouveau senseur calcique appelé Junctate. Il a été démontré que Junctate interagit avec le récepteur à l’inositol-1,4,5-trisphosphate (IP3R) ainsi qu’avec d’autres canaux perméables au calcium localisés dans la membrane plasmique tel que TRPC3. De plus, Junctate peut recruter STIM1 au niveau des sites de contact RE-MP grâce à sa partie luminale et sa surexpression entraîne une augmentation du SOCE dans les cellules T primaires. Récemment, Nunes et al. ont montré que STIM1 est capable de rapprocher les saccules du RE à proximité de la membrane phagocytyque permettant son interaction avec les canaux SOCE, créant ainsi des microdomaines calciques autour...
du phagosome qui stimulent l’activité phagocyttaire. Puisqu’il a été montré que les sites de contact RE-phagosome (RE-Ph) peuvent se former en l’absence de STIM1, nous avons cherché à déterminer si Junctate est capable de moduler la formation des sites de contacts RE-Ph. Junctate-YFP a été exprimé dans des cellules fibroblastiques embryonnaires (MEFs) STIM1−/− et STIM1−/−;STIM2−/− qui présentent des propriétés phagocytaires grâce à l’expression ectopique du récepteur Feγ-RIIA. Nous avons ensuite évalué l’efficacité phagocytique, la signalisation calcique globale et locale et la formation de sites de contact membranaires par microscopie confocale, par visualisation des sondes calciques Fura-2 et Fluo-8 et par microscopie électronique, respectivement. De façon étonnante, la surexpression de Junctate-YFP dans les cellules STIM1−/−, STIM1−/−;STIM2−/− et les MEFs sauvages a révélé que Junctate peut augmenter la phagocytose dans ces cellules ainsi que le nombre de sites de contact autour des phagosomes de manière indépendante de STIM1. La surexpression de Junctate n’augmente pas la concentration calcique globale ni dans les cellules sauvages ni dans les MEFs STIM1−/−. Cependant, l’imagerie en temps réel du calcium a montré que Junctate augmente la fréquence des microdomaines periphagosomaux aussi bien dans les cellules STIM1−/− que dans les cellules STIM1−/−;STIM2−/−. De plus, l’utilisation d’inhibiteurs de l’IP3R et des canaux calciques a prouvé que Junctate induit le relargage de Ca2+ depuis les stocks intracellulaires plutôt que le relargage de Ca2+ phagosomal contrairement à STIM1. Finalement, la microscopie électronique a montré que la surexpression de Junctate augmente la longueur des sites de contact membranaires autour des phagosomes dans les cellules sauvages et les cellules STIM1−/−. Ces résultats indiquent que Junctate peut remplacer la fonction pro-phagocytique de STIM1 en générant des microdomaines de Ca2+ autour des phagosomes dus à la déplétion des stocks intra-cellulaires.
Introduction

Phagocytosis

Phagocytosis is a fundamental mechanism used by both innate and adaptive immune cells as defence against bacteria but it also has a role in the clearance of apoptotic cells, which is important for tissue homeostasis and remodelling. Even if Elie Metchnikoff observed phagocytosis for the first time in 1884, some of the mechanisms that characterize this immune process are still poorly understood. Only specialized immune cells like macrophages, neutrophils and dendritic cells, also called professional phagocytes, are able to perform phagocytosis to kill bacteria or fungi from infectious sites, thereby contributing to the first line of immune defence. Phagocytosis is also used to activate the adaptive immune response by presenting the antigens derived from the destruction of the foreign particle to lymphoid cells. Innate immune cells can also activate adaptive immunity by the release of pro-inflammatory cytokines or, in contrast, release anti-inflammatory mediators when they are involved in the clearance of dead cells. Phagocytosis is regulated by different types of receptors, which can interact directly with foreign bodies or through the intermediation with other molecules called opsonins.

Bacteria or fungi possess different types of molecules that are not present in our organism, called pathogen-associated molecular patterns (PAMPs). These PAMPs can be recognized directly by different types of receptors called pattern recognition receptors (PRRs). PRRs can be divided into receptors able to mediate phagocytosis, such as scavenger and mannose receptors, and other receptors that can only trigger an inflammatory signal transduction, like Toll-like receptors. For instance, lipopolysaccharide (LPS) that characterizes Gram-negative bacteria can be detected by scavenger receptor A. Other receptors recognize only opsonized foreign bodies. These opsonins are circulating blood molecules like immunoglobulins or components of the complement cascade, which can recognize foreign antigens rendering the external particle visible to professional phagocytes. In nature, phagocytosis is simultaneously triggered by more than one receptor, producing
in this way a synergistic response. Many pathogens have developed different mechanisms to avoid phagocytosis, like interfering with opsonin or receptor binding by using polysaccharide-based capsules acting as barrier, by expressing specific surface proteins that can avoid the interaction with the receptor and by binding extracellular matrix proteins, which protects from complement opsonisation. For example, group A streptococci are able to produce M proteins, or \textit{Yersinia enterocolitica} can synthetize adhesin YadaA to escape complement-mediated phagocytosis. Moreover, \textit{Staphylococcus aureus} is able to bind the Fc portion of the IgG through protein A resident in the plasma membrane, thereby avoiding the interaction with Fc\(\gamma\) receptors. A better understanding of the mechanisms underlining phagocytosis could lead to the creation of novel therapies important to defeat these pathogens.

\textbf{Phagosome Formation}
Phagocytosis can mainly be divided in two parts: phagosome formation and phagosome maturation. Different types of events and molecules define phagosome formation. The first step is the interaction between one of the phagocytic receptors resident in the plasma membrane and the foreign body, that can be opsonized or not as mentioned above. Several receptors are involved in the regulation of phagocytosis and they differ because of the nature of the ligand, the structure of the receptor and the pathway that they trigger.

\textbf{Fc\(\gamma\) Receptor mediated phagocytosis.}
Phagocytosis is driven by Fc\(\gamma\)Rs only when an IgG-opsonized foreign body is involved. The Fc portion of the immunoglobulin is recognized by the extracellular part of the Fc receptor, which can lead to the activation of different pathways inside the cell. Notably, many receptors interact sequentially with the same opsonized target in a process called zipper-mediated engulfment, where the actin-dependent formation of pseudopods facilitates the interaction between new receptors and the next uncoupled ligands. Inside the cell, Fc\(\gamma\)Rs cluster through their cytosolic domain, the immunoreceptor tyrosine-based activation motif (ITAM), which is then phosphorylated by tyrosine kinase of the Src family, like HCK, LYN and Fgr. Interestingly, the clustering of the Fc-receptor
could be favoured by the presence of cholesterol-enriched lipid rafts in the plasma membrane, where Scr family kinases (SFKs) are enriched. There is evidence showing that after crosslinking activation, FcγRIIA can be found in detergent-resistant membranes (DRMs) \(^{15,16}\) and that receptor phosphorylation is inhibited upon cholesterol depletion by methyl-β-cyclodextrin \(^{15}\). Since it is not clear whether DRMs can be representative or not of intact membrane and since methyl-β-cyclodextrin might impair cell physiology, the correlation between the clustering of the receptor and cholesterol-enriched lipid rafts has to be taken with caution \(^{17}\). The phosphorylated tyrosines are then engaged by the spleen tyrosine kinase (Syk) by its two Scr homology domains (SH2) \(^{18}\) (Figure 1). Syk kinase has been demonstrated to be required for an efficient Fc-receptor mediated phagocytosis in macrophages \(^{19,20}\).

![Figure 1. Signalling cascade during Fcγ-mediated phagosome formation.](image)


Figure 1. Signalling cascade during Fcγ-mediated phagosome formation. After receptor clustering, the immunoreceptor tyrosine-based activation motif (ITAM) is phosphorylated by Scr family kinase (SFK), thereby activating the spleen tyrosine kinase (Syk) (orange) and the recruitment of adaptor proteins (green). Consecutively, a series of lipid-modification enzymes (blue) and small GTPases are activated (brown), leading to actin polymerization via the actin nucleation complex Arp2/3 (red) \(^{2}\).
The subsequent phosphorylation of Syk kinase activates the recruitment of different adaptor proteins to the activated Fc receptor, which are important to recruit other downstream signalling components. The transmembrane protein linker of activated T cells (LAT) once phosphorylated by Syk \(^{21,22}\) can recruit the additional protein Grb2, which binds LAT through its SH2 domain. Grb2 also recruits its associated binding partner Gab2, that is further stabilized at the level of the plasma membrane by its interaction with phosphatidylinositol-3,4,5-trisphosphate \(\text{PI}(3,4,5)\text{P}_3\) through its pleckstrin homology (PH) domain. Knocking out genes that code for the adaptors can impair phagocytosis \(^{23}\), they are important for the downstream regulation of Fc-receptor pathway.

IgG is the major opsonin binding to the corresponding FcγRs, however there are other immunoglobulins that participate in phagocytosis. For instance, IgA and IgE interact with FcαR and FcεR respectively \(^{24,25}\). Moreover, different types of FcγRs can trigger phagocytosis, like FcγRI \(^{26}\), FcγRIIA \(^{27}\) and FcγRIIIA \(^{28}\). In contrast, FcγRIIB is an isoform of FcγRIIA that can negatively regulate phagocytosis \(^{29}\). There is evidence showing that overexpression of FcγRIIB can block FcγR-mediated phagocytosis and dysfunction of this receptor is associated with autoimmune diseases like lupus erythematosus \(^{30}\). FcγRIIB accomplishes its inhibitory role by the immunoreceptor tyrosine-based inhibition motif (ITIM). After being phosphorylated, ITIM can recruit the phosphatase Src homology 2 domain-containing inositol 5'-phosphatase (SHIP) and the Src homology 2 domain-containing protein tyrosine phosphatase (SHP) 1 and 2. SHIP is able to hydrolyse \(\text{PI}(3,4,5)\text{P}_3\) into phosphatidylinositol-3,4-bisphosphate \(\text{PI}(3,4)\text{P}_2\), thereby blocking phagocytosis \(^{31}\). Despite the evidence that SHP-1 overexpression inhibits phagocytosis, the mechanisms underlining its action are not completely understood.

**Complement mediated phagocytosis**

The complement system, evolutionarily speaking, is much older than other types of immunity but still represents a valid resource of the innate immune system also in higher organisms, like humans \(^{32}\). Even if actin polymerisation plays a role, complement mediated phagocytosis differs from FcRs
mediated phagocytosis, since there is a minimal membrane protrusion, and complement-mediated
internalization does not always lead to an inflammatory response.

Different types of receptors can modulate the complement-mediated immune response. Complement
receptor 1, 3 and 4 (CR1, CR3 and CR4) 33, which are expressed both on macrophages and
neutrophils, can be activated by microbial macromolecules or by IgM and IgG opsonized targets.
Upon activation, a serine that localizes in the β-chain is phosphorylated in a PKC dependent manner
34,35 and it appears to be important for both opsonized and non-opsonized complement-mediated
phagocytosis 36,37. Once the receptor is activated, it is able to associate with components of the
cytoskeleton like α-actinin through its cytosolic domain 38, thereby starting the engulfment process.
CR3 is the most well-known complement receptor and there is evidence showing that it can bind
several ligands 39. For instance, CR3 interactions with intracellular adhesion molecules (ICAM-1,2)
40,41, with platelet glycoprotein Ibα 42 or matrix molecules (fibrin, fibrinogen, collagen) 43,44 are
important for the regulation of cell spreading and chemotaxis. In addition, CR3 can mediate non-
opsonic complement phagocytosis by binding β-glucan 45. After complement activation, C3b is
produced and it acts as an opsonin interacting with hydroxyl or amino groups present on the surface
of the foreign particles. Moreover, C3b-opsonized targets are directly recognized by CR1 or by CR3,
after C3b has been modified by plasma Factors H and I in iC3b 46.

**Phagocytosis during tissue remodelling**
Cells undergoing apoptosis release different types of molecules, like ATP, fractalkine, sphingosine-
1-phosphate and lysophosphatidylcoline that act as chemoattractants, thereby recruiting professional
phagocytes 47. Moreover, apoptotic cells exhibit on their surface different molecules which represent
the “eat-me” signals highlighting them as unhealthy cells.

One of the most common markers of apoptotic cells is phosphatidylserine (PS), which resides in the
inner leaflet of the plasma membrane in healthy cells. In contrast, apoptotic cells present PS in the
outer leaflet of the plasma membrane and it can be bound by several receptors of the T cell
immunoglobulin mucin (TIM) family like BAI1 and Stabilin-2\textsuperscript{48-50}. Interestingly, there is a threshold driving the “eat-me” signal that avoids healthy cells from being eaten. In fact, healthy cells still present PS but at concentrations 300-fold lower than apoptotic cells\textsuperscript{51}. Phosphatidylserine can also interact with phagocytic receptors by the crosslinking action of a third molecule. For instance, MFG-F8\textsuperscript{52} bridges PS to αvβ3, and protein Gas6 and protein S\textsuperscript{53} link PS to Tyro3, Axland Mer (TAM) phagocytic receptors family members. Furthermore, some phagocytic receptors like CD36 and CD68 can bind oxidized PS\textsuperscript{54}. Under physiological conditions healthy cells, like activated T and B cells, can also increase the amount of PS that resides on the outer leaflet of their plasma membrane, but they are able to escape phagocytosis by the expression of “don’t eat me” signals. Specifically, CD31 can inhibit phagocytosis when it undergoes oligomerization, in order to discriminate between apoptotic and healthy cells\textsuperscript{55}. Moreover, cells that express CD47 avoid being phagocytosed by the interaction with the signal regulatory protein α (SIRPα) that causes the activation of tyrosine phosphates, thereby triggering a dephosphorylation cascade able to block phagocytosis\textsuperscript{56}.

The role of Actin
Actin remodelling is fundamental for maintaining cellular morphology and it plays an important role as a primary driving force during phagocytosis. However, there is still a big gap in our knowledge in the regulation of F-actin filaments, which modulate the shape of the cell and the engulfment process. The changes in morphology occurring during phagocytosis are driven by the Arp2/3 complex that activates actin polymerization\textsuperscript{57,58} (Figure 1)\textsuperscript{2}. Normally, Arp2/3 is kept in an inactive state until it is activated by nucleation-promoting factors (NPFs), which thereby promotes the creation of new actin filaments. The Wiskott-Aldrich syndrome protein (WASP) family is the most studied NPFs and macrophages harvested from WAS patients present with deficient FcγR-mediated phagocytosis, indicating the pivotal role of WASP for the immune system. This family is characterized by several members but only WASP expressed by hematopoietic cells, neuronal WASP (N-WASP) and WASP family verprolin-homologous proteins (WAVE) 2 have been associated with phagocytosis in macrophages\textsuperscript{59}. They are characterized by a verprolin homology, central hydrophobic and acid
regions (VCA domain), which are implicated in the binding and activation of the Arp2/3 complex \[60\]. A binding site for SH3 domain-containing proteins is close to the VCA domain and the N-terminus presents a WASP homology domain (WH1) and a GTPase-binding domain (GBD), which interacts with Cdc42. WAVE proteins display a different N-terminus domain composed by the WAVE homology domain (WH2), a basic domain that is important to interact with PI(3,4,5)P\(_3\), a proline rich domain to bind proteins with SH3 domain and a C-terminus with the common VCA domain \[59\]. In particular, after FcγR engagement WASP proteins are recruited and activated, thus triggering Arp2/3-mediated F-actin polymerization. WASP proteins have been proposed to be recruited at the phagocytic cup by formin-binding protein 17 (FBP17), which contains a BAR domain that is able to recognize lipid rafts in the plasma membrane at the site of FcγR target interaction \[61\]. Several factors are involved in WASP activation, like RhoGTPases and PI(4,5)P\(_2\). Cdc42 is a well-studied RhoGTPase that has been shown to have a central role in WASP activation \[62-64\]. F-actin polymerization is also maintained by Ena/VASP, which is a profilin ligand able to recruit monomeric actin at the polymerization site, or by coronin that has been described to localize at the phagocytic cup in Dictyostelium \[65\], mice \[66\] and human \[67\]. Interestingly, the human isoform hcoronin1 can also interact with NOX2, thus proposing a role in the regulation of the oxidative burst during phagocytosis \[67\]. The formation of the phagocytic cup is followed by myosin contractility, which is important for the creation of pseudopods involved in phagosome sealing \[68\]. The delivery of new membrane is necessary for pseudopods growth and there is evidence showing that it occurs at the base of the cup \[69,70\]. For instance, VAMP3 vesicles are recruited to the phagocytic base by Arf6 \[71,72\]. Simultaneously, cargo delivery and membrane fusion require F-actin depolymerisation, which is mediated by several factors, like gelsolin \[73\] and cofillin \[74\].

As described above, FcγR and complement mediated phagocytosis are morphologically different. In contrast to FcγR mediated phagocytosis, Cdc42 and WASP effectors have been shown to not be involved in complement-triggered phagocytosis \[62,64\]. Rac1/2 seems to have a role in F-actin
accumulation at the phagocytic cup that is not completely understood \cite{64,75}. The formin mDia, which is an effector of RhoA important in the regulation of actin dynamics, is recruited at site of phagocytosis only upon CR3 binding and its reduction causes a deficit in phagocytosis efficiency \cite{76}.

**Phagosome Maturation**
The formation process ends with the creation of a novel organelle called a phagosome, which is not able to kill the phagocytosed foreign particle since its contents still resemble the extracellular milieu. After the formation step, the phagosome undergoes several biochemical modifications, characterized by a series of membrane fusion and fission events with early endosomes, late endosomes and lysosomes. These modifications alter the phagosome composition, leaving on the other hand its dimension quite constant \cite{77}. Presently, there is no clear evidence whether phagosome membrane trafficking events involve a complete fusion with endosomal membranes or whether they represent transient and reversible interactions that define the so called kiss-and-run model \cite{78}. The biochemical modifications occurring during the phagosome maturation are cell type-dependent. Specifically, macrophages produce phagosomes mildly oxidative and acid (pH <5.0), due to V-ATPase activity, neutrophils form phagosomes highly oxidative, owing to a higher NADPH oxidase activity, with a neutral/alkaline phagosomal lumen (pH 7.2-7.8). Phagosomal pH regulation in dendritic cells is still controversial, since both acidification and alkalinisation have been reported in the literature \cite{79}.

![Figure 2. Representative scheme of phagosome maturation in macrophages.](image)

After receptor engagement, the newly formed phagosome undergoes a series of biochemical and molecular changes, characterized by several fusion events with intracellular vesicles \cite{80}. 

11 | Page
**Early phagosomes**

Phagosome maturation is a well-defined mechanism. In fact, nascent phagosomes are more prone to interact first with early endosomes rather than late endosomes or lysosomes. The early phagosome is generated by changes in the membrane of the new organelle, which are mediated by vesicular traffic to and from the phagosomal vacuole. For instance, neutrophils contain several granules that fuse with the phagosome, like the primary and secondary granules called azurophilic and specific respectively, the tertiary and the secretory vesicles. This membrane trafficking is regulated by Rab GTPase family proteins that alternate between the GTP-bound active state and the GDP-bound inactive state, like all the GTPases family proteins, and they are controlled by several factors such as, GDIs, GEFs and GAPs. Upon activation, Rab proteins are able to shuttle to/from membranes, to ensure the correct site and time of fusion and to interact with a series of effector molecules involved in vesicular trafficking, fusion and fission. In particular, the presence of Rab5 is an exclusive characteristic of early phagosome (Figure 2). Rab5 is already located at the plasma membrane prior to the end of the engulfment process, but its density on the phagosomal membrane increases overtime, meaning that new Rab5 proteins are recruited during this phase. The role of Rab5 is to coordinate the fusion of newly formed phagosomes with early endosomes and its malfunctioning can lead to an improper phagosome maturation, avert the formation of late phagosomes and phagolysosomes. The early endosomal antigen 1 (EEA1), the p150-Vps34 complex and Mon1a/b are some of the Rab5 effectors. Specifically, mVps34 is a class III PI3K that catalyses the production of phosphatidylinositol 3-phosphate [PI(3)P] using as substrate phosphatidylinositol. The relevance of both mVps34 and [PI(3)P] for phagosome progression has been proven using anti-Vps34 antibodies, which severely impairs phagosome maturation. mVps34 recruitment is enhanced by the interaction with the Vps15-like serine/threonine kinase p150, forming together a complex with Rab5. In addition, EEA1 is recruited to early phagosome through a simultaneous interaction with Rab5, via its N-terminal CH2 zinc-finger domain, and with PI(3)P. Once EEA1 is recruited, it can assert its function of boosting docking and fusion with early endosomes. EEA1 mediates its role in part by the interaction...
with syntaxin 13 that is a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE). It is well established that SNARE proteins are involved in membrane fusion, acting by the creation of hairpin-like complexes and they can be divided in two groups: the arginine-containing SNARE (R-SNARE) and the glutamine-containing SNARE (Q-SNARE). The early phagosome membrane also contains components that need to be brought back to the plasma membrane. The recycling pathways that coordinate this process are regulated by Rab4, Rab 11, the hetero-oligomeric complex COP, the GTPase adenosine ribosylation factor and the retromer complex, which are all involved in the retrieval of phagosomal proteins either to the plasma membrane or to the trans-Golgi network (TGN). The retromer complex is necessary for the retrieval of specific endosomal proteins, for instance the cation-independent M6PR, to the TGN. Several proteins define the retromer complex, like the vacuolar protein sorting-associated protein 26 (Vps26), Vps29, Vps35 and the sorting nexin (SNX) subcomplex 1/2 and 5/6. Rab5 has a key role in the activation of mVps24 and the production of PI(3)P, which are necessary for the recruitment of SNX complexes.

**Late phagosomes**

Three major biochemical events underlie late phagosome maturation: the loss of Rab5, the acquisition of Rab7 and the acidification of the phagosomal lumen with a pH of 5.5-6.0 in macrophages (Figure 2). The transition between Rab5-early phagosome to Rab7-late phagosome is mediated by the protein Mon1a/b, which can interact only with active Rab5. Mon1 leads to the recruitment of Ccz-1, a Rab7-binding protein that acts as a tether for GDP-bound Rab7. In addition, Mon1 is also capable of displacing from the phagosome membrane the Rab5 GFP-exchange factor (GEF) (Rabex-5), leading to the inactivation of all Rab5-mediated events. Moreover, the homotypic fusion and protein sorting (HOPS) complex is another factor that has been shown to be required for Rab7 recruitment to late phagosomes and it is involved in nucleotide exchange and so Rab7 activation. HOPS consists of several proteins, such as Vps21, Vps16, Vps18 and Vps33 and once it is recruited to phagosomes HOPS can interact with Vps41 and Vps39 that possesses Rab7 GEF activity. In summary, Mon1 is able to recruit Rab7 to early phagosome through Ccz-1 and then activates Rab7 and modulates
nucleotide exchange after the association with the HOPS complex. Once Rab7 has been recruited to the late phagosome, different effectors accumulate in a Rab7-dependent manner. For instance, the Rab7-interacting lysosomal protein (RILP) and the oxysterol-binding protein-related protein 1 (ORPL1) are two Rab7-dependent effectors, which are responsible for promoting microtubule-dependent vesicular traffic by direct interaction with the dynein/dynactin motor complex. Lysosome-associated membrane protein 1 and 2 (LAMP-1 and LAMP-2) are other factors involved in an efficient Rab7 recruitment to late phagosomes. The crucial role of these heavily glycosylated integral membrane proteins is clear in Lamp deficient cells that are ineffective to eradicate Neisseria gonorrhoeae, showing their involvement in the gain of phagosome microbial functions.

**Phagolysosomes**

During the last step of phagocytosis, the phagosome acquires a complete arsenal of microbicidal and hydrolytic features, like hydrolytic enzymes, oxidants and cationic peptides, to be able to digest proteins, lipids and carbohydrates. The ability to degrade is not the same among all the different phagocytic cells, but is correlated to the final purpose, like total pathogen destruction in the case of neutrophils and antigen presentation for dendritic cells. The final step for the complete maturation of the phagosome is represented by the fusion with lysosomal compartments, which is mediated in part by a SNARE complex that consist of syntaxin 7 and VAMP7. For instance, the phagolysosome in macrophages is biochemically characterized by a highly acidic lumen with a pH of 4.5-5, the recruitment of active cathepsins and the loss of PI(3)P, M6PR and lyso-bisphosphatidic acid (LBPA).

Several factors regulate the acidification of the phagosomal lumen, such as the vacuolar-ATPase (V-ATPase) and a reduced proton permeability, which allow the accumulation of protons. The former is a large protein complex composed of the cytosolic V₁ complex, which is comprised of eight different proteins and controls ATP hydrolysis, and the V₀ complex that constitutes the pore in the phagosomal membrane, allowing the ATP-dependent proton flux into the phagosome. In order to maintain the acidic pH inside the phagosomal lumen without causing an excessively high electrical potential,
several conductive pathways are involved in the inward flux of anions like Cl\(^{-}\) and efflux of cations such as K\(^{+}\) and Na\(^{+}\) to reduce the lumen positive potential \(^{111,112}\). The acidity of the phagosome has an antimicrobial role, since it is able to directly boost the degradative capability of the phagosome through the activation of proteolytic enzymes like cathepsins D and L and indirectly reduce microbial growth by activating natural resistance-associated macrophages protein 1 (NRAMP) \(^{113,114}\). NRAMP1 is a transmembrane protein highly abundant in the phagosome membrane and exerts its bacteriostatic and antiparasitic roles by extruding from the phagosomes divalent cations, such as Zn\(^{2+}\) and Mn\(^{2+}\), essential for the bacterial growth \(^{115}\). As mentioned above, the microbicidal activity is linked to the functions of phagocytic cells, for instance macrophages phagosomes are highly acidic to be effectively antimicrobial immune cells. In contrast, the phagosomal pH of dendritic cells is still controversial \(^{79}\) (Figure 3)\(^{79}\).

**Figure 3. Cell type-dependent phagosomal pH and ROS production.** During phagolysosome formation, several components are involved in phagosomal pH establishment in a cell type-dependent manner. In macrophages, phagosomes acidify rapidly due to high V-ATPase activity. Conversely, neutrophils exhibit an almost neutral phagosomal pH, since the NADH oxidase-dependent electrochemical imbalance is compensated by Hv1 that pumps protons inside the phagosome. The regulation of phagosomal pH in dendritic cells (DCs) is still controversial and it could be associated to different DC subpopulations \(^{79}\).
Intraphagosomal protons are also involved in the production of toxic reactive oxygen species (ROS) that play a key role in the bacterial degradation. Upon inflammatory stimuli, NOX2 is assembled and transfers electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to molecular oxygen, thereby producing superoxide anion (O$_2^-$). NOX2 is a multimeric protein complex composed by the transmembrane proteins gp91phox and gp22phox, which are component of the flavocytochrome b$_{558}$ and the cytosolic proteins p40phox, p47phox and p67phox. The cytosolic components are important for the activation of flavocytochrome b$_{558}$, which is mediated by p47phox and p67phox at the phagocytic cups and p40phox, whose function is Vps34 and PI(3)P dependent, regulates NOX2 activity in sealed phagosomes. Once NOX2 is activated, the cytosolic components can interact with the small GTPases Rac1 and Rac2 and together with the flavocytochrome b$_{558}$ start the production of O$_2^-$. O$_2^-$ is already a cytotoxic species, but it can be used to form H$_2$O$_2$ that in turn is used by the granular enzyme myeloperoxidase to convert chloride ions into hypochlorous acid in neutrophils. It is now clear that the voltage-gated proton channel (Hv1) is involved in the charge compensation caused by the NADPH oxidase electrogenic activity in neutrophils. In fact, the accumulation of electrons inside the phagosomal lumen, mediated by the NADPH oxidase, creates an electrochemical imbalance that is compensated by the flow of protons across Hv1 from the cytosol into the phagosome. The extreme importance of a proper functional NOX2 is evident in patients affected by chronic granulomatous disease (CGD), who are severely sensitive to bacterial and fungal infections. CGD is caused by different genetic forms: the principal one is X-linked and it is characterized by mutations in the CYBB gene, which encodes NOX2. The other genetic forms are represented by autosomal recessive mutations in CYBA, NCF1, NCF2 and NCF4, gene that code for p22phox, p47phox, p67phox and p40phox respectively. In addition to ROS, reactive nitrogen intermediates (RNI) also contribute to pathogen killing with its ability to damage cellular proteins, lipids and nucleic acids. The synthesis of RNI during phagocytosis, like nitrous oxide (NO), is mediated by nitrous oxide synthase 2, also called inducible NOS (iNOS). In contrast to the rapid ROS production, RNI synthesis is delayed since iNOS gene is not expressed under basal condition.
but its transcription and translation is stimulated by the activation of several factors, like p38 mitogen-activated protein kinase, nuclear factor kB and the Janus-activated-kinase–signal transducer and activator of transcription–interferon regulatory factor 1 (JAK-STAT-IRF1) pathway \(^{123-125}\). Moreover, proinflammatory cytokines and pathogen associated molecules, such as LPS and lipoteichoic acid, are involved in the regulation of iNOS expression. Members of the ROS and RNI family can interact and produce more toxic reactive species, such as peroxynitrite (ONOO\(^-\)) and also react with several proteins and DNA to impair pathogen replication. For instance, ONOO\(^-\) is able to cause DNA double stranded breaks leading to instability of the bacterial genome, and NO is able to block the electron transport of the respiratory chain, thus causing ATP biosynthesis deficiency, in many bacteria and in some eukaryotic parasites, like *Leishmania major* and *Trypanosoma* spp.\(^{126,127}\).

Immune cells possess other tools in order to eradicate pathogens, such as antimicrobial proteins and peptides. Some immune cells, like neutrophils, are more equipped compared to others, like macrophages, as they display specialized secretory organelles inside the cytosol called granules, which enclose a plethora of different microbicidal and digestive proteins. Neutrophils present antimicrobial factors that are capable of blocking bacterial grow by depriving them of essential nutrients. As described above for NRAMP1, which is involved in the extrusion of Zn\(^{2+}\) and Mn\(^{2+}\) from the phagosome in macrophages, neutrophils display other proteins able to restrict essential metal availability. For instance, thanks to the fusion between phagosome and granules, neutrophils can diminish phagosomal iron concentration using lactoferrin, which is an iron (Fe\(^{3+}\))-binding protein, thereby leading to an arrest in bacterial growth since iron is essential for several bacterial mechanisms, like DNA replication \(^{128,129}\). Furthermore, phagosomes contain additional antimicrobial proteins that can directly interact with membranes, carbohydrates and proteins to destabilize the structural integrity of the ingested pathogen. Immune cells have developed mechanisms to combat both Gram-positive and Gram-negative bacteria, for example lysozymes are able to hydrolyse \(\beta\) glycosidic linkages that sustain the bacterial peptidoglycan layer, thus compromising bacterial integrity. In contrast, Gram-
negative bacteria presents an outer membrane that protects against lysozyme, but phagocytes can overcome it using cationic antimicrobial peptides (CAP). CAPs, such as defensins, are able to bind negatively charged bacterial membranes and create pores that cause diffusion of ions inside the bacteria, thereby leading to an osmotic imbalance\textsuperscript{130,131}. Finally, in order to completely degrade foreign particles the phagosomes harbour several enzymes, such as serine proteases (cathepsins A and G), aspartate proteases (cathepsins D and E) and cysteine proteases (cathepsins B, C, F, H, K, L, O, S, V, X and W), which are highly regulated by biochemical conditions of the phagosomal lumen, like acidity and the redox state\textsuperscript{132}.

**How microbes can escape phagocytic killing**

As already mentioned before, pathogens have been able to develop several strategies to avoid phagocytic killing and one of the most common strategies is to hamper opsonin binding. For instance, there are fungi or bacteria able to synthetize polysaccharide-based capsules that prevent them from being opsonized. Another strategy is to express specific proteins on their surface aiming to inhibit the binding with the receptor or that are able to bind proteins of the extracellular matrix, like fibronectin, albumin and plasminogen, and thus avoid complement-dependent opsonisation. Among them we can find *Yersinia enterocolitica* expressing the adhesin YadA to bind fibrinogen and collagen to prevent the deposition of iC3b\textsuperscript{9}. Moreover, Staphylococcus aureus exhibits protein A that is able to interact with the Fc portion of IgG, thereby escaping Fcγ-mediated phagocytosis\textsuperscript{10}; similar to group A streptococci that can bypass CR-dependent phagocytosis via protein M\textsuperscript{8}. In addition, *Yersinia* spp., *Pseudomonas aeruginosa* and *Escherichia coli* can escape phagocytosis via inhibition of host signalling pathways that regulate the immune process. A syringe-like molecular system, called type III secretion system, is used to insert several effectors into the immune cells, aiming to inhibit Rho-family GTPases or tyrosine phosphorylation and PI3K-dependent signalling. *Helicobacter pylori* presents a type IV secretion system to prevent phagocytosis\textsuperscript{133}, but the effectors are still unknown. Conversely, other pathogens like HIV and *Haemophilus ducreyi* use a different system called a Trojan
horse-like strategy, where their effectors stop phagocytosis once the pathogen is internalized\textsuperscript{134,135}. Finally, \textit{Mycobacterium} \textit{tuberculosis} and \textit{Leishmania} can also subvert phagocytic killing via Ca\textsuperscript{2+} signal inhibition. There is evidence showing a decrease in both SK1 recruitment to phagosomes and the subsequent intracellular calcium elevation in macrophages containing \textit{M}. \textit{tuberculosis}, thereby blocking phagosome acidification and lysosomal markers acquisition\textsuperscript{136–138}. Phagosome maturation is impaired also in \textit{Leishmania}-containing phagosome that present thicker actin rings around the phagosomes and a reduced PKCa recruitment\textsuperscript{139}.

\textbf{Lipid signalling during phagocytosis}

Different classes of lipids are involved in the regulation of the phagocytic process. Several lipids, such as sphingolipids, glycerophospholipids and cholesterol, are able to drive phagocytosis through the modulation of signalling microdomains, the production of second messengers, the remodelling of actin and the regulation of membrane traffic.

\textbf{Phosphoinositides}

In the very early stage of phagocytosis a transient increase in the level of phosphatidylinositol-4,5-bisphosphate [PI(4,5)P\textsubscript{2}] occurs, which is essential for cup formation\textsuperscript{140}. This local synthesis is mediated by phosphatidylinositol 4-phosphate 5-kinase (PI4P-5K), whose inhibition totally blocks phagocytosis, showing how both PI(4,5)P\textsubscript{2} and PI4P-5K are key molecules of this process\textsuperscript{141}. There are four isoforms of PI4P-5K, among them PI4P-5K\textgamma and PI4P-5K\textalpha are the most studied. A lack of the first isoform seems to affect more the ingestion phase on the other hand, a deficiency of the latter disturbs particle binding\textsuperscript{142}. The complete mechanism of how PI4P-5K is activated is not completely understood, even if there is evidence that small GTPases, like ARF6 and Rac1, and phosphatidic acid (PA) are involved in other contexts\textsuperscript{143–145}.

At the end of cup formation, just before phagosome sealing, a rapid drop in the level of PI(4,5)P\textsubscript{2} takes place. Two major enzymes play a role during this reduction: the phosphoinositide-specific phospholipase C\textgamma (PLC\textgamma) and the class I phosphatidylinositol 3-kinase (PI3K). Once PLC\textgamma is
phosphorylated and recruited to the phagocytic cup, in a Syk-dependent manner, it can bind PI(4,5)P$_2$ through its PH domain. Then PLC$\gamma$ hydrolyses PI(4,5)P$_2$ producing diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). DAG remains attached to the membrane where it can recruit other key phagocytic enzymes, whereas IP3 diffuses inside the cell and interacts with a specific receptor present in the endoplasmic reticulum (ER) membrane, thereby allowing the release of Ca$^{2+}$ from this store. Both second messengers are important for the recruitment and activation of protein kinase C (PKC), which boosts phagocytosis independently of actin polymerization$^{146}$. Furthermore, PI(4,5)P$_2$ can be also phosphorylated by PI3K yielding phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P$_3$] which then replaces PI(4,5)P$_2$ at the phagocytic cup$^{147,148}$. PI3K requires p85, a regulatory subunit that is recruited to phagosomes by Syk and Gab2 and then activated through phosphorylation. Gab2 is also stabilized by PI(3,4,5)P$_3$ creating a signalling amplification loop$^{23,149}$. Moreover, there is evidence showing PI(3,4,5)P$_3$ acts as an important factor for the recruitment of myosin X to the phagocytic cup, which is a motor protein involved in the formation of the pseudopods important for the sealing of the phagosome.

PI(3,4,5)P$_3$ or PI(3,5)P$_2$ are used as substrate by 4 and 5 phosphates and Vps34, a class III PI3K, uses PI to synthetize PI(3)P, another important lipid involved in phagocytosis$^{150,151}$. PI(3)P seems to be excluded from the phagosome membrane at early stage, but accumulates 1 min after phagosome formation, suggesting its involvement in phagosome maturation. In fact upon inhibition of PI(3)P production by both antibodies and wortmannin, phagosome fusion with late endosomes and lysosomes is prevented$^{85,152}$. A clear evidence of the key role that PI(3)P plays during phagocytosis was shown by Vergne et al., reporting the ability of $M$. tuberculosis to reduce PI(3)P concentration through the secretion of a phosphoinositide-3-phosphatase, called the secretory acid phosphatase (SapM), leading to the block of phagosome maturation$^{153}$. Moreover, as already mentioned above the p40 subunit of NADPH oxidase is recruited to phagosome through the interaction with PI(3)P.
Glycerophospholipids
Other types of lipids can contribute to have an efficient Fc-mediated phagocytosis, like glycerophospholipids. These lipids are generated when the phospholipase D (PLD) is recruited at the phagocytic cup, where it can hydrolyse phosphatidylcholine (PC) to produce PA. There is evidence showing that PA is important for the regulation of PI4P-5K and to recruit sphingosine kinase 1 (SK1). Furthermore, due to its cone shape PA may probably cause a negative curvature of the membrane, thereby favouring membrane fission. PLD presents two isoforms, PLD1 and PLD2 and during phagocytosis in macrophages the former is localized in late endosomes and lysosomes, whereas the latter is present in the plasma membrane. PI(4,5)P\(_2\) is required for an optimal activity of both isoforms, but in contrast to PLD2 that can be active even in the absence of further cofactors, PLD1 activity requires GTPases, like Rho, Ral and Arf and PKC. During Fc\(\gamma\)-mediated phagocytosis in macrophages, the local synthesis of PA at the phagocytic cup is primarily mediated by PLD2, which is localized at the base of the nascent phagosome and in the extending pseudopods, then it is rapidly lost during the sealing process. Conversely, PLD1 may have a role during phagosome maturation, since PLD1-containing vesicles are still recruited to early phagosomes.

PS is another member of the glycerophospholipids group and is mostly located in the inner leaflet of the plasma membrane. In addition, PS is synthesized in the so called mitochondria-associated membranes of the endoplasmic reticulum, by two enzymes: phosphatidylserine synthase 1 (PSS1) and phosphatidylserine synthase 2 (PSS2). PS is involved in phagocytosis of apoptotic bodies, where PS is externalized to the outer leaflet of the plasma membrane of apoptotic cells to play the role of an “eat-me” signalling molecule. Interestingly, studies have shown that this PS externalization occurs also in the plasma membrane of macrophages during phagocytosis of dead cells. Specifically, the adenosine triphosphate-binding cassette transporter 1 (ABC1) has been reported to regulate the transbilayer reorganization of PS in macrophages. In fact, ABC1 deficient macrophages are not able to externalize PS, thereby leading to a deficiency in phagocytosis of apoptotic cells. Currently, how the externalization of PS in the membrane of macrophages drives phagocytosis of apoptotic
bodies is still not understood. Moreover, PS could also be associated to PKC recruitment to phagosomes, since PKC possesses a C2 domain that can interact with anionic phospholipids, such as PS. Moreover, PS could also be associated to PKC recruitment to phagosomes, since PKC possesses a C2 domain that can interact with anionic phospholipids, such as PS. The last member of this group is arachidonic acid (AA) that is produced by phospholipase A2 (PLA2) and associated with several physiological and pathological mechanisms, such as inflammation and asthma, where AA is used as a substrate to produce leukotrienes and prostaglandins to mediate proinflammatory responses. AA has an important role during the early stage of phagocytosis and has been suggested to be implicated in the recruitment and activation of NADPH oxidase subunits. The accepted mechanism comprises first the oxidase assembling and then the PLA2 recruitment to the phagosome membrane, leading to the AA production needed for NADPH allosteric activation. There are 15 groups and subgroups belonging to the PLA2 superfamily and all of them can be categorized into five different types of isoforms: secreted, cytosolic, calcium-dependent, lysosomal and the platelet-activating factor acetylhydrolases. The cPLA2, which can bind PC-rich membranes through its calcium-dependent C2 domain, is the one that plays a major role during phagocytosis. A serine phosphorylation, which can be mediated by MAPK, calcium/calmodulin-dependent protein kinase II and MAPK-interacting kinase I, regulates cPLA2 activity.

**Cholesterol and Sphingolipids**
The greater portion of cellular cholesterol derived from diet is first esterified and then taken up into the cells by receptor-mediated internalization of a lipoprotein complex. Esterified cholesterol is hydrolysed in the endocytic pathway and then redistributed to secretory compartments like the ER and to the plasma membrane, where it can be extruded from the cell by the ABCA1 transporter. In addition, cholesterol can be synthesised de novo in the ER, using acetate as substrate and following approximately 30 steps. Since cholesterol is a planar and rigid molecule, it is able to distribute into the phospholipid bilayer, between the long and saturated acyl chains of the phospholipid and the sphingosine backbones, where it is stabilized by van der Waals interaction. In contrast, unesterified cholesterol accumulates in phagosomes, but its role is not well understood. Studies have...
suggested that unesterified cholesterol may mediate the retention of Rab-family GTPases in the phagosome membrane\textsuperscript{168}. Specifically, an increase in Rab7 and Rab9 concentration in the phagosome membrane was detected upon pharmacological cholesterol accumulation induced by U18666A\textsuperscript{169,170}.

In addition to cholesterol, also glycosphingolipids, sphingomyelin (SM) and their precursors confer rigidity to the phospholipid bilayer of the plasma membrane. The sphingolipid family is composed of about 400 compounds, which present a long carbon chain called sphyngoid base, such as sphingosine and sphinganine that can be further modified to produce ceramide and SM\textsuperscript{171,172}. Once ceramides are produced in the ER and then delivered to the Golgi apparatus, they can be used to synthetize glucosylceramides by glucosyltransferases. Furthermore, glucosylceramides are metabolized by luminal Golgi enzymes to obtain several glycosphingolipids, which are then delivered to the plasma membrane\textsuperscript{172,173}.

The strong interaction in the plasma membrane between cholesterol and sphingolipids leads to the creation of the so called lipid rafts, one of the most controversial subjects in biological membranes. In the most recent definition, lipid rafts are considered as nanometer sized and transient domains that are able to interact with and favour transmembrane protein clustering during receptor stimulation\textsuperscript{174,175}. In the context of Fcγ-mediated phagocytosis, Fcγ receptors have been suggested to be recruited into lipid rafts that can favour receptor clustering. In accordance with this view, there is evidence showing that Src family kinases are recruited in lipid rafts after receptor stimulation\textsuperscript{176}. Since cholesterol is believed to play a major role in lipid raft maintenance, several studies have been using β-methyl cyclodextrin to extract cholesterol from the membrane and nystatin or filipin to induce cholesterol sequestration in the plasma membrane\textsuperscript{177,178}. The reliability of these tools has been questioned because of their potential deleterious side effects on cells. In fact, massive cholesterol extraction has been reported to modify the PI(4,5)P\textsubscript{2} organization, thereby causing actin remodelling or even to deplete intracellular calcium stores\textsuperscript{179,180}. Recently, Barnes et al. used an alternative approach that aims to manipulate the molecular determinants of receptor-lipid raft association. The
researchers showed that the FcγRIIA receptor requires a palmitoylation on cysteine 208, which is located in the cytoplasmic tail close to the membrane, for association with lipid rafts. Surprisingly, even if the mutation impairs the ability of the FcγRIIA receptor to cluster into lipid rafts, only a slight reduced and delayed tyrosine receptor phosphorylation has been detected\textsuperscript{181}. The same modest effects have been reported with alanine 224, another fundamental residue linked to Fc receptor-lipid raft association, suggesting that lipid rafts are not essential for FcγRIIA-mediated phagocytosis\textsuperscript{182}, even if further experiments would be needed to avoid any compensatory effects between residues. In contrast to FcγRIIA, the association with lipid rafts seems to be more crucial for other Fc receptors, such as FcγRIIB that possesses an isoleucine at position 232 that is mutated in patients with lupus. In healthy subjects, this receptor negatively affects phagocytosis, but when the isoleucine of FcγRIIB is substituted with a threonine, the receptor is no longer able to associate within lipid rafts, thereby causing an increased phagocytic index and higher major histocompatibility complex expression in the plasma membrane. In this specific case, the failed receptor-lipid raft association leads to a FcγRIIB malfunction that results in an abnormally high activity of FcγRIIA and FcγRI receptors\textsuperscript{30}. Furthermore, studies have shown that the raft-associated protein flotillin-1 accumulates in the phagosome membrane during phagosome maturation. Dermine et al. proposed that lipid rafts can have a role in the fusion between phagosomes and late endosomes, since they have shown that the lack of flotillin-1 in phagosomes containing Leishmania causes a defect in late endosome fusion with phagosomes\textsuperscript{183}.

Sphingosine-1-phosphate (S1P), which is synthetized by sphingosine kinase, is another member of the sphingolipid family that is involved in phagocytosis. There is evidence showing S1P as a fundamental extracellular ligand of five GPCRs, the S1PR\textsubscript{1-5}\textsuperscript{184}, and also that it displays antiapoptotic effects and regulates angiogenesis and vascular maturation\textsuperscript{185}. Moreover, S1P is able to triggers calcium signals inside the cell by the interaction with its receptor on the cell surface and also acting as an intracellular second messenger that binds calcium-permeable channels resident in the ER
membrane\textsuperscript{186–188}. During phagocytosis in macrophages, SK1 is recruited at the phagocytic cup\textsuperscript{138} and an in vitro study has shown that SK1 has nanomolar affinity for anionic phospholipids, like phosphatidylserine, which are enriched in the plasma membrane\textsuperscript{189}. In addition, calcium signals occurring during the early phase of phagocytosis could be involved in SK1 recruitment, which contains a calmodulin domain that promotes the membrane translocation of the kinase\textsuperscript{184,189}. Kusner et al. found that the inhibition of SK1 by dihydrosphingosine leads to an abolishment of the intracellular calcium elevation, thereby causing a substantial reduction on the acquisition of lysosomal markers, arresting phagosome maturation. Surprisingly, the researchers noted an impairment in SK1 recruitment and activation, when the prey was represented by Mycobacterium tuberculosis, suggesting this mechanism is a means to subvert phagocytic killing that prolongs the intracellular survival of the bacteria\textsuperscript{190}.

**Lipid diffusion limiting barrier**

By now, it is clear that phagocytosis is a very defined and regulated mechanism and that the phagosome membrane undergoes a series of lipid modifications. However, it is still not clear how this lipid redistribution occurs, despite the continuity of the nascent phagosome with the plasma membrane. In fact, several types of lipids have been detected only at the phagocytic cup, such as PI(4,5)P\textsubscript{2}, and there is evidence showing that during membrane remodelling there is no lateral diffusion between the phagocytic cup and the remaining plasma membrane\textsuperscript{140}. One hypothesis consists of a locally continuous generation and fast degradation called “focal source-peripheral degradation” model, but there is no direct evidence supporting it. Interestingly, there is a second hypothesis based on the concept of a diffusional barrier that would acts as a fence to confine lipid modifications within the phagocytic cup. In fact, a study has reported that the lateral diffusion was drastically reduced at the level of the nascent phagosome compared with the closer plasma membrane. In conclusion, the lipid modifications that characterize the phagosome formation and maturation are still poorly understood, so further studies are needed to understand the mechanisms that regulate the creation of these lipid gradients during the phagocytic process.
The Role of Calcium during phagocytosis

Ca\(^{2+}\) is a ubiquitous second messenger that plays a pivotal role in different physiological mechanisms, such as proliferation and differentiation and can regulate fundamental steps of the immune response, like chemotaxis, adhesion and cytokine secretion. The first evidence that cytosolic Ca\(^{2+}\) elevations may regulate phagocytosis was shown by Stossel et al. in 1973\(^{191}\). Even if it is now broadly accepted that the phagosome formation is mostly a Ca\(^{2+}\)-independent process, with neutrophils being the only exception, and, in contrast, Ca\(^{2+}\) is extremely important for phagosome maturation, there is still a lot to discover and many molecular mechanisms underlying phagocytosis remain to be understood. Ca\(^{2+}\) signals occurring during phagocytosis have different spatio-temporal features based on the type of cell or receptor being engaged. For instance, IgG-mediated phagocytosis leads to local periphagosomal Ca\(^{2+}\) microdomains, whereas C3b-dependent phagocytosis is characterized by global Ca\(^{2+}\) elevation both in neutrophils\(^{192,193}\) and macrophages\(^{194}\). The influence of Ca\(^{2+}\) on phagocytosis becomes more evident after taking into consideration its therapeutic implications. Moreover, there are some intracellular pathogens, such as Mycobacterium tuberculosis and Leishmania that are able to subvert phagocytic killing by suppressing Ca\(^{2+}\) signals after being phagocytosed\(^{190,195}\).

FcγR-mediated calcium signals

Ca\(^{2+}\) signals occurring during phagocytosis are regulated by different types of pathways, depending on which phagocytic receptor triggers the immune process. Cytoplasmic Ca\(^{2+}\) elevations are modulated both by the release of calcium from intracellular stores, mostly the ER, and Ca\(^{2+}\) influx from the extracellular environment through the opening of permeable Ca\(^{2+}\) channels resident in the plasma membrane. Specifically, these two calcium sources are involved in the regulation of a crucial mechanism called Store Operated Calcium Entry (SOCE), which is essential for many immune functions\(^{196}\). Upon FcγRs engagement, two major pathways can be activated: PLC and PLD pathways with different isoforms involved that are cell types and receptors dependent. For instance, both PLC\(\gamma\)-1 and 2 are activated in monocytes after hFcγRI and hFcγRIIA ligation\(^{197–199}\), while hFcγRIIIA alone can activate both PLC\(\gamma\) isoforms in natural killer cells (NK)\(^{200,201}\). Conversely, in
other cell types only one isoform is stimulated, such as transfected murine macrophages or in non-phagocytic cells as Jurkat cells and platelets. hFcγRIIA activates PLCγ1 202–204, whereas hFcγRIIIA or mFcγRIII/ mFcγRIV engagement activates PLCγ2 22197,205. As previously mentioned, PLCγ is able to generate inositol-1,4,5-trisphosphate and diacylglycerol using PI(4,5)P2 as substrate. Consecutively, InsP3 can diffuse inside the cell and interact with its specific receptor resident in the ER membrane, called inositol trisphosphate receptor (IP3R) 206, thereby causing the calcium release from the store in the cytoplasm. During FcγR-mediated phagocytosis, InsP3-independent Ca2+ signals also occur via PLD-mediated activation of SK, which generates S1P. The mechanisms regulating Ca2+ signals mediated by S1P are still poorly understood. Since S1P is a membrane-bound lipid second messenger, researchers have proposed that S1P could be involved in the regulation of local Ca2+ microdomains close to the plasma or phagosome membrane 207. In addition, S1P is believed to promote global calcium elevations by the interaction with SCaMPER, a receptor present in the ER membrane, but this hypothesis remains controversial 208. Until now, studies to understand how S1P can modulates Ca2+ release from intracellular stores have been problematic due to S1P specific receptors localized in the plasma membrane, which can trigger Ca2+ signals through the PLCβ/InsP3 pathway 209,210.

The influence of the PLD pathway on phagocytosis has been detected in several cell types, such as in neutrophils in response to IgG-mediated phagocytosis 211,212 and in monocyte-derived macrophages 213. There is evidence showing a complex cross-talk between Fc-receptors in order to preferentially activate PLD or PLC pathways. Specifically, the use of dbcAMP to differentiate monocyte cell line into macrophages-like cells leads to an increase of hFcγRIIA expression level, thereby causing an increment in InsP3 concentration and prolonged and oscillatory Ca2+ signals 214,215. In contrast, hFcγRI is primarily expressed in monocytes that have been primed with IFN-γ, leading to shorter Ca2+ spikes but with higher amplitude, which are correlated with SK activation 216,217. These data suggest that hFcγRIIA signals via PLC and hFcγRI triggers phagocytosis by PLD. Moreover, in
human neutrophils coengagement of hFcγRIIA and hFcγRIIIB instead of altering PLCγ1 activity extends the duration of the Ca²⁺ signals, resulting in increased phagocytic index. Interestingly, only hFcγRIIIB activation generates Ca²⁺ signals in an InsP3-independent manner, since there is no InsP3 production and the use of PLD or SK inhibitors prevents the synergistic effect between hFcγRIIA and hFcγRIIIB receptors.

**Complement-mediated Ca²⁺ signals**
Ca²⁺ signals appear to also be involved in complement-mediated phagocytosis. There is evidence showing the presence of Ca²⁺ transients upon ingestion of serum-opsonized yeasts, which are principally internalized via complement receptor (CR) engagement. In contrast to Fc-mediated phagocytosis, which is mediated by both PLC and PLD pathways, CR-dependent phagocytosis appears to be almost entirely driven via the PLD pathway. CR3 and CR1 ligation lead to PLD activation, whose inhibition abolishes CR-mediated phagocytosis.

There are many examples of cooperation between FcR and CR-dependent phagocytosis. For instance, phagocytosis of IgG-opsonized prey mediated by hFcγRIIIB occurs only when CR3 is also coexpressed in fibroblasts. In neutrophils, the lack of CR3 causes a decrease in the phagocytic index, a lower amplitude of the Ca²⁺ transients and a reduction in superoxide generation after immune complex stimulation. Furthermore, FcR cross-linking has been shown to boost CR3 mobility and clustering at the level of the nascent phagosome in macrophages.

**Ca²⁺ signals during phagosome formation**
Whether Ca²⁺ signals are strictly required for phagosome formation is still controversial. A tight control of cytosolic Ca²⁺ concentration has been reported to be important for adequate phagocytic rates, but there is evidence showing discordant results in murine macrophages. In fact, some studies have shown an impaired phagocytosis of serum-opsonized erythrocytes (RBCs) as a consequence of intracellular Ca²⁺ chelation. On the other hand, a normal phagocytic rate of IgG-coated RBC was reported in several studies under low intracellular Ca²⁺ conditions. These clashing results are not cell type related, since also in
human neutrophils an efficient phagocytosis has been reported to be both Ca\(^{2+}\)-dependent \(^{222}\) and Ca\(^{2+}\)-independent \(^{242}\).

Several reasons can be taken into account to explain these conflicting studies. In fact, the efficiency of the phagocytic process relies on different factors, such as the presence of LPS, immune complexes, chemotactic peptides and also neuroendocrine hormones \(^ {243}\). Moreover, the materials and methods used in the studies can vary, for example differences in the method used to isolate cells and the number of target per cells that have been used can lead to discrepancy among results. Finally, the multiple engagement of different receptors at the same time could also explain Ca\(^{2+}\) requirement inconsistency, since some receptors have been shown to be more Ca\(^{2+}\)-dependent then others. In human neutrophils, Ca\(^{2+}\) is necessary for IgG-opsonized RBCs, in contrast to IgG-zymosan that can interact also with mannose receptors in addition to hFc\(\gamma\)RIIA \(^{244,245}\).

**Ca\(^{2+}\) signals during phagosome maturation**

As mentioned above, phagosome maturation is a fundamental process of phagocytosis that is characterized by a series of fusion events of the phagosome with endosomes and lysosomes, which aim to render the novel organelle able to kill the foreign particles or to degrade it in order to present antigens to cells of the adaptive immune system. In contrast to phagosome formation, studies related to phagosome maturation are more congruent in concluding that Ca\(^{2+}\) signals play an important role during this process \(^ {243}\). During these fusion events, the new phagosome acquires proteolytic enzymes and the capability to generate ROS. Studies have shown that ROS production is regulated by Ca\(^{2+}\) signals, by using both non-specific Ca\(^{2+}\) channel blockers and Ca\(^{2+}\)/CaM inhibitors \(^ {233,246}\), or through intracellular Ca\(^{2+}\) chelation during CR-dependent \(^ {247}\) and FcR-mediated phagocytosis in neutrophils \(^ {248}\). NADPH oxidase is able to depolarize both the plasma and the phagosome membrane, thus diminishing the Ca\(^{2+}\) driving force inside the cell. Immune cells, like neutrophils, possesses a voltage-gated proton channel, called VSOP/Hv1, which is able to compensate the electrical activity of the oxidase. In fact, it has been shown that the proton channel is critical for both a proper Ca\(^{2+}\) entry in neutrophils and for the consequent periphagosomal actin shedding \(^ {249}\). Furthermore, there is evidence
showing that Ca$^{2+}$ signals are crucial for lactoferrin delivery to phagosomes both using C3bi-
opsonized yeast$^{250}$ or IgG-coated RBCs$^{248}$. Interestingly, in neutrophils the fusion of azurophilic and
specific granules takes place during phagosome formation$^{251,252}$ and has been reported to be affected
by intracellular Ca$^{2+}$ chelation$^{253}$.

Another key Ca$^{2+}$-dependent step during phagosome maturation is represented by the remodelling of
the actin cytoskeleton. As previously mentioned, phagocytosis is an extremely dynamic biological
process that requires actin remodelling during pseudopods extension, closure of the nascent
phagosome and also during phagosome maturation$^{46}$. In fact, a thick meshwork of actin has been
observed around the newly formed phagosomes, which needs to be dissolved in order to promote
phagosome maturation. Gelsolin is a Ca$^{2+}$-dependent protein that is recruited at the phagocytic cup to
mediate the shedding of the actin coat$^{254,255}$. Moreover, there is recent evidence showing actin, PKC$\alpha$
and CaM be involved in mediating docking and fusion between phagosomes and lysosomes in a Ca$^{2+}$-
dependent manner$^{256,257}$. CaM has been reported to be necessary for ROS production and
phagolysosome fusion in macrophages$^{258}$. Furthermore, annexins have been detected to translocate
to the phagosome membrane in a Ca$^{2+}$-dependent way$^{259}$ and since they are phospholipid-binding
proteins able to promote membrane fusion, they have been proposed to integrate calcium signals with
cytoskeletal rearrangements$^{260}$.

Ca$^{2+}$ signals and lipid remodelling are extremely interconnected during phagocytosis, since the lipid
composition of the phagosome membrane modulates the possibility to generate local calcium
elevations. For instance during the early phase of phagocytosis, PI(4,5)P$_2$ accumulates in the
phagosome membrane to be then degraded via PLC and cause cytosolic calcium elevations. In
addition, early endosomes are enriched in PA that is used to generate S1P-depedent Ca$^{2+}$ signals.
During later phases of phagocytosis, PI(4,5)P$_2$ is depleted from the phagosome membrane, thus
excluding PLC activity in generating periphagosomal calcium microdomains. This suggests that other
mechanism might be involved during this stage, such as the recruitment of calcium stores close to
phagosome membrane or the opening of phagosomal calcium permeable channels. The first evidence showing store recruitment close to phagosome membrane in neutrophils was made by immunostaining using ER markers, such as sarco/ER calcium-adenosine trisphosphatase (SERCA) and calreticulin. Conversely, there are few evidences of endogenous calcium permeable channels resident in the phagosome membrane of professional phagocytes, but TRPC1 was shown to cluster into lipid rafts during phagocytosis when overexpressed in COS cells and all Orai isoforms have been detected in the phagosome membrane upon ectopic expression. Interestingly, TRPC3 or TRPC5 might be present in the phagosome membrane since are activated by S1P that is already been shown to be recruited at the phagocytic cup. TRPV2 is another Ca\(^{2+}\) permeable channel resident in the phagosome membrane involved in the particle binding, the first step of phagocytosis. Link et al. proposed that the entry of sodium through the channel causes the depolarisation of the phagosome membrane, thereby increasing the production of PI(4,5)P\(_2\), which then boosts phagocytosis.

In summary, Fc\(\gamma\)Rs drive phagocytosis via both PLC and PLD pathway, on the other hand CR-mediated phagocytosis is regulated by PLD pathway. In both case, the stimulation of the receptor is followed by Ca\(^{2+}\) release from the ER via InsP3 or SK/S1P, thereby causing the activation of SOCE mechanism both at the level of the plasma and phagosome membrane.

**Store Operated Ca\(^{2+}\) Entry**

The hypothesis that cells harbour intracellular calcium stores, which are able to release this calcium in response to specific physiological agonists and also that store depletion causes a mobilization of calcium from the extracellular environment, aimed to refill the stores, was firmly established by the early 1980s. Researchers have since described InsP3 as one of the second messengers able to drive calcium release from the stores and that its production is PLC-dependent. Evidence suggesting a connection between Ca\(^{2+}\) sensing in the stores and Ca\(^{2+}\) influx via plasma membrane calcium channels further define the SOCE model. Finally, electrophysiological experiments, describing the calcium release-activated calcium (CRAC) current in mast cells and T cells and
more physiological data linking CRAC currents with Fce engagement and ER calcium depletion
272,273, strongly establish a connection between SOCE and immune function.

Even if the above mentioned evidence came out at the end of the 1990s, the first data describing the molecular players of SOCE date back only to 2005. Specifically, two groups used genome-wide RNAi screening both in Drosophila S2 cells and in HeLa cells to show the role of stromal interaction molecule 1 (STIM1) in the regulation of store-operated calcium entry274,275. One year later, the same type of screening was used to identify the Ca\(^{2+}\) permeable channel ORAI in Drosophila, using as readout calcium entry or the activation of the calcium-dependent transcription factor NFAT276–278. Moreover, parallel human genetic mapping experiments were used to associate a mutation present in the ORAI1 gene, which impairs calcium influx in T cells, to Severe Combined Immunodeficiency (SCID) in humans278. There is now evidence reporting that STIM1 or ORAI1 mutations result in immunodeficiency, impairment of platelet function, tubular aggregate myopathy and Stormorken syndrome, dental defects thus further confirming the pivotal role of SOCE for a correct regulation of several physiological mechanisms279.

In humans there are 3 different STIM proteins, STIM1, STIM2, the inhibitory STIM2\textbeta\ isoform and STIM1L that are resident in the ER membrane (Figure 4)280. All isoforms have their N-terminus in the lumen of the ER where they present the calcium binding (EF)-hand domain and the sterile alpha motif (SAM) domain. After the transmembrane (TM) domain, STIM proteins display the coiled-coil domains 1 and 3 (CC-1-CC-3), the CRAC activation domain (CAD), the STIM-Orai activating region (SOAR) and the polybasic domain (PBD) in the cytosol, which represent the C-terminus. Also ORAI has 3 different isoforms in humans, ORAI1, 2 and 3 and each ORAI monomer presents four transmembrane domains with both N- and C-termini resident in the cytosol, both harboring STIM1-binding sites279.
Figure 4. Schematic organization of STIM proteins. Both functional and structural domains are divided in coloured regions. Coloured lines represent mutations that cause STIM inhibition (red), STIM activation (yellow), alter CDI (orange), SOCE inhibition during mitosis (blue) and inhibit EB1 binding (purple). Arrows indicate alternative splicing insertion for both STIM proteins (STIM1L and STIM2β) 280.

During the resting state, the EF-hand domain of STIM1 interacts with Ca^{2+} present in the lumen of the ER and the CC1α1-CC3 interaction, keeps STIM1 in an inactive state which maintains the CAD/SOAR domains unable to interact with the channel 281. Upon store depletion, STIM1 undergoes a conformational change that releases the CAD/SOAR region and the PBD, allowing it to interact with ORAI channels and PI(4,5)P\textsubscript{2} lipids resident in the plasma membrane 281. During this active state, STIM1 is able to oligomerizes via CC3-CC3 interactions and moves within the ER to reach Endoplasmic reticulum – Plasma membrane (ER-PM) contact sites, where it gates ORAI channels and allows Ca^{2+} influx from the extracellular environment. ORAI channels diffuse across the plasma membrane to reach the ER-PM contact sites where STIM1 is located after store depletion (Figure 5) 282. There is evidence showing that the ORAI N-terminus is fundamental for gating 283–285 and the ORAI C-terminus needs to undergoes a modest rearrangement required for STIM1 binding 286.
Figure 5. Representation of STIM1 activation and recruitment at ER-PM membrane contact sites. In resting state STIM1 displays a homodimer conformation (1). Upon agonist stimulation, phospholipase C (PLC) is activated in order to generate inositol-1,4,5-trisphosphate (IP3) (2), thereby stimulating the release of calcium from the endoplasmic reticulum (ER) (3). The store depletion induces STIM1 rearrangement (4a), thus exposing both SOAR domain and polybasic domain (K-rich domain) (4b). After STIM1 oligomerization (5a), it can be recruited at the level of the plasma membrane and interact with PI(4,5)P₂ (5b). Ora1 diffuse in the plasma membrane (6-7) in order to interact with STIM1, thereby activating SOCE (7). Finally, SERCA pumps the calcium into the ER to refill the store (8).

Store operated calcium entry can be regulated through different mechanisms. For instance, there are two types of calcium-dependent negative feedback: fast calcium-dependent inactivation and slow calcium-dependent inactivation, which occur on a time course of 10 to 100 milliseconds and 10 to 100 seconds respectively. In addition, external pH can inhibit ORAI channels, with a strong block of the CRAC current at pH 6.0 – 6.5. Interestingly, the heteromeric arachidonic acid-activated (ARC) channel formed by ORAI1/ORAI3 is less affected by pH than the CRAC channel. Phosphorylation appears to be another mechanism by which SOCE can be negatively modulated, since STIM1 can be phosphorylated at the level of Ser/Thr-Pro sequence located at the C-terminus by proline-directed kinases like ERK1/2, p38 MAPK and mitotic kinases. Moreover, ORAI1 phosphorylation at the N-terminus seems also to inhibit SOCE. Finally, some proteins can regulate SOCE via direct interaction.
with STIM or ORAI proteins, such as CRAC2A, that potentiates Ca\(^{2+}\) entry via interaction with the N-terminus of ORAI, or SARAF that is also resident in the ER and is capable of inhibiting STIM1-ORAI interactions through the interaction with STIM1 in the ER membrane\(^{279}\).

Several types of Ca\(^{2+}\) signals occur during phagocytosis, such as calcium release from intracellular stores and calcium influx from the extracellular environment. Normally, store operated calcium entry is a highly regulated mechanism that takes place at the level of ER-PM membrane contact sites, but during phagocytosis SOCE can be detected also around the phagosome at the level of endoplasmic reticulum-phagosome (ER-Ph) membrane contact sites, in order to generate periphagosomal calcium microdomains able to boost phagocytosis. Recently, the group of Demaurex described the recruitment of STIM1 in this specific membrane contact site and its role in regulating periphagosomal Ca\(^{2+}\) hotspots. Nunes et al. were able to show how STIM1 regulates phagocytosis via a SOCE-dependent periphagosomal Ca\(^{2+}\) elevation, thereby regulating actin shedding. Moreover, mouse embryonic fibroblasts (MEFs) overexpressing STIM1 displayed an increase in both number and size of ER-Ph membrane contact sites. These data define the key role of STIM1 in the regulation of the phagocytic process (Figure 6)\(^{264}\).

**Figure 6. SOCE at the level of ER-Ph MCS.** STIM1 is able to recruit the ER close to the phagosome membrane, in order to open store-operated calcium channels to generate periphagosomal Ca\(^{2+}\) hotspots that boost phagocytosis\(^{264}\).
**Junctate**

Recently, junctate, has been discovered to be involved in the regulation of the store operated calcium entry. Junctate is an ER transmembrane protein of 33-kDa expressed in several human tissues, such as pancreas, brain, liver, heart, placenta and kidney, that possesses an EF-hand domain inside the lumen of the ER, through which it can sense the depletion of the store. Junctate is encoded by the human AβH-J-J locus that codes for 4 different proteins: the enzyme aspartyl-ß-hydroxylase (AβH), junctin that is a structural protein of the sarcoplasmic reticulum, and 2 types of junctate. Two different promoters can regulate the AβH-J-J locus, P1 and P2, where P1 ubiquitously regulates the expression of AβH and junctate via alternative splicing, with the two proteins sharing the amino acid sequence of their cytosolic portion. Conversely, P2 is activated only in excitable cells and codes for junctin and junctate by a different alternative splicing, with junctate sharing the same cytoplasmic portion of junctin (Figure 7).

![Figure 7. Schematic representation of junctate alternative splicing isoforms.](image)

**Figure 7. Schematic representation of junctate alternative splicing isoforms.** The AβH-J-J locus is under the regulation of two promoters, P1 and P2. P1 is ubiquitously activated and regulates the expression of aspartyl-ß-hydroxylase (AβH), which is characterized by a catalytic domain (purple) and shares the highly charged domain (blue), the transmembrane domain (black) and the cytosolic portion (green) with the ubiquitously expressed junctate. P2 regulates junctate and junctin expression only in excitable cells. Junctin displays a basic luminal region (red) and junctin-specific cytosolic region (yellow).

Junctate has been described to interact with STIM1 via its luminal domain and facilitates STIM1 recruitment at the level of ER-PM contact sites (Figure 8). Moreover, there is evidence reporting
junctate populating ER cisternae close to the plasma membrane during the resting state and the recruitment was mediated by its N-terminus, which resides in the cytosol. In addition, the EF-mutated version of junctate recruited STIM1 at the plasma membrane contact sites in resting cells and when overexpressed efficiently increased SOCE in primary T cells. Junctate can interact with all 3 isoforms of the inositol-1,4,5-trisphosphate receptor (InsP3R) and TRPC channels via its cytoplasmic domain (Figure 8). Upon junctate overexpression, mice develop myocardial hypertrophy and it can also interact with sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase 2a (SERCA2a) in mouse cardiomyocytes, thus regulating the sarcoplasmic reticulum Ca\(^{2+}\) cycling. There is evidence showing junctate as an important player in calcium homeostasis, i.e. junctate overexpression leads to an increase in the size and number of ER-PM contact sites and also in the amount of Ca\(^{2+}\) released from the store. Furthermore, the C-terminus of junctate has been described to be a new way of STIM1 recruitment in a phosphoinositide- and ORAI-independent manners.

**Figure 8. Partners of junctate at ER-PM MCS.** Junctate can recruit STIM1 at the level of ER-PM MCS via the luminal domain. Moreover, it can also interact with IP3Rs and calcium permeable channels, like TRPC3, through its cytosolic portion.
Purpose of the project
A better understanding of both the molecular mechanisms regulating phagocytosis and the strategies used by pathogens to escape phagocytic killing could lead to the creation of novel therapies aimed at helping our immune system to defeat pathogens. Nunes et al. were still able to detect both periphagosomal calcium microdomains and membrane contact sites between the phagosome membrane and the endoplasmic reticulum in STIM1 KO MEFs, suggesting that other proteins are mediating ER-Ph membrane contact sites. Junctate was investigated as a potential candidate due to its role in calcium homeostasis regulation. In fact, junctate is able to regulate SOCE not only as STIM1 partner, but also as a modulator of ER-PM contact sites.
RESEARCH ARTICLE

Junctate boosts phagocytosis by recruiting endoplasmic reticulum Ca\textsuperscript{2+} stores near phagosomes

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ABSTRACT

Local intracellular Ca\textsuperscript{2+} elevations increase the efficiency of phagocytosis, a process that is essential for innate and adaptive immunity. These local Ca\textsuperscript{2+} elevations are generated in part by the store-operated Ca\textsuperscript{2+} entry (SOCE) sensor STIM1, which recruits endoplasmic reticulum (ER) cisternae to phagosomes and opens phagosomal Ca\textsuperscript{2+} channels at ER-phagosome junctions. However, residual ER–phagosome contacts and phagosomal Ca\textsuperscript{2+} hotspots remain in Stim\textsuperscript{−/−} cells. Here, we tested whether junctate (also called ASPH isoform 8), a molecule that targets STIM1 to ER–plasma-membrane contacts upon Ca\textsuperscript{2+}-store depletion, cooperates with STIM1 at phagosome junctions. Junctate expression in Stim\textsuperscript{−/−} and Stim\textsuperscript{+/-}; Stim\textsuperscript{−/-} phagocytic fibroblasts increased phagocytosis and perphagosomal Ca\textsuperscript{2+} elevations, yet with only a minimal impact on global SOCE. These Ca\textsuperscript{2+} hotspots were only marginally reduced by the SOCE channel blocker lanthanum chloride (La\textsuperscript{3+}) but were abrogated by inositol trisphosphate receptor inhibitors 2-APB and xestospongin-C, revealing that unlike Stim1-mediated hotspots, junctate-mediated Ca\textsuperscript{2+} originates predominantly from perphagosomal Ca\textsuperscript{2+} stores. Accordingly, junctate accumulates near phagosomes and elongates ER–phagosome junctions in Stim\textsuperscript{−/−} cells. Thus, junctate mediates an alternative mechanism for generating localized Ca\textsuperscript{2+} elevations within cells, promoting Ca\textsuperscript{2+} release from internal stores recruited to phagosomes, thereby boosting phagocytosis.

KEY WORDS: Ca\textsuperscript{2+}, Capacitative calcium entry, Ion channel, Junctate, Signal transduction, Membrane contact site, Phagocytosis

INTRODUCTION

Phagocytosis, the engulfment of foreign particles into a membrane-enclosed vacuole known as the phagosome, is a fundamental cellular process that is essential for bacterial killing and antigen presentation by innate immune cells. The phagocytic process comprises two phases, an ingestion phase that is characterized by the recognition of the particle and its actin-driven engulfment into a new membrane-derived organelle, as well as a maturation phase during which the phagosome acquires oxidative and lytic properties through fusion with endosomes and lysosomes (Adenium and Underhill, 1999). The kinetics and efficiency of phagosome maturation is promoted by both global as well as localized Ca\textsuperscript{2+} elevations occurring during phagocytosis that drive the shedding of the actin coat (Bengtsson et al., 1993), the assembly and activation of the phagocytic NADPH oxidase (Dewitt et al., 2003), and the fusion with lysosomes (Jaconi et al., 1990), reviewed by Nunes and Demaurex (2010). Global Ca\textsuperscript{2+} elevations occur during phagocytosis as a result of phagocytic receptor ligation and phospholipase-C or phospholipase-D-driven store-operated Ca\textsuperscript{2+} entry (SOCE) (Nunes and Demaurex, 2010). Localized perphagosomal Ca\textsuperscript{2+} elevations can be generated in two ways: (i) by the opening of Ca\textsuperscript{2+}-permeable channels upon Ca\textsuperscript{2+} stores located in the vicinity of phagosomes or by the opening of Ca\textsuperscript{2+} channels present on phagosomes through mechanisms that have only recently begun to be uncovered. We have previously shown that the transmembrane endoplasmic reticulum (ER) Ca\textsuperscript{2+} sensor stromal interaction molecule 1 (STIM1) recruits ER cisternae to phagosomes and promotes the opening of perphagosomal Ca\textsuperscript{2+} channels (Nunes et al., 2012). STIM1 mediates the ubiquitous SOCE mechanism (Liu et al., 2005; Roos et al., 2005; Zhang et al., 2005) by acting as an intracellular ligand for plasma membrane Ca\textsuperscript{2+}-permeable channels of the ORAI (Feske et al., 2006; Vig et al., 2006; Zhang et al., 2006) and transient receptor potential canonical (TRPC) families (Huang et al., 2006; reviewed in Hogan et al., 2010), together referred to as store-operated Ca\textsuperscript{2+} (SOCE) channels. Upon ER Ca\textsuperscript{2+} depletion, STIM1 oligomerizes and accumulates in cortical contact sites between the ER and plasma membrane (Luik et al., 2008; Lur et al., 2009; Orei et al., 2009; Wu et al., 2006), where its channel activating domain (CAD) directly interacts with ORAI to promote channel opening (Luik et al., 2006; Park et al., 2009; Xu et al., 2006; Zhou et al., 2010). Three independent synergistic mechanisms promote STIM1 localization at ER–plasma-membrane junctions: (1) STIM1 binding to phosphoinositides through the exposed polylysine C-terminal tail (Walsh et al., 2010), (2) binding of the STIM1 cytoplasmic CAD domain to ORAI1 (Huang et al., 2006; Muik et al., 2009; Park et al., 2009; Yuan et al., 2009), and (3) Ca\textsuperscript{2+}-regulated binding of STIM1 through its luminal domain to junctate (Srikanth et al., 2012).

Junctate is a ubiquitously expressed 33-kDa single-pass transmembrane ER protein bearing a luminal EF-hand motif, generated by alternative splicing of the gene encoding junctin and aspartate-β-hydroxylase (ASPH isoform 8) (Dinckuk et al., 2000; Hong et al., 2001; Treves et al., 2000). Junctate and STIM1 interact through their luminal domains, thereby facilitating STIM1 recruitment to membrane contact sites (Srikanth et al., 2012), and interacts with the inositol 1,4,5 trisphosphate receptor (InsP3R) and with TRPC channels through its cystolic N-terminus (Treves et al., 2004, 2010). Junctate also interacts with sarco-endoplasmic reticulum Ca\textsuperscript{2+} ATPase 2a (SERCA2a) in cardiomyocytes to regulate sarcoplasmic reticulum Ca\textsuperscript{2+} cycling (Kwoon and Kim, 2009), and mice overexpressing junctate develop myocardial hypertrophy (Hong et al., 2008), highlighting the importance of junctate in Ca\textsuperscript{2+} homeostasis. Junctate populates plasma membrane clusters in nestin cells, indicating that it is a structural component of ER–plasma-membrane contact sites, and junctate overexpression...
elongates and stabilizes ER-plasma-membrane junctions and increases the amount of Ca²⁺ released from intracellular Ca²⁺ stores (Treves et al., 2004, 2010). The N-terminus of junctate is devoid of lipid-binding motifs but is required for junctate accumulation in plasma membrane clusters, suggesting that junctate provides an alternative mechanism of STIM1 recruitment that is independent of phosphoinositides and Orai1 (Srikanth et al., 2012). Such a mechanism might be important for the targeting of STIM1 to membranes that have distinct phosphoinositide compositions, or importantly, for the delivery of Ca²⁺ stores to target membranes independently of STIM1.

Phagosomes, although derived from the plasma membrane, have a very distinct lipid and protein composition because extensive sorting occurs during phagosome engulfment. In a previous study, we detected a significant number of ER-phagosome junctions, as well as residual Ca²⁺ hotspots (also called microdomains), in primary neutrophils as well as in mouse embryonic fibroblasts (MEFs) that had been engineered to be phagocytic and isolated from mice in which STIM1 had been genetically ablated. These results suggest that proteins other than STIM1 recruit ER cisternae and mediate localized Ca²⁺ signaling near phagosomes. We therefore postulated that junctate cooperates with STIM1 to recruit ER cisternae to phagosomes, thereby providing an alternative mechanism for generating periphagosomal Ca²⁺ hotspots. We tested this hypothesis by expressing junctate in fibroblasts, which had been rendered phagocytic through expression of FcγRIIA receptors (also known as FCGR2A), from STIM1-knockout mice. Our data show that junctate recruits ER Ca²⁺ stores to phagosomes and promotes localized Ca²⁺ elevations that sustain high-efficiency phagocytosis.

**RESULTS**

**Junctate is recruited to phagosomes independently of STIM1**

To assess whether junctate is recruited to phagosomes, we quantified the percentage of phagosomes that were decorated by yellow fluorescent protein (YFP)-junctate, by green fluorescent protein (GFP)-KDEL as a control ER marker, or by mCherry-STIM1, which we have previously shown to be recruited to phagosomes (Nunes et al., 2012). The proteins were expressed in wild-type, Stim1−/− and Stim1−/−, Stim2−/− MEFs that had been rendered phagocytic through co-expression with FcγRIIA c-Myc receptors, and cells were then exposed to IgG-opsonized red blood cells (RBCs). To synchronize phagocytosis, RBCs were centrifuged onto cells that had been seeded on coverslips, and cells were allowed to phagocytose for 10 min before fixation. Coverslips were then immunostained to reveal c-Myc tagged Fc receptors, and imaged by using confocal microscopy. Quantification through visual inspection of confocal z-stacks revealed that both GFP-KDEL and YFP-junctate fluorescent clusters (or puncta) were observed around ~40% of phagosomes found in wild-type cells (Fig. 1A). In Stim1−/− cells, no discernible periphagosomal GFP-KDEL clusters could be detected, whereas YFP-junctate and mCherry-STIM1 clusters decorated 31% and 57% of phagosomes, respectively (Fig. 1B). When the proteins were expressed in Stim1−/−, Stim2−/− cells, YFP-junctate and mCherry-STIM1 clusters decorated 57% and 68% of phagosomes compared with 16% of phagosomes for GFP-KDEL (Fig. 1C). The absence of GFP-KDEL clusters in Stim1−/− but not Stim1−/−, Stim2−/− cells might reflect the difficulty to identify clusters owing to the low and more finely reticular expression pattern of the ER marker in Stim1−/− cells (compare

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**Fig. 1. Junctate is recruited to phagosomes in a STIM1-independent manner.** (A) Three-dimensional projections of confocal z-stacks show that in wild-type MEFs, GFP-KDEL puncta (left panel, green), as well as YFP-junctate puncta (middle panel, yellow) are observed near red blood cell (RBC)-containing phagosomes (red) at similar frequencies (right panel). Arrows indicate periphagosomal puncta. (B) Contrary to wild-type MEFs, GFP-KDEL puncta (left panel, green) are not detectable around phagosomes in Stim1−/− MEFs. In contrast, YFP-junctate (middle panel, yellow) still localized near to phagosomes in the absence of STIM1, albeit at a lower frequency than mCherry-STIM1 puncta (right panel). (C) YFP-junctate (upper left panel, yellow) and mCherry-STIM1 (middle panel, red) are recruited to phagosomes (red in left panel, green in middle panel) in Stim1−/−, Stim2−/− MEFs at 3- to 3.5-fold higher frequencies (right panel) than GFP-KDEL (green, lower left panel). (D) YFP-junctate (green) and mCherry-STIM1 (red) colocalize in periphagosomal puncta when co-expressed in Stim1−/− MEFs. Scale bars: 3 µm. Data are means±s.e.m. of three independent experiments, comprising the following number of cells, phagosomes, puncta. Wild type: KDEL, 121, 247, 02: junctate, 155, 150, 63: Stim1−/−:KDEL, 189, 271, 0: junctate, 103, 404, 127: STIM1, 154, 242, 120: Stim1−/−:Stim2−/−: KDEL, 99, 36, 11: junctate, 91, 83, 45: STIM1, 95, 119, 62:**P<0.05, ***P<0.01, ****P<0.001.
Fig. 1B left panel and Fig. 1C lower left panel). Alternatively, an altered number of residual contact sites in cells expressing only STIM2 could account for this difference. Interestingly, when co-expressed in Stim1−/− MEFs, YFP-junctate and STIM1-mCherry both localized in the same peripherogosomal clusters (Fig. 1D). These data indicate that juncate is recruited to phagosomes independently of STIM proteins but might interact with STIM1 at ER–phagosome contact sites.

**Junctate expression increases the size of phagosome-ER junctions**

To establish whether juncate recruits ER cisternae to the vicinity of phagosomes and/or alters the morphology of ER–phagosome-membrane contact sites, we quantified the extent of ER cisternae recruitment to phagosomes by using electron microscopy in 50-nm slices. To normalize for the cross-sectional sampling, junctional ER was quantified as the average contact length divided by the perimeter of the phagosome (Fig. 2, arrows). Contacts were defined as the contiguous length of an ER cisterna that discernibly remained ≤30 nm from the membrane of a phagosome. These juxtaposed ER structures were detected in 23–29% of phagosomes forming in Stim1−/− and wild-type phagocytic MEFs, regardless of juncate expression (Fig. 2), and the length of the recruited ER structures averaged ~250 nm (268±51 nm for YFP-junctate and 231±58 nm for GFP-KDEL, corresponding to 2.84±0.54% and 2.45±0.62% of the phagosome perimeter, n=20 and 28, respectively; mean±s.e.m.; Fig. 2). By contrast, YFP-junctate expression doubled the length of the recruited cisternae in Stim1−/− cells (231±36 nm vs 115±19 nm for GFP-KDEL, corresponding to 2.45±0.38% vs 1.22±0.20% of the phagosome perimeter, n=24 for each; Fig. 2). Qualitative differences were also apparent as the juxtaposed ER cisternae were thinner in Stim1−/− cells expressing YFP-junctate (Fig. 2, arrows). These data indicate that juncate, when expressed in the absence of STIM1, can promote the elongation of peripherogosomal ER cisternae.

**Junctate mediates store-operated Ca2+ entry in the absence of STIM proteins**

Junctate has been previously reported to populate STIM1-Oral1 membrane contact sites and to interact with TRPC channels, suggesting that this protein might modulate the activity of Ca2+ entry channels. To test this hypothesis, we measured Ca2+ entry rates following store depletion with the SERCA inhibitor thapsigargin in cells expressing either YFP-junctate, GFP-KDEL or mCherry–STIM1 as controls. Juncate expression had no impact on Ca2+ entry rates in wild-type or Stim1−/− MEFs, but surprisingly, juncate increased Ca2+ entry rates in Stim1−/−; Stim2−/− MEFs by twofold. In comparison, STIM1 re-expression in Stim1−/− and Stim1−/−; Stim2−/− MEFs increased Ca2+ entry rates by fivefold and eightfold, respectively, to values near to 70% of wild-type levels (Fig. 3A,C). The small levels of Ca2+ entry mediated by juncate alone, in Stim1−/−; Stim2−/− MEFs, was abolished in the presence of the non-specific Ca2+ channel inhibitor lanthanum chloride (La3+), similar to the STIM1-mediated entry, indicating that the source of the signal was extracellular (Fig. 3A,C). In addition, no significant change in intracellular Ca2+ was detected during Ca2+ removal and add-back in the absence of thapsigargin (Fig. 3B), indicating that the juncate-mediated entry was dependent on the emptying of ER Ca2+ stores. Taken together, these data indicate that juncate can have a small impact on global SOCE that is only revealed when both STIM proteins are absent.

**Junctate boosts phagocytosis independently of STIM proteins**

We next tested whether juncate expression impacts the ability of cells to phagocytose, using a high target:cell ratio (10:1) to increase the reliability of the phagocytic process on C3a signals (Nunes et al., 2012). To synchronize the time of exposure to targets, RBCs were centrifuged onto cells that had been seeded onto coverslips, and cells were allowed to phagocytose for 30 min before fixation. This single timepoint snapshot provides an estimate of the phagocytic rate as cells reached their maximum phagocytic capacity, ~2 h after target centrifugation at this target:cell ratio. Coverslips were immunostained to reveal e-Myc-tagged Fc receptors to facilitate phagosome identification, and imaged by using confocal microscopy. Quantification of confocal z-stacks through visual inspection revealed that the average number of phagocytosed particles per cell (the phagocytic index) during this 30-min time interval doubled in cells that expressed juncate compared to cells that expressed GFP-KDEL [wild type, 2.05±0.14 (GFP-KDEL)]
and 3.76±0.47 (YFP-juncate) phagosomes per cell, mean±s.e.m. 
n=4 for each; Stim1−/−, 1.58±0.21 and 3.03±0.35 phagosomes per cell, 
n=6 for each; Stim1−/−; Stim2−/−, 1.45±0.2 and 4.27±0.65 
phagosomes per cell, n=6 for each; Fig. 4). mCherry–STIM1 re-
expression further boosted the phagocytic index (Stim1−/−), to 4.1± 
0.79 phagosomes per cell, n=6; Stim1−/−; Stim2−/−, to 5.97±1.08 
per cell, n=6; Fig. 4). These data suggest that juncate can increase 
the phagocytic capability of cells in the absence of STIM proteins 
despite having only a marginal effect on SOCE. The fact that 
Stim1−/−; Stim2−/− cells showed similar or higher phagocytic rates 
and recruitment to phagosomes upon juncate overexpression 
indicates that juncate does not simply recruit STIM2 as an 
alternative mechanism to gate SOCE channels, and that, if anything, 
interaction with STIM2 could hinder juncate function, possibly by 
sequestering juncate elsewhere.

Juncate increases the frequency of periphagosomal Ca2+
hotspots by promoting Ca2+ release from internal stores

Because STIM1 boosts phagocytosis by increasing the frequency 
and duration of local Ca2+ elevations around phagosomes, we 
assessed whether juncate also promotes periphagosomal Ca2+
elevations by using live confocal imaging and the Ca2+-sensitive 
dye Fluo-8 (Fig. 5A). Ca2+ hotspots (arrows) were detected within a 
750-nm perimeter (red region of interest, middle panel) in 6-s 
nsnapshots taken 25–30 min after the addition of phagocytic targets. 
Changes in hotspot occurrence in these snapshots might reflect 
either an increased frequency or duration of these events, both of 
which would reflect increased signaling. Although we did not 
observe changes in the temporal dynamics of hotspots (see 
Movie 1), approximately 39% of phagosomes in Stim1−/− cells 
that expressed red fluorescent protein (RFP)-KDEL as a control ER 
protein displayed periphagosomal Ca2+ hotspots, and this
percentage increased to 76.0% and 82.0% upon expression of RFP-junctate and mCherry–STIM1, respectively (n=5 for all conditions; Fig. 5A, bottom panel). Thus, junctate is as efficient as STIM1 in promoting phagosomal Ca\(^{2+}\) elevations. Interestingly, although the hotspots promoted by junctate covered a similar fraction of the phagosomal surface as the STIM1-generated hotspots (28% vs 27%, n=47 and 64 phagosomes, respectively), they extended more frequently beyond the 750-nm wide phagosomal space (84±5.0% vs 59±4.5%, n=3), prompting us to further explore the source of the Ca\(^{2+}\) signal. The intracellular Ca\(^{2+}\) hotspots that form around phagosomes can reflect either Ca\(^{2+}\) release from the ER or Ca\(^{2+}\) release from phagosomes, which initially contains a high concentration of Ca\(^{2+}\) that is derived from the extracellular space. To distinguish between these two sources of Ca\(^{2+}\), we repeated the experiment either in Ca\(^{2+}\)-free medium or in the presence of SOCE channel inhibitor La\(^{3+}\), which abolished global SOCE in Stim\(^{1-/-}\) and Stim\(^{3-/-}\); Stim2\(^{-/-}\) cells (Fig. 3A, C). This generic Ca\(^{2+}\) channel blocker is internalized into the lumen of forming phagosomes and minimizes the contribution of Ca\(^{2+}\) flowing across both plasma-membrane and phagosomal channels, allowing us to isolate the Ca\(^{2+}\) signals originating from internal stores. Ca\(^{2+}\) removal from the external medium decreased the percentage of phagosomes that were associated with local Ca\(^{2+}\) elevations to 14% and 22% in RFP–KDEL and mCherry–STIM1 cells, respectively, but to only 32% in cells that expressed RFP–junctate, which exhibited significantly more periphagosomal Ca\(^{2+}\) microdomains than cells that expressed the other two proteins (Fig. 5B, C). This difference was further increased in the presence of La\(^{3+}\), which had only a mild effect on the activity recorded in RFP–junctate-expressing cells but decreased the activity of mCherry–STIM1-expressing cells to control levels (55% for RFP–junctate versus 34% and 36% for RFP–KDEL and mCherry–STIM1, respectively; Fig. 5B, C).

To confirm that junctate regulates periphagosomal Ca\(^{2+}\) hotspots by recruiting functional Ca\(^{2+}\) stores, periphagosomal Ca\(^{2+}\) was measured in the presence of the InsP3R inhibitor 2-APB. Because 2-APB also blocks ORAI channels, La\(^{3+}\) was simultaneously applied to eliminate the contribution of any SOCE channel, including ORAI-family proteins. This treatment decreased the frequency of periphagosomal Ca\(^{2+}\) hotspots to control levels in both RFP–junctate- and mCherry–STIM1-expressing cells (12% for RFP–junctate vs 19% for RFP–KDEL and 13% for mCherry–STIM1; Fig. 5B, C). Finally, to control for the contribution of residual STIM2-mediated channel gating, periphagosomal Ca\(^{2+}\) hotspots were also quantified in Stim\(^{1-/-}\); Stim2\(^{-/-}\) MEFs. Similar to Stim\(^{1-/-}\) cells, YFP–junctate and mCherry–STIM1 expression increased hotspot frequency by twofold, to 51% and 53% respectively, compared to 25% for RFP–KDEL (Fig. 5B). Xestospongin-C, a more specific InsP3R blocker that suppressed ATP-induced Ca\(^{2+}\) signals in Stim\(^{1-/-}\); Stim2\(^{-/-}\) MEFs (Fig. S1), had no effect on hotspot frequency in cells that expressed RFP–KDEL but did abrogate the increase in periphagosomal Ca\(^{2+}\) hotspots evoked by junctate expression and decreased the frequency of the STIM1-induced hotspots to 26% (Fig. 5D). The residual Ca\(^{2+}\) hotspots persisting in the presence of inhibitors might reflect incomplete inhibition of Ca\(^{2+}\) influx and release channels, or release from InsP3R-independent Ca\(^{2+}\) stores. Taken together, these data confirm that STIM1-mediated hotspots are generated by both phagosomal and ER-store release components, as previously suggested, and show that the store release component is InsP3R-dependent. Moreover, these results indicate that junctate promotes periphagosomal Ca\(^{2+}\) elevations primarily by recruiting functional Ca\(^{2+}\) stores to phagosomes rather than by interacting with Ca\(^{2+}\)-permeable channels that are present in the plasma membrane.

**Junctate promotes phagosomal actin shedding**

Periphagosomal Ca\(^{2+}\) hotspots have been previously shown to promote shedding of the actin coat that forms during phagocytic ingestion, which is considered to be an important stage during early phagosome maturation (Nunes and Demlaux, 2010; Nunes et al.,
2012). Reduced actin shedding might limit global phagocytic rates by hindering phagosome–lysosome fusion as well as fission events that allow recycling of Fc receptors back to the cell surface. The availability of actin, and particularly rapid actin dynamics, could be additionally important for higher speeds of subsequent actin-driven membrane remodeling as the cell internalizes new targets. We thus further investigated whether junctionate, like STIM1, influences periphagosomal actin structures. Both in Stim1−/− and Stim1+/−; Stim2−/− MEFs, the overexpression of RFP–junctionate and mCherry–STIM1 decreased periphagosomal filamentous F-actin rings compared to that in RFP–KDEL controls (Fig. 6). These data indicate that junctionate regulates phagocytic ingestion rates by promoting periphagosomal actin shedding.

**DISCUSSION**

In this study, we show that junctionate is an important signaling protein that can determine the efficiency of the phagocytic process. We provide morphological evidence that junctionate is recruited to phagosomes and increases the length of juxtaposed ER structures, and functional evidence that junctionate significantly increases the efficiency of phagocytosis independently of STIM1. We could link the pro-phagocytic effects of junctionate to an increased frequency of periphagosomal Ca2+ elevations occurring near phagosomes, as well as to increased actin shedding during early phagosome maturation, and show that the junctionate-associated Ca2+ hotspots or microdomains are much less affected by Ca2+ removal and by the SOCE channel inhibitor La3+ than STIM1-associated Ca2+ microdomains. We further show that the junctionate-associated Ca2+ microdomains are almost abrogated by the InsP3R inhibitor 2-APB in the presence of La3+, as well as by the more specific InsP3R inhibitor xestospongin-C alone, indicating that they essentially reflect Ca2+ release from inositol-triphosphate-sensitive stores. This suggests that, unlike STIM1, junctionate does not promote the opening of phagosomal Ca2+ channels but instead recruits InsP3R-containing ER Ca2+ stores near phagosomes. Junctionate is more efficient than STIM1 in recruiting functional ER Ca2+ stores, whereas STIM1 is more efficient in gating phagosomal Ca2+ channels, allowing the two proteins to cooperate to promote the generation of local Ca2+ elevations that boost phagocytosis.

Junctionate has previously been shown to play an important role in Ca2+ homeostasis and to interact with several components of the Ca2+ ‘toolkit’. Initial studies reported that junctionate interacts through its cytosolic N-terminal domain with the InsP3R and TRPC channels, and that junctionate overexpression increases agonist-induced Ca2+ elevations, whereas silencing has the opposite effect (Treves et al., 2004). Those authors have subsequently shown that junctionate stabilizes ER–plasma-membrane junctions and promotes agonist-activated Ca2+ entry across TRPC3 channels (Treves et al., 2010). Junctionate also interacts with SERCA2a in cardiomyocytes (Kwon and Kim, 2009), and with TRPC2 and TRPC5 channels in rodent sperm (Stamboulis et al., 2005). More recently, junctionate has been shown to interact through its luminal domain with STIM1 and to provide an alternative mechanism for STIM1 recruitment to Ca2+ entry sites at the plasma membrane (Srikant et al., 2012). In this study, junctionate overexpression in primary T cells caused a marked increase in SOCE, whereas a junctionate ER-hand mutant promoted the accumulation of STIM1 in ER–plasma-membrane clusters and caused a marked increase in SOCE. These data indicate that junctionate acts as an ER sensor that facilitates STIM1 clustering at ER–plasma-membrane junctions, but they do not exclude the possibility that junctionate acts as a ligand for membrane channels on its own in response to store depletion. We observed that when SOCE is maximally activated, junctionate overexpression does not further increase global Ca2+ entry rates in wild-type MEFs, nor does it rescue the residual SOCE of Stim1−/− cells. By contrast, it can induce a small but significant SOCE in the complete absence of STIM proteins. This surprising result suggests that junctionate can gate SOCE channels independently of STIM proteins. This observation not only lends further insight into the function of junctionate, but adds another layer to the complex regulation of store-operated Ca2+ entry by identifying an additional ER Ca2+ sensor, beyond the three known STIM protein isoforms STIM1, STIM1L and STIM2. Because junctionate is known to bind to TRPC-family channels, and because the non-specific Ca2+ channel blocker La3+ abolished junctionate-mediated influx, TRPCs are the prime candidates for serving as junctionate partners, and further study will be required to identify the exact partner channels as well as whether other physiological functions are linked to this additional influx mechanism. Junctionate–TRPC interactions could explain the fact that, in RFP–junctionate–expressing cells, La3+ slightly decreased the fraction of phagosomes that had Ca2+ microdomains (from 76% to 57%; Fig. 5A,B) in Stim1−/− cells. However, this could also reflect reduced Ca2+ release from stores, because La3+ could prevent store refilling by inhibiting plasma membrane Ca2+ channels during the phagocytic process. In any case, La3+ was much more effective in STIM1–expressing cells, impacting the majority of the Ca2+ signaling around those phagosomes (from 82% to 36%; Fig. 5B). Thus, the STIM1–Orai interactions that take place at the ER–phagosome interface are much more efficient than the putative junctionate–TRPC interactions in promoting the opening of phagosomal Ca2+ channels.
Our Stim1−/− phagocytic cells provided a useful model system to highlight the similarities and differences between STIM1 and junctate. Both STIM1 and junctate were recruited to phagosomes in Stim1−/− cells, demonstrating that junctate is recruited independently of STIM1, and the two proteins populated the same periphagosomal structures when co-expressed. Junctate is localized at ER-plasma-membrane contact sites before store depletion through its N-terminal domain (Srikantan et al., 2012; Treves et al., 2010), but whether junctate is ‘pre-addressed’ to contact sites through this targeting motif or responds to smaller or localized store depletion compared to STIM1 is unclear. The STIM1 luminal domain has been estimated to have a Ca2+-binding Kd of 200–600 μM in vitro (Stathopulos et al., 2006), and 169–210 μM by using fluorescence and patch-clamp recordings (Brandman et al., 2007; Luik et al., 2008). The Kd of junctate has been estimated to be around 217±20 μM by using fluorescence imaging (Treves et al., 2000). We cannot establish whether junctate is recruited to phagosomes independently of store depletion because the Ca2+ concentration within the ER lumen decreases as soon as phagocytic receptors are engaged, which could initiate the recruitment of junctate to its target membranes. Whether an additional signal triggers the accumulation of junctate at the ER–phagosome interface or alternatively, whether phagosomes form preferentially around pre-existing junctate-containing ER–plasma-membrane contact sites therefore remains to be determined.

Our electron microscopy data also establish that junctate is an ER-shaping protein that can elongate ER cisternae that are apposed to phagosomes, causing a doubling in their length (from 115 to 231 nm; Fig. 2). This effect was most apparent in the absence of STIM1 because junctate expression in wild-type fibroblasts only marginally increased the already larger size of the recruited ER structures (from 231 to 268 nm; Fig. 2). Previous studies have reported that junctate expression extends ER–plasma-membrane contacts by up to 55% (from 218 to 339 nm) in HEK-293 cells (Treves et al., 2004). We confirm here that junctate is indeed able to elongate ER structures and further establish that this effect is independent of STIM1. In wild-type MEFs, juxtaposed ER might already be near to maximal length owing to high levels of endogenous STIM1, but differences in junctate localization and overexpression efficiency might also explain the lack of lengthening effect in these cells. Compared to STIM1, junctate was less efficient in remodeling the ER because its expression did not promote the appearance of new ER structures that were apposed to phagosomes, only their extension. It should be noted however that we only quantified ER structures that were located less than 30 nm from the phagosomal membrane. Junctate might, in fact, be more efficient than STIM1 in recruiting ER structures at some distance from the phagosomes, as suggested by the fluorescence pattern of periphagosomal YFP-junctate. The physiological role of the ER extension mediated by STIM1 and junctate is unclear. The enlargement of ER–plasma-membrane junctions that is mediated by different STIM1 isoforms does not correlate with an increase in SOCE or in the efficiency of ER Ca2+ refilling (Saté et al., 2015), suggesting that elongation is not related to the Ca2+-signaling function of STIM1 molecules. The enlargement of juxtaposed ER structures might provide more stable signaling platforms for interactions of ER proteins with their ligands at the plasma or phagosomal membrane, or for lipid exchange between the two juxtaposed membranes (Toulmay and Prinz, 2011). At the ER–phagosome interface, the extension might promote the cross-presentation of antigens processed within the phagosomes, which relies on the communication between phagosomes and the ER-resident antigen loading machinery in a manner that is still poorly understood (Blum et al., 2013).

To conclude, our data further the understanding of junctate function as an important component of ER membrane contact sites, platforms that allow cells to precisely localize Ca2+ signals within cells. We showed that junctate complements STIM1 function, independently increasing contact lengths and Ca2+ release from stores near to phagosomes, thereby increasing phagocytic efficiency. Although our work is focused within the context of an important process of immune cell function – that of phagocytosis – in a more general sense, our data illustrate that, when global Ca2+ signals are abrogated, increasing localized Ca2+ signals through Ca2+ release from stores can provide an effective compensatory mechanism when SOCE is naturally, pathologically or therapeutically inhibited. This might be a concept with repercussions beyond the field of phagocytosis because Ca2+ signaling is important for many cell types and for a variety of cellular functions, including cell growth, apoptosis, secretion, contraction and motility. Furthermore, because SOCE channels have been proposed as drug targets in therapeutic avenues for diseases including cancer and autoimmune disorders, a better understanding of complementary or compensatory mechanisms of Ca2+ signaling will undoubtedly lead to more intelligent therapeutic designs.

**MATERIALS AND METHODS**

**Reagents**

Stim1−/− MEFs, generated through targeted gene disruption (Prins et al., 2011), and wild-type MEF control cells were a kind gift from Dr Marek Michalak (University of Alberta, Canada). YFP-tagged junctate was a kind gift from Dr Susan Treves (University of Basel, Switzerland). Dalbacco's modified Eagle's medium (DMEM; high glucose, catalog number 31966), heat-inactivated fetal calf serum (FCS), penicillin–streptomycin mixture (pen-strep, catalog number 15140), Fura-2-AM, BAPTA-AM, Lipofectamine 2000 transfection reagent, goat-anti-mouse conjugated to Alexa-Fluor-647 (1:1000) and SlowFade mounting medium were obtained from Life Technologies. Quest Fluor-8-AM was purchased from AAT Bioquest (Sunnyvale, CA). Mouse anti-Myec-tag antibody (9B11, 1:100) was purchased from Cell Signaling. Gluteraldehyde-stabilized sheep red blood cells (sRBCs), rabbit anti-sRBCs (1:200) and all other chemicals were obtained from Sigma-Aldrich. To reduce autofluorescence, gluteraldehyde-stabilized sRBCs were treated with bubbling 0.6% NaI2H4 in PBS with agitation for 1 h, washed three times in PBS and stored frozen.

**Cell culture and transfection**

MEFs were grown in DMEM containing 10% FCS and 0.5% pen–strept at 37°C under 5% CO2 and were passaged twice a week. Cells were used between passages 5 and 50. Transfections were performed with Lipofectamine 2000 with cells at 50–60% confluence in high glucose (4.5 g/l) DMEM. Transfected cells were allowed to recover for 24 h before manipulation.

**Phagocytosis and ER protein recruitment to phagosomes**

Oxpsenization was performed the same day as phagocytosis experiments. RBCs were opsonized by rabbit-anti-sheep RBCs at 37°C for 1 h and washed three times in PBS. Targets were added directly to cells that had been seeded on 12-mm coverslips in 24-well plates at 10:1 target:cell ratio in serum-containing medium. Plates containing coverslips were centrifuged at 600 g for 1 min. Cells were then incubated at 37°C under 5% CO2 for 10 min in the case of ER recruitment experiments and 30 min for phagocytosis experiments, before fixation and immunolabeling.

**Immunolabeling**

Cells were fixed in 4% paraformaldehyde in PBS. Fixed cells were permeabilized and blocked in 0.3% Triton X-100 with 1% BSA in PBS, and
incubated overnight at 4°C in primary antibody (mouse antibody against Myc tag, 1:100). The following day, cells were incubated for 1 h in secondary antibody (goat anti-mouse, conjugated to Alexa-Fluo-647, 1:1000) and then washed three times with PBS. Coverslips were mounted in SlowFade mounting medium containing 1 µg/ml Hoechst 33342.

**Electron microscopy**

Cells were fixed for 1 h in 2% glutaraldehyde with 0.1 M NaOH, pH 7.4, and scraped, and the pellets were washed once in 0.1 M sodium phosphate buffer, pH 7.4. En bloc staining with uranyl acetate, postfixation with osmium tetroxide, dehydration in ethanol, embedding in Epon and sectioning were performed by the Poli Facultaire de Microscopie Electronique core facility at the University of Geneva. Two grids per individual sample containing 8–10 50-nm sections were observed using a Tecnai transmission electron microscope (FEI, Eindhoven, The Netherlands). Of these, all phagosomes that could be identified in two to three randomly selected intact sections were examined. Quantification was performed using XT Pro software (Soft Imaging System GmbH, Germany).

**Imaging**

All fixed-cell imaging was performed using a confocal laser scanning microscope (LSM 700, Carl Zeiss AG) equipped with a 60× objective. Fura-2 imaging was performed using a wide-field fluorescence microscope equipped with a 40× objective, polychromator illumination, 430/DCPL dichroic and 510/BS40 emission filter (VisiRon Systems GmbH). Here, the fluorescence of FYP and GFP, or of RFP tags in transfected cells was simultaneously examined, and cells expressing very high levels of transfected proteins or showing signs of pre-activation or toxicity were eliminated from the analysis. Fluor-4 imaging was performed using a Nikon A1R inverted confocal microscope system equipped with 60× objective and a resonant scanner, and maintained at 37°C by a microscope temperature control system (Life Imaging Services, Basel, Switzerland). Fluoro-4 fluorescence was imaged using the 488-nm laser line, whereas the autofluorescence of RBCs was imaged using the far-red 633-nm laser. Experiments were performed in physiological buffer – 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 20 mM Hepes, 10 mM glucose, adjusted to pH 7.4, with NaOH. Ca²⁺-free medium contained 1 mM EGTA instead of 2 mM CaCl₂. Fura-2-AM (2 µM) was loaded in physiological buffer with 0.02% pluronic and visualized using 340/380-nm alternate excitation and 510/40-nm emission frames. Frames were acquired every 2 s. Fluoro-4-AM (4 µM) was loaded in physiological buffer with 250 µM sodiumpyruvate at 37°C for 30 min. This was followed by incubation for 20 min at room temperature and subsequent addition of BAPTA-AM (5 µM) for 30 min. For quantification of phagocytosis, ER protein recruitment and periphagosomes Ca²⁺ hotspots, the same protocol that has been previously described for STIM1 was used (Nunes et al., 2012), except for Ca²⁺-free experiments, where instead of using 3 mM EDTA to chelate extracellular 2 mM Ca²⁺, the experiment was performed in Ca²⁺-free medium. Briefly, for phagocytosis and ER protein recruitment studies, at least three confocal z-stacks of equal dimensions (≈15 µm thickness at 0.5 µm steps) per coverslip were imaged, and at least n=3 independent experiments were quantified for each condition. RBCs completely enclosed by cellular borders were defined as phagosomes. For RBCs found near the cell periphery, in the case of incomplete enclosure, only RBCs with ≥75% of their surface surrounded by Fc receptor immunoreactivity were counted as phagosomes. ER proteins were considered to be recruited to the vicinity of phagosomes when bright fluorescent clusters ( puncta) could be detected by visual inspection in the space within 3 pixels (≈750 nm) of phagosomes. Ca²⁺ hotspots were defined as regions of an area ≥500 nm² (4 pixels) within a distance of ≤750 nm (3 pixels) from the phagosomal border displaying fluorescence at least 2 s.d. higher than the average cytoplasmic Fluoro-4 intensity. At least five snapshots per coverslip and at least n=3 independent coverslips were quantified for each condition.

**Statistics**

All statistical analyses were performed using Prism software (GraphPad). Significance between two sets of experiments was determined using an unpaired Student’s t-test.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

P.N. and N.D. designed research; D.G. and P.N. performed research and analyzed data; D.G., P.N. and N.D. wrote the paper. N.D. and P.N. contributed equally as senior authors to this manuscript.

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**Supplementary information**

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allow formation of STIM1 puncta and segregation of STIM1 from IP3 receptors. Curr. Biol. 19, 1648-1653.
Discussion

Global and local Ca\(^{2+}\) signals are able to boost phagocytosis, a process involved in both innate and adaptive immunity. Recently, STIM1 has been described as an active regulator of store operated calcium entry at the level of ER-Ph membrane contact sites, through ER cistern recruitment to phagosomes and via the opening Ca\(^{2+}\) permeable channels resident in the phagosome membrane. Since periphagosomal calcium microdomains and ER-Ph membrane contact sites were still detectable in *Stim1*\(^{-/-}\) cells, we tested whether junctate could have a role in the regulation of this specific membrane contact site. Indeed, we were able to show that junctate colonizes STIM1-enriched ER-Ph junctions and that junctate was able to recruit ER cisternae to phagosomes independently of both STIM proteins, as shown in both *Stim1*\(^{-/-}\) and *Stim1*\(^{-/-}\); *Stim2*\(^{-/-}\) MEFs. Moreover, functional evidences point out the ability of junctate in boosting phagocytosis in a STIM protein-independent manner. The increased phagocytic index exhibited by cells overexpressing junctate was linked to its ability to regulate periphagosomal calcium microdomains, thus leading to augmented actin shedding. In fact, both *Stim1*\(^{-/-}\) and *Stim1*\(^{-/-}\); *Stim2*\(^{-/-}\) MEFs overexpressing junctate displayed a decreased actin ring around phagosomes, demonstrating junctate’s role in phagosome maturation independently of STIM proteins. In contrast to what was previously shown for STIM1, further investigations of the nature of these local microdomains have revealed that junctate generates them with a major Ca\(^{2+}\) component arising from the endoplasmic reticulum rather than from the phagosome. Specifically, the use of two InsP3R inhibitors, 2-APB and xestospongion C almost completely abolished the percentage of phagosomes that presented periphagosomal calcium microdomains, which indicates an InsP3R-dependent calcium release from the store. Conversely, junctate function in the regulation of local calcium signals was not drastically altered when La3+ was used to block phagosomal Ca\(^{2+}\) permeable channels. This evidence indicates that junctate is not as efficient as STIM1 in gating phagosomal channels, however junctate is more efficient in recruiting InsP3R-enriched ER stores close to the phagosome membrane. We delineate a mechanism where both STIM1 and junctate cooperate in periphagosomal calcium microdomain regulation, aimed at boosting phagocytosis. Morphological
evidence was provided via electron microscopy showing the ability of junctate to enlarge the size of ER juxtaposed to phagosome membranes. Specifically, junctate was more efficient as an ER-shaping protein in the absence of STIM1, since in WT MEFs junctate overexpression only marginally increased the size of ER cisternae interacting with the phagosome membrane. The capability of junctate in extending the size of the juxtaposed ER at the level of the plasma membrane was already published by Treves et al. in 2004. Here we show additionally that junctate can exert its ER reshaping capability in a STIM1-independent manner and in the context of ER-Ph membrane contact sites.

The function of junctate was discovered by Treves et al. in 2000 and it was presented as an alternative splice form of the AβH-J-J locus that could interact with important components of the store operated calcium entry machinery, such as InsP3R and TRPC3 channel, via its cytosolic domain. Moreover, junctate was shown to possess a calcium binding domain through which it can sense the depletion of the store and when overexpressed is able to increase agonist-induced Ca\(^{2+}\) elevation. In contrast, the silencing of junctate negatively affects calcium homeostasis \(^{290}\). In our study, junctate overexpression did not produce any further increase in SOCE, nor was it able to rescue STIM1 deficiency in \(\text{Stim}1^{-/-}\) MEFs. Interestingly, junctate significantly increased global calcium entry rates when both STIM proteins were absent, indicating that junctate can gate Ca\(^{2+}\) permeable channels resident in the plasma membrane in a STIM-independent manner. This result proposes junctate as a new calcium sensor protein that can modulate SOCE even when both STIM1 and STIM2 are genetically deleted. There is evidence proposing TRPC channels as the most likely candidate partner channel since TRPC3 \(^{293}\) was shown to interact with junctate in humans and TRPC2 and TRPC5 in mice \(^{294}\). Concerning our results, junctate-TRPC interaction could explain the 20% decrease mediated by the use of La\(^{3+}\) in the amount of phagosomes presenting periphagosomal calcium microdomains when junctate is overexpressed. However, further experiments are required to narrow down the exact channels that partner with junctate in the modulation of local Ca\(^{2+}\) signals around the phagosomes. Lately, the group of Gwack has described junctate as a new STIM1 recruiter at the level of ER-PM contact sites via its
intraluminal domain independently of phospholipid binding. For future studies, it would be interesting to investigate whether the lack of junctate, via siRNA or genetic deletion using the new CRISPR/Cas9 technique, could have any negative effect on immune cell functions, like phagosome maturation.

In conclusion, our results propose for the first time that junctate is a new calcium sensor protein able to modulate both global and local Ca\textsuperscript{2+} signals even in the absence of STIM proteins. Specifically, junctate can modulate periphagosomal calcium microdomains either cooperating with STIM1 or acting alone, thus boosting phagocytosis. The incremented ability to phagocytose exerted by cells overexpressing junctate is mediated by the increased actin shedding around the phagosome, an important early phase of phagosome maturation. STIM1 appears to be more efficient than junctate in recruiting new ER cisternae close to phagosome membrane, but junctate could be more adequate to bring calcium stores at a distance further than 30 nm, as suggested by the fluorescence images, and these new ER cisternae might participate in the regulation of periphagosomal calcium microdomains.

The ability of junctate to increase the size of the ER that interacts with the phagosome membrane could be important in establishing a more stable signalling environment and could favour lipid exchange between the membranes. In fact, it would be very intriguing to investigate whether those cortical ER enriched in junctate could have a role in modulating the creation of certain lipid environments that favour receptor clustering or even the recruitment of other proteins involved in phagocytosis regulation. Interestingly, these data on junctate suggest a new compensatory mechanism that could be used as a new therapeutic avenue. Once SOCE is inhibited by extracellular pathogens or compromised by genetic mutations, such as the one characteristic of Severe Combined Immunodeficiency, an increased calcium release from stores could overcome the genetic deficit of CRAC family members or provide compensatory way in order to destroy foreign particles.

The Mycobacterium tuberculosis strategy to avoid the phagocytic killing is one of the greatest examples of the relevance of Ca\textsuperscript{2+} signalling in the regulation of phagocytosis. Tuberculosis is still a
global health problem with enormous impact on both human morbidity and mortality. Phagocytosis of M. tuberculosis is preferentially complement-mediated, with CR3 being the primary receptor involved in humans. After being phagocytosed, this extremely dangerous pathogen is able to block the calcium signals necessary for phagosome maturation. Specifically, M. tuberculosis can inhibit both SK1 activity and translocation to the phagosome, thereby affecting the local calcium signals important for phagosome-lysosome fusion. Interestingly, the ability of M. tuberculosis to block phagosome maturation is receptor-dependent, inasmuch as a phagosomes formed via FcR-mediated phagocytosis can undergo full maturation, thereby decreasing the survival of the pathogen inside the phagosomes. The first evidence reporting the obstruction of calcium signals being the cause of M. tuberculosis survival was made using calcium ionophores. The incubation of complement-opsonized M. tuberculosis with Ca²⁺ ionophores leads to an increment of cytosolic calcium concentration, thereby causing a decrease in the percentage of intracellular survival of the pathogen. Upon Ca²⁺ ionophores addition, the researchers further reported an increase in the percentage of phagosomes positive for markers of phagosome maturation, such as LAMP1, cathepsins D and CD63, finally supporting the hypothesis of M. tuberculosis being able to block local Ca²⁺ signals fundamental for phagosome lysosome fusion, in order to escape phagocytic killing. Taking into consideration the novel role of junctate in the regulation of phagocytosis and its ability to increase calcium signals in T cells, it would be fascinating to examine whether junctate overexpression could also modulate CR-mediated phagocytosis in macrophages, thereby compensating SK1 inhibition caused by M. tuberculosis.

The second messenger Ca²⁺ is profoundly involved in the regulation of cell proliferation, migration and in fundamental processes like vasculogenesis and angiogenesis. Currently, the involvement of CRAC channels and store operated calcium entry in the regulation of both the development and progression of cancer and immune function is broadly accepted. Cancer is characterized by two different types of mutations: driver mutations and passenger mutations. The
former are causally related to cancer development, on the other hand passenger mutations are not oncogenic but are able to boost cancer development and progression. Since Ca$^{2+}$ signals are involved in cancer development, proliferation and metastasis only if the minimal cancer driver mutations are present, STIM/Orai and TRP genes mutations are listed as passengers. Moreover, SOCE-mediated calcium influx is affected by several factors, such as STIM-Orai ratios where too low or too high Orai expression can negatively affect intracellular calcium concentrations. Similarly, an increase in the Orai3/Orai1 ratio can decrease the Ca$^{2+}$ current, and the recently discovered STIM2 splice variant was reported to decrease Orai activity. The tumor microenvironment is also a fundamental factor able to modulate tumor growth, which is composed, among others, of immune cells like cytotoxic cells, tumor-associated macrophages, neutrophils and dendritic cells. These specific cells are attracted by tumor cells via cytokine secretion and have an immunosuppressive effect that helps tumor progression. As mentioned above, CRAC channels are crucial in the regulation of several immune functions. In the case of cancer, both the production of ROS and pH acidification are CRAC-dependent and lead to the inhibition of SOCE in T cells, thus contributing to immune suppression. Furthermore, CRAC channels have been reported to interfere with cancer therapy. In fact, the use of rituximab promotes STIM/Orai clusters, which then generates a Ca$^{2+}$ influx in B lymphoma cell lines, thereby negatively affecting rituximab-mediated apoptosis. It is clear that both the inhibition of Ca$^{2+}$ signals in tumor cells as cancer therapy and the consequent effect on immune cell functions have to be taken into consideration in future studies to find more effective multimodal cancer therapies.
Figure 9. The involvement of STIM and Orai proteins in cancer. STIM and Orai family proteins have been reported to be involved in the regulation of several types of tumors. In brown is represented the male tissue (prostate) and in violet are illustrated the female tissues (breast, ovary and cervix) 399.

The microenvironment surrounding the neoplastic mass is characterized among others by a low pH, hypoxia and a high concentration of grow factor and cytokines, which play a crucial role during the development of several tumors. The drastic decrease in oxygen tension leads to the activation of hypoxia-inducible factor-1α (HIF-1α), thereby initiating the vascularization of the tumor, a mechanism called angiogenic switch 300,301. In fact, a neoplastic mass cannot increase its dimension beyond 1-2 mm³ without any access to blood vessels, which sustain tumor grow by supplying oxygen and nutrients and clearing out its metabolic waste 301,302. Moreover, this new blood vessel network is used by cancer cells in order to move from the primary lesion site to other sites in the human body,
thereby developing secondary metastasis. There is evidence showing that both local endothelial cells and endothelial progenitor cells (EPCs) are able to regulate the angiogenic switch during tumor growth and metastatic progression. HIF-1α is a transcription factor able to induce the expression of several growth factors, such as vascular endothelial growth factor (VEGF) and stromal derived factor-1α (SDF-1α), which consecutively activate the mobilization of EPCs from the bone marrow into both primary tumor lesion and secondary pre-metastatic niches. The phenotype of EPCs is still not completely established, since there are no specific surface markers, thus making impossible to classify the EPCs as a unique cell population. However, EPCs can be divided in two subgroups: hematopoietic and non-hematopoietic (Figure 10). The former subgroup is composed by colony forming unit-endothelial cells (CFU-ECs) and circulating angiogenic cells (CACs), which are recruited towards the neoplastic mass without being incorporated into neovessels. On the contrary, the non-hematopoietic subgroup comprises the endothelial colony forming cells (ECFCs) that can differentiate into mature endothelial cells and they are capable of forming capillary-like structures both in vitro and in vivo. There is evidence showing VEGF as a stimulator of ECFCs proliferation, homing and tubulogenesis both in vitro and in vivo. In fact, upon an ischemic insult ECFCs are guided to the lesion site in a VEGF-dependent manner. VEGF binds VEGF receptor-2 (VEGFR-2) leading to the activation of several proteins, such as PLCγ, PKC and cytosolic PLA2, thus generating Ca²⁺ signals that promote ECFCs proliferation and tubulogenesis. Specifically, PLCγ cleaves PI(4,5)P₂ in order to produce InsP₃ that causes the calcium release from the ER through the gating of InsP₃R₃s resident in the ER membrane, thereby activating SOCE. In ECFCs harvested from healthy patients, VEGF is able to induce Ca²⁺ oscillation to stimulate the Ca²⁺-sensitive transcription factor NF-kB, thus boosting ECFC proliferation. In contrast, both VEGF-dependent Ca²⁺ oscillations and its related gene expression were absent in ECFCs harvested from patients with metastatic renal cellular carcinoma (mRCC), showing a different regulation of the calcium homeostasis. Despite the development of monoclonal anti-VEGF antibodies, such as Bevacizumab, or inhibitor of tyrosine kinase receptor, like Sunitinib and Pazopanib, anti-VEGF drugs
are not giving considerable results, since patients develop resistance and dye because of the formation of secondary metastatic lesions \textsuperscript{320,321}. Lodola et al. showed that ECFCs derived from mRCC patients are able to replicate in a more aggressive and VEGF-independent way, which could explain why anti-VEGF drugs do not increase the overall survival (OS) in these patients. Interestingly, the researchers showed a remodelling in the component machinery of SOCE, with STIM1, Orai1 and TRPC1 being overexpressed. Since both in vitro proliferation and tubulogenesis were SOCE-dependent in ECFCs collected from patients affected by mRCC \textsuperscript{322}, specific CRAC channels blockers could be more suitable for preventing tumor growth. Moreover, it would be interesting to investigate the role of junctate in the regulation of calcium signals that are fundamental for ECFCs functions. In mRCC-ECFCs, the amount of calcium released from the ER is significantly reduced compare to their healthy correspondent, using both a physiological agonist or SERCA blockers in order to deplete the store. In contrast, the ECFCs harvested from mRCC patients display a slower calcium rate recovery to the baseline compare to normal ECFCs, only when the depletion of the store is stimulated by ATP. The reduced intracellular calcium reservoir and the incremented amount of time for the removal of cytosolic calcium suggest an impaired SERCA activity. As mentioned above, junctate is able to interact with SERCA2a in murine cardiomyocyte and increases its activity, thereby leading to a severe hypertrophy. Therefore, the decreased SERCA-dependent Ca\textsuperscript{2+} uptake in mRCC-ECFCs could be related to a reduction in the expression of junctate, which could be a novel player in the Ca\textsuperscript{2+} homeostasis regulation in ECFCs.
Figure 10. Endothelial progenitor cell (EPC) subgroups. EPCs can be isolated from both peripheral and umbilical cord blood. Colony forming unit-endothelial cells (CFU-ECs) are obtained after seeding mononuclear cells (MNCs) on fibronectin-coated dishes with 20% fetal bovine serum (FBS) and VEGF. Circulating angiogenic cells (CACs) can be isolated from MNCs after seeding them for 4 days in endothelial condition. Endothelial colony forming cells (ECFCs) must be seeded in collagen-coated dishes in the presence of endothelial growth medium.

Conclusion
Ca\(^{2+}\) entry has been the central point in most of the studies aimed at finding a link between Ca\(^{2+}\) signals and tumor development and progression. In our study, we had reported Ca\(^{2+}\) release from stores as a complementary calcium signal able to cooperate with SOCE in order to boost phagocytosis. In addition, the data on junctate describe a novel mechanism that is able to regulate the phagocytic process independently of both STIM proteins, suggesting that calcium release alone is enough to exert an effect on this immune process. This novel point of view has to be taken in consideration to increase the phagocytic efficiency of immune cells when SOCE is altered or completely abolished, but also in the development of new cancer therapies, where the release of calcium from stores could be used by tumor cells to develop resistance, especially considering that
CRAC channels blockers, like BTP-2, do not completely abolish cell proliferation. In future studies, it would be interesting to test whether junctate is involved in the modulation of the augmented calcium signals characteristic of several types of cancers, as has already been described for STIM and Orai proteins. In prostate cancer, Orai1 expression has been reported to be lower but at the same time TRPV6, which is another Ca\(^{2+}\)-selective channel, is overexpressed. As the higher expression of TRPV6 compensate the loss of Orai1 in the tumor growth, it would be extremely interesting to investigate whether junctate could have the same role in other types of cancer, where its compensation would be represented by increased calcium release from the ER. The ability of junctate to interact with SERCA2a and InsP3Rs could be fundamental to create a system that generates a continuous loop between calcium release from the store and calcium uptake by the ER Ca\(^{2+}\) pump.
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